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QUENCHING OF CHLOROPHYLL FLUORESCENCE AND PRIMARY PHOTOCHEMISTRY IN CHLOROPLASTS BY DIBROMOTHYMOQUINONE

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SUMMARY

The quenching action of dibromothymoquinone on fluorescence and on primary photochemistry was examined in chloroplasts at −196 °C. Both the initial (F_0) and final (F_M) levels of fluorescence as well as the fluorescence of variable yield ($F_V = F_M - F_0$) were quenched at −196 °C to a degree which depended on the concentration of dibromothymoquinone added prior to freezing. The initial rate of photoreduction of C-550 at −196 °C, which was assumed to be proportional to maximum yield for primary photochemistry, φ_{P_0}, was also decreased in the presence of dibromothymoquinone. Simple theory predicts that the ratio F_V/F_M should equal φ_{P_0}. Excellent agreement was found in a comparison of relative values of φ_{P_0} with relative values of F_V/F_M at various degrees of quenching by dibromothymoquinone. These results are taken to indicate that F_0 and F_V are the same type of fluorescence, both emanating from the bulk chlorophyll of Photosystem II.

Dibromothymoquinone appears to create quenching centers in the bulk chlorophyll of Photosystem II which compete with the reaction centers for excitation energy. The rate constant for the quenching of excitation energy by dibromothymoquinone is directly proportional to the concentration of the quencher. Rate constants for the de-excitation of excited chlorophyll molecules by fluorescence, k_F, by nonradiative decay processes, k_D, by photochemistry, k_P, and by the specific quenching of dibromothymoquinone, k_Q, were calculated assuming the absolute yield of fluorescence at F_0 to be either 0.02 or 0.05.

INTRODUCTION

It was found in a previous investigation [1] of the site of inhibition of dibromothymoquinone (DBMIB) on photosynthetic electron transport that the oxidized form of the inhibitor was an effective quencher of chlorophyll fluorescence. The quenching of fluorescence does not appear to be related to the inhibitory action of DBMIB: both the oxidized and the reduced forms of the inhibitor block electron

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transport (at a site on the Photosystem I side of plastoquinone [1, 2]) while only the oxidized form quenches fluorescence; also, the quenching of fluorescence appears to be due to a direct interaction between DBMIB and the photochemical apparatus since the quenching is manifest at −196 °C as well as at room temperature [1]. The quenching of fluorescence has proved to be a useful tool to eliminate fluorescence yield artifacts from measurements of light minus dark difference spectra of chloroplasts at −196 °C [3]. The observation that DBMIB is highly effective in quenching fluorescence at low temperature also raises the related question of whether the primary photochemistry is quenched as well. The purpose of the work reported here was to compare the effects of DBMIB on fluorescence yield and on the primary photochemistry, as indicated by the photoreduction of C-550, at liquid nitrogen temperature.

MATERIALS AND METHODS

Chloroplasts, prepared by methods described previously [4], were suspended at a concentration of about 3.0 mg chlorophyll per ml in a medium containing 0.4 M sucrose, 0.01 M NaCl and 0.05 M Tris–HCl, pH 7.8, and kept near 0 °C. Just before an experiment, 0.2 ml of the concentrated chloroplast suspension were diluted with a glycerol–buffer medium (65 % glycerol, 35 % of the above suspending buffer) to make the final concentration 250 μg chlorophyll per ml. DBMIB, dissolved at various concentrations in ethanol, was added to the diluted chloroplast suspension to give the specified concentration but never in a volume which exceeded 1 % of the final volume. The ratio of moles of DBMIB to moles of chlorophyll appears to be a more significant index for fluorescence quenching than the molar concentration of DBMIB so that care was taken to maintain equal chlorophyll concentrations throughout these experiments. Samples (0.3 ml) of the glycerol suspended chloroplasts were frozen to liquid nitrogen temperature in complete darkness in a vertical cuvette and Dewar system [5]. The samples, which had an optical path of about 2 mm, were used only if they remained as a clear optical glass, without cracking, throughout the experiment.

