

Chlorophyll *a* Fluorescence Measurements of Isolated Spinach Thylakoids Obtained by Using Single-Laser-Based Flow Cytometry¹

Chunhe Xu, Julie Auger, and Govindjee

Departments of Physiology and Biophysics and Plant Biology (C.X., G.) and Cell Science Center, Biotechnology Center (J.A.), University of Illinois at Urbana-Champaign, Urbana Illinois 61801

Received for publication May 30, 1989; accepted October 20, 1989

Flow cytometry data of spinach thylakoid membrane preparations indicate the presence of a homogeneous thylakoid population. Fluorescence data from a flow cytometer and comparison with data from two other fluorometers show that chlorophyll *a* fluorescence detected with a flow cytometer has the character of maximum fluorescence (F_{max}), not of the constant component (F_0). This conclusion is important since F_0 measures fluorescence that is affected mostly by changes in excitation energy transfer and $F_{max}-F_0$ (the variable fluorescence) by changes in photochemistry. This was demonstrated by: 1) The light intensity as well as diffusion rate dependence of the quenching effect of various quinones (p-benzoquinone, phenyl-benzoquinone, and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone, DBMIB) on fluorescence yield; quenching for the same concentration of these quinones was lower at the

higher than at the lower light intensities. 2) Temperature dependence of the fluorescence yield; increasing the temperature from 20 to 70°C did not show an increase in fluorescence yield using a flow cytometer in contrast to measurements with weak excitation light, but similar to those obtained for F_{max} . 3) Addition of an inhibitor diuron up to 100 μ M did not change the fluorescence intensity. A comparison of quenching of fluorescence by various quinones obtained by flow cytometry with those by other fluorometers suggests that the high intensity used in the cytometry produces unique results: the rate of reduction of quinones is much larger than the rate of equilibration with the bulk quinones.

Key terms: Constant fluorescence F_0 , maximum fluorescence F_{max} , photosynthesis, quenching of fluorescence, quinones, diuron, ferricyanide (spinach)

Laser-activated flow cytometry has become an important experimental tool in various areas of animal cell physiology and microbiology. Important advantages of this technique are the capacity for rapid identification, quantification, and resolution of discrete subpopulations in heterogeneous cell samples on the basis of expression of cell surface marker or internal metabolic capabilities (9,23,26,31). Although chlorophyll (Chl) *a* fluorescence and light-scattering signals from higher plant and algal cells can be easily detected by this sophisticated technique, reports on the application of flow cytometry to marine biology and plant biology, particularly photosynthesis, are scarce (3).

In order for the readers to appreciate the significance of Chl *a* fluorescence in photosynthesis research, a brief background is provided (see the diagram). Photo-

synthesis is driven by two photosystems, I and II, leading to the oxidation of H_2O to molecular O_2 , to the reduction of a pyridine nucleotide, and to the production of ATP (7). Chlorophyll *a* fluorescence is particu-

¹This work was supported by funds from the McKnight Interdisciplinary Photosynthesis Research Program, and in part by Biomedical Shared Instrument Grant #S10 RR02277 from National Institute of Health, which provided a flow cytometer/cell sorter to the Cell Science Laboratory, Biotechnology Center, University of Illinois.