Fluorescence was excited by broad band blue light (defined by Corning filters 5543 and 5433, an Optics Technology 500 nm shortpass interference filter, and a Calflex C heat-reflecting filter) at 100 μW/cm^2 at the top of the sample. The fluorescence was measured from the bottom of the sample through a filter combination (2 Balzers' 690 nm interference filters and 2 Corning 9830 filters) which blocked completely the blue exciting light and passed a band with a maximum at 690 nm and a half-width of 7 nm. Measurements of fluorescence were also made at 730 nm with interference filters and blocking filters giving a half-width of 7 nm. The photocurrent from an EMI 9558C phototube was measured and stored as a function of time with a Fabri-Tek 1072 computer.

Absorption spectra of the frozen samples were measured with our computerized single-beam spectrophotometer [5]. Spectra were measured on a given sample before irradiation, after each of a sequence of irradiations (typically 3, 5, 7, 10 and 15 s] with the broad band blue light (100 μW/cm^2) and after a saturating irradiation with 1 min of white light. Each absorption spectrum is the sum of two spectra measured sequentially. The data were accepted only if the difference between the two sequential spectra showed no differences other than white noise. Such procedures eliminate

spurious changes which occasionally occur during a scan. The extent of C-550 reduction induced by given irradiation periods was determined from light-minus-dark difference spectra by the absorbance difference between 543 and 546 nm [3]. The extent of P_{700} oxidation was similarly determined by the absorbance difference between 702 and 697 nm.

RESULTS

Fluorescence-induction curves (intensity of fluorescence at 690 nm as a function of time following the onset of the blue exciting light) for chloroplasts at −196 °C are shown in Fig. 1 for several concentrations of DBMIB. The latter part of these curves is plotted on a slower time scale so that both the initial, F_0, and final, F_M, levels can be ascertained. The ratio F_M/F_0 for the chloroplasts used in the work reported here was 4.5. That ratio varied among the different chloroplast preparations but generally fell within the range of 4.0–5.0. It is apparent from Fig. 1 that DBMIB quenches both the F_M and F_0 levels of fluorescence at −196 °C. Amesz and Fork [6] showed similar fluorescence quenching data for a number of oxidized quinones.

The rates of photoreduction of C-550 at −196 °C at various concentrations of DBMIB are shown in Fig. 2. The total extent of C-550 reduction achieved by a saturating irradiation with white light was constant over the range of DBMIB concentrations shown. The extent of C-550 reduction induced by various periods of irradiation with blue light (100 μW/cm^2) is plotted as a function of irradiation time. The curves are extrapolated through zero and the initial rate of photoreduction, R_0, is taken as the relative amount of C-550 reduced during the first second of irradiation. The signal-to-noise ratio at the smallest absorbance change measured (3 s irradiation in the presence of 10^{-4} M DBMIB) was approximately 10 and was proportionately larger for the larger absorbance changes. The measurement of the R_0 is less precise than the measurement of the levels of fluorescence, F_0 and F_M, but the extrapolation through zero and the fitting of values R_0 versus DBMIB concentration to a smooth curve in effect averages the results of a number of experimental determinations. Fig. 3 shows F_0, F_M and R_0 plotted as a function of DBMIB concentration. DBMIB at a concentration of 10^{-7} M has no measurable effect on these measurements of fluorescence and C-550 (i.e. at −196 °C and at a chlorophyll concentration of 250 μg/ml).

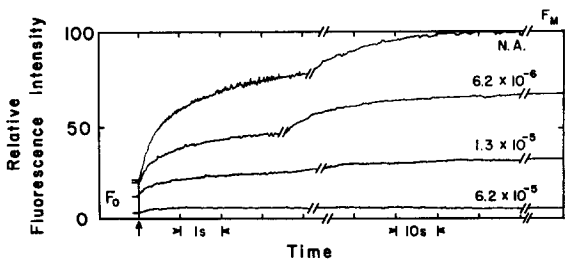

Fig. 1. Relative fluorescence (690 nm) of chloroplasts at −196 °C as a function of time after the onset of irradiation with blue light (100 μW/cm^2). Chloroplasts (250 μg/ml in 60 % glycerol) were frozen to −196 °C after addition of DBMIB to the concentration indicated. The initial levels of fluorescence, F_0, and the final levels, F_M, are indicated at the onset and end of irradiation.

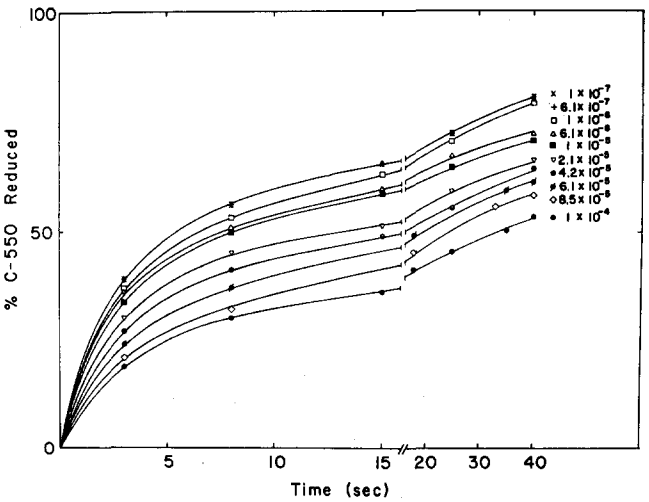

Fig. 2. Relative amount of C-550 reduced at −196 °C as a function of irradiation time with blue light (100 μW/cm^2). Chloroplasts (250 μg/ml in 60 % glycerol) were frozen to −196 °C after addition of DBMIB to the concentration indicated. (in moles/l).

It is apparent from Fig. 3 that fluorescence is more sensitive than photochemistry to quenching by DBMIB.

The relative values of the fluorescence yield at 690 nm were measured at −196 °C and at room temperature in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Both F_0 and F_M increased 2.7-fold on cooling to −196 °C (data not shown).

The effect of DBMIB on Photosystem I activity was investigated by measuring fluorescence-induction curves at 730 nm and the rate of oxidation of P_{700} at −196 °C. The Photosystem I parameters appeared somewhat less sensitive to DBMIB than the Photosystem II parameters. Table I gives the DBMIB concentrations which resulted

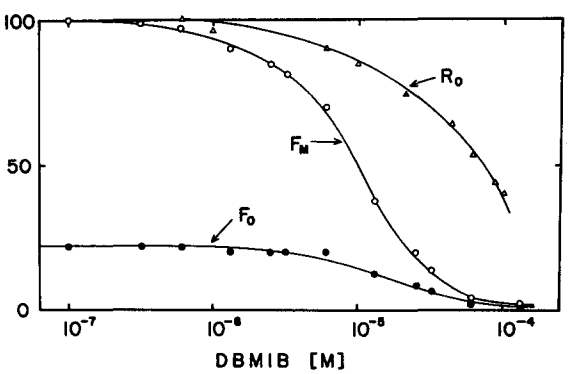

Fig. 3. F_0 and F_M (determined from plots such as those in Fig. 1) and R_0 (the initial rate of photoreduction of C-550, determined from Fig. 2, as the amount reduced during the first second of irradiation) as a function of the concentration of DBMIB plotted on a relative scale (100 represents the value without DBMIB).

TABLE I

CONCENTRATIONS OF DBMIB FOR 50 % INHIBITION OF FLUORESCENCE AND PHOTOCHEMISTRY

Photosystem		Photochemical yield (φ_P)		Fluorescence yield (φ_F)		
				F_0	F_M	F_V
II	C-550:	$6.2 \cdot 10^{-5}$	690 nm:	$1.5 \cdot 10^{-5}$	$1.0 \cdot 10^{-5}$	$1.0 \cdot 10^{-5}$
I	P_{700}:	$2.5 \cdot 10^{-4}$	730 nm:	$2.0 \cdot 10^{-5}$	$1.25 \cdot 10^{-5}$	$1.0 \cdot 10^{-5}$

in 50 % inhibition of these Photosystem II and Photosystem I parameters. At high concentrations of DBMIB the extent of the P_{700} change was increased about 2-fold (Table II) even though the rate of change was inhibited. It was shown previously [7] that about half of the P_{700} oxidized by light at −196 °C was reversibly reduced in the dark (probably by a backreaction between the oxidized P_{700} and the reduced primary electron acceptor). High concentrations of DBMIB appear to inhibit that backreaction at −196 °C so that all of the P_{700} is photooxidized irreversibly.

TABLE II

TOTAL EXTENT OF THE C-550 CHANGE ($A_{546}-A_{543}$) AND THE P_{700} CHANGE ($A_{702}-A_{697}$) BY SATURATING IRRADIATION WITH WHITE LIGHT

ΔA at 10^{-4} M DBMIB taken as 100.

DBMIB	Total change C-550	P_{700}
$1 \cdot 10^{-4}$ M	100	100
$5 \cdot 10^{-4}$ M	80	190
$1 \cdot 10^{-3}$ M	80	210

DISCUSSION

The quenching of fluorescence and of photochemistry can be analyzed in terms of the rate constants for the depopulation of the first excited singlet state of chlorophyll by fluorescence, k_F by nonradiative decay processes, k_D, by photochemistry, k_P, and by the specific quenching of DBMIB, k_Q. Two models representing limiting cases for energy transfer within the photosynthetic apparatus can be considered. One model assumes that the apparatus consists of independent photosynthetic units with one reaction center per unit and no energy transfer between units. If we let A represent the fraction of units which have "open" reaction centers (or the fraction of the primary acceptor molecules in the oxidized state), the quantum yields for fluorescence and photochemistry can be written as:

$$\varphi_F = \frac{k_F(A)}{k_F+k_D+k_Q+k_P} + \frac{k_F(1-A)}{k_F+k_D+k_Q}$$

$$\varphi_P = \frac{k_P(A)}{k_F+k_D+k_Q+k_P}$$

The other model assumes complete energy transfer between units; essentially a matrix in which all reaction centers belong to the matrix. In such a model:

$$\varphi_F = \frac{k_F}{k_F + k_D + k_Q + k_P}$$

$$\varphi_P = \frac{k_P}{k_F + k_D + k_Q + k_P}$$

where $k_P = K_P(A)$. Although these models show different kinetic behavior, they give identical expressions for the quantum yields of fluorescence and photochemistry at the initial and final states represented by F_0 and F_M in the low temperature irradiation experiments. At the onset of irradiation when fluorescence is at the F_0 level, $A = 1$ (experiments show that C-550 is fully oxidized in dark adapted chloroplasts), so that with either model:

$$\varphi_{F_0} = \frac{k_F}{k_F + k_D + k_Q + k_P} \tag{1}$$

$$\varphi_{P_0} = \frac{k_P}{k_F + k_D + k_Q + k_P} \tag{2}$$

At the end of the irradiation period when fluorescence is at the F_M level, the primary electron acceptor is fully reduced so that $A = 0$, $\varphi_P = 0$ and

$$\varphi_{F_M} = \frac{k_F}{k_F + k_D + k_Q} \tag{3}$$

Thus, the derivations based on φ_{F_0}, φ_{F_M} and φ_{P_0} which follow should be valid for any degree of energy transfer between units in the photosynthetic apparatus.

The ratio of the fluorescence of variable yield ($F_V = F_M - F_0$) to the total fluorescence (F_M) bears a simple and useful relationship to the yield of photochemistry. It can be shown from Eqns 1, 2, and 3 that:

$$\frac{F_V}{F_M} = \frac{\varphi_{F_M} - \varphi_{F_0}}{\varphi_{F_M}} = \frac{k_P}{k_F + k_D + k_Q + k_P} = \varphi_{P_0} \tag{4}$$

This relationship has been derived in somewhat different form previously [8]. Eqn 4 provides the basis for a direct experimental test since F_V, F_M and φ_{P_0} are all measurable quantities, at least on a relative scale, as a function of the quenching by DBMIB.

The yield for quenching by a specific concentration of DBMIB

$$\varphi_Q = \frac{k_Q}{k_F + k_D + k_Q + k_P} \tag{5}$$

can also be determined experimentally from a comparison of measurements made in the absence (Qo) and in the presence (Q) of the quencher. From Eqn 4 with $k_Q = 0$ when $Q = 0$:

$$\left(\frac{F_V}{F_M}\right)_{Q_0} - \left(\frac{F_V}{F_M}\right)_{Q} = \frac{k_P k_Q}{(k_F+k_D+k_P)(k_F+k_D+k_Q+k_P)} = (\varphi_{P_0})_{Q_0}\varphi_{Q_0}$$

or

$$\varphi_Q = \frac{\left(\frac{F_V}{F_M}\right)_{Q_0} - \left(\frac{F_V}{F_M}\right)_{Q}}{\left(\frac{F_V}{F_M}\right)_{Q_0}} = \frac{(\varphi_{P_0})_{Q_0}-(\varphi_{P_0})_{Q}}{(\varphi_{P_0})_{Q_0}} \tag{6}$$

Thus, φ_Q for various concentrations of DBMIB can be determined experimentally from either fluorescence measurements or from measurements of the initial rate of photoreduction of C-550.

It is assumed that the initial rate of photoreduction of C-550 is proportional to the quantum yield of the primary photochemical reaction, φ_{P_0}, so that relative values of φ_{P_0} can be compared with relative values of F_V/F_M (in accordance with Eqn 4) at various degrees of quenching by DBMIB. The kinetic data in Fig. 3 on the initial rate of reduction of C-550 is plotted in Fig. 4 on a relative scale as a function of DBMIB concentration. The fluorescence of variable yield as a function of DBMIB concentration is also plotted in Fig. 4 on the same relative scale. The open circles in Fig. 4 indicate the relative values of the F_V/F_M ratios at various DBMIB concentrations. The agreement between the calculated values of φ_{P_0} (from F_V/F_M) and the measured values (from the initial rates of photoreduction of C-550) supports completely the theoretical prediction from Eqn 4.

DBMIB appears to create quenching centers which dissipate the excitation energy. If we assume that the number of quenching centers is proportional to the concentration of DBMIB, $[Q]$, we can write $k_Q = K_Q[Q]$ or from Eqn 5:

$$\frac{1}{\varphi_Q} = 1 + \frac{k_F+k_D+k_P}{K_Q[Q]} \tag{7}$$

Thus a plot of $1/\varphi_Q$ vs $1/[Q]$ should give a straight line, which extrapolates to unity as

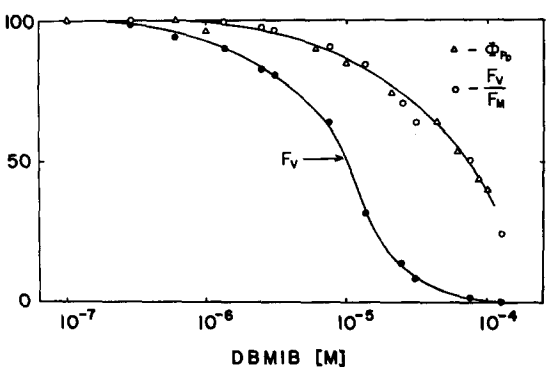

Fig. 4. F_V, ●, (taken from the fluorescence data as $F_M - F_0$), φ_{P_0}, △, (the same plot as R_0 in Fig. 3) and the ratio F_V/F_M, ○, as a function of the concentration of DBMIB plotted on a relative scale (100 represents the value without DBMIB).

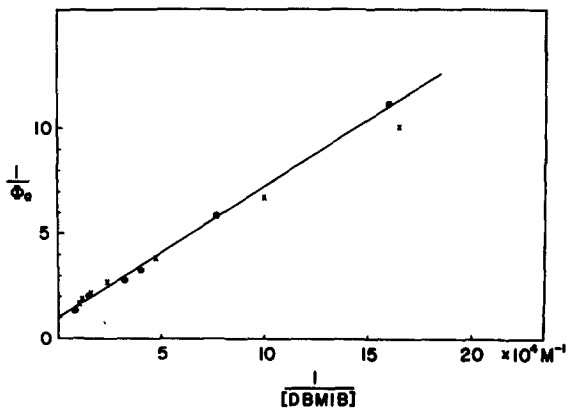

Fig. 5. $1/\varphi_Q$ (determined from measurements of F_V/F_M, ●, or from measurements of φ_{F_0}. ×, as described in the text) versus the reciprocal of the concentration of DBMIB.

$1/[Q]$ goes to zero. Fig. 5 shows such a plot where φ_Q was determined from the fluorescence data (solid points) or from the initial rates of reduction of C-550 (crosses) according to Eqn 6 for various concentrations of DBMIB. The fluorescence data (F_V/F_M) are more precise than the initial rates of C-550 reduction for determining the slope of the plot in Fig. 5. These results support the simple assumption that the number of quenching centers is proportional to the concentration of DBMIB and suggest that the quenching centers consist of a DBMIB–chlorophyll complex.

The rate constants for de-excitation of the first excited-singlet state of chlorophyll can be calculated if we take values of k_F and φ_{F_0} from the literature. The natural lifetime of chlorophyll fluorescence, τ_0 (the lifetime of fluorescence if there were no other competing processes for the excitation energy, i.e. k_D and $k_P = 0$) is about 20 ns [9]. $k_F = 1/\tau_0 = 5 \cdot 10^7\ s^{-1}$. Latimer et al. [10] measured the absolute value of the fluorescence yield of *Chlorella* cells, extrapolated to very weak exciting light (φ_{F_0}), to be 0.02 at room temperature. The fluorescence yield of chloroplasts (at both the F_0 and F_M levels) increases about 2.5 fold on cooling to −196 °C so that φ_{F_0} at −196 °C should be about 0.05. In the measurements reported here $\varphi_{F_M} = 4.5\ \varphi_{F_0}$ both at room temperature and at low temperature. Given values of φ_{F_M} and k_F, k_D can be calculated from Eqn 3 with $k_Q = 0$ (no DBMIB present) and with that value of k_D, k_P can be calculated from Eqn 1. From the slope of the plot in Fig. 5,

$$\text{slope} = \frac{k_F + k_D + k_P}{K_Q} = 6.25 \times 10^{-5}\ \text{M}$$

and the values of the k_F, k_D and k_P, K_Q can be calculated for a given value of φ_{F_0}. Such values of K_Q are not particularly meaningful at a molecular level because no distinction was made between the bound DBMIB and that in solution but they are useful to calculate rate constants for quenching at various concentrations of DBMIB. Values for the various rate constants to depopulate the first excited singlet state are given in Table III for two assumed values of φ_{F_0}.

It was implicitly assumed in the theoretical framework for the analysis of the measurements of Photosystem II parameters (i.e. fluorescence at 690 nm and C-550)

TABLE III

RATE CONSTANTS FOR DE-EXCITATION OF EXCITED CHLOROPHYLL MOLECULES AND THE YIELD OF QUENCHING BY 10^{-4} M DBMIB

	$\varphi_{F_0} = 0.02$	$\varphi_{F_0} = 0.05$
k_F	$5 \cdot 10^7\ s^{-1}$	$5 \cdot 10^7\ s^{-1}$
k_D	$50 \cdot 10^7\ s^{-1}$	$17 \cdot 10^7\ s^{-1}$
k_P	$195 \cdot 10^7\ s^{-1}$	$78 \cdot 10^7\ s^{-1}$
K_Q	$40 \cdot 10^{12}\ M^{-1} \cdot s^{-1}$	$16 \cdot 10^{12}\ M^{-1} \cdot s^{-1}$
k_Q (10^{-4} M)	$400 \cdot 10^7\ s^{-1}$	$160 \cdot 10^7\ s^{-1}$
φ_Q (10^{-4} M)	0.615	0.615

that there was only one origin of fluorescence, k_F, emanating from the bulk chlorophyll of Photosystem II. This assumption of a single kind of fluorescence at 690 nm is in marked contrast to the usual assumption that most of F_0 is "dead fluorescence" or a fluorescence of constant yield emanating from Photosystem I and that the fluorescence of variable yield, F_V, from Photosystem II begins at or near zero for dark adapted chloroplasts and increases in direct proportion to the reduction of the primary electron acceptor of Photosystem II. However, we regard the experimental verification of Eqn 4 in Fig. 4 (i.e. the correlation between relative values of φ_{P_0} determined from the initial rates of photoreduction of C-550 and the relative values of F_V/F_M for varying degrees of quenching by DBMIB) and the experimental verification of Eqn 7 in Fig. 5 to be strong presumptive evidence supporting the theoretical basis of the analysis and, therefore, that a major part of F_0 has the same origin as F_V, i.e. from Photosystem II chlorophyll.

Etienne et al. [11] recently published data on the quenching of fluorescence and the inhibition of photosynthetic electron transport by dinitrobenzene in chloroplasts and *Chlorella* cells at room temperature. They assumed, for the analysis of their results, that Photosystem II fluorescence was very close to zero for dark adapted chloroplasts and that most of the fluorescence at the F_0 level was due to Photosystem I. However, the data obtained by Etienne et al. with dinitrobenzene appeared similar to those obtained by us with DBMIB in that both the F_0 and F_M levels of fluorescence were quenched and the fluorescence was more sensitive to the quencher than the electron transport. We have taken the data from their Fig. 1A, which gives the relative quenching of F_0 and F_V, and from their Figs 2 and 4 which give the rate of O_2 evolution with *Chlorella* cells and the rate of dichlorophenolindophenol (DCIP) reduction with chloroplasts, respectively, in order to compare the ratio F_V/F_M with the rate of photochemistry at various concentrations of dinitrobenzene. The only assumption we make, because the datum was not specified, is the ratio F_M/F_0 in the absence of dinitrobenzene. Table IV shows the comparison between the ratio F_V/F_M (assuming $F_M/F_0 = 4.0$ or 5.0) and the rates of photochemistry. The agreement is especially good for chloroplasts (photoreduction of DCIP assuming $F_M/F_0 = 5.0$. We conclude that the quenching induced by dinitrobenzene is the same as that induced by DBMIB and that the data of Etienne et al. are consistent with our view that most of F_0 has some of the same origin as F_V.

We are aware that a number of fluorescence-quenching reagents or treatments give results which are quite different from those obtained with DBMIB or dinitro-

TABLE IV

ANALYSIS OF DATA FROM REF. 11

Fluorescence 685 nm: *Chlorella* (25 μg chlorophyll/ml) from Fig. 1A. DCIP reduction: chloroplasts (6.3 μg chlorophyll/ml) from Fig. 4, curve A. O_2 evolution: *Chlorella* (39 μg chlorophyll/ml) from Fig. 2 average of curves A and B.

DNB conc. [M]	Fluorescence (F_V/F_M) (assuming)		Rate of photochemistry (φ_P)	
	$F_M/F_0 = 4$	$F_M/F_0 = 5$	DCIP	O_2 evolution
0	100	100	100	100
$2.15 \cdot 10^{-5}$	97	98	100	—
4.7	94	95	97	100
$1 \cdot 10^{-4}$	90	91	93	100
2.15	77	82	85	87
4.7	59	65	71	70
$1 \cdot 10^{-3}$	44	50	53	32
2.15	23	26	31	—

benzene in that F_V is quenched specifically while F_0 is little affected. Both types of quenching, however, must be explained within the framework of the photochemical apparatus. A model to explain both kinds of results is presented in the accompanying paper [12].

The primary photochemistry of Photosystem I (photooxidation of P_{700} at −196 °C) was also quenched by DBMIB but at significantly higher concentrations than were required to quench the photochemistry of Photosystem II. The analysis of Photosystem I photochemistry and fluorescence in whole chloroplasts, however, is complicated by the possibility of energy transfer from Photosystem II to Photosystem I. In that case the quenching of Photosystem II by DBMIB would also quench the energy transfer to Photosystem I. We interpret the fluorescence of variable yield measured at 730 nm at −196 °C to be due to energy transfer from Photosystem II to Photosystem I since Photosystem I particles (free from Photosystem II) show no fluorescence of variable yield. The observations that F_V at 730 nm shows the same sensitivity to DBMIB as F_v at 690 nm (Table I) is consistent with the view that F_V at 730 nm is due to energy transfer from Photosystem II to Photosystem I. Murata [13] concluded previously, on the basis of kinetic data, that the fluorescence of variable yield at 730 nm at −196 °C was due to energy transfer from Photosystem II. So far as Photosystem II is concerned, energy transfer from the bulk chlorophyll to Photosystem I could be included as one of the k_D processes.

ACKNOWLEDGEMENT

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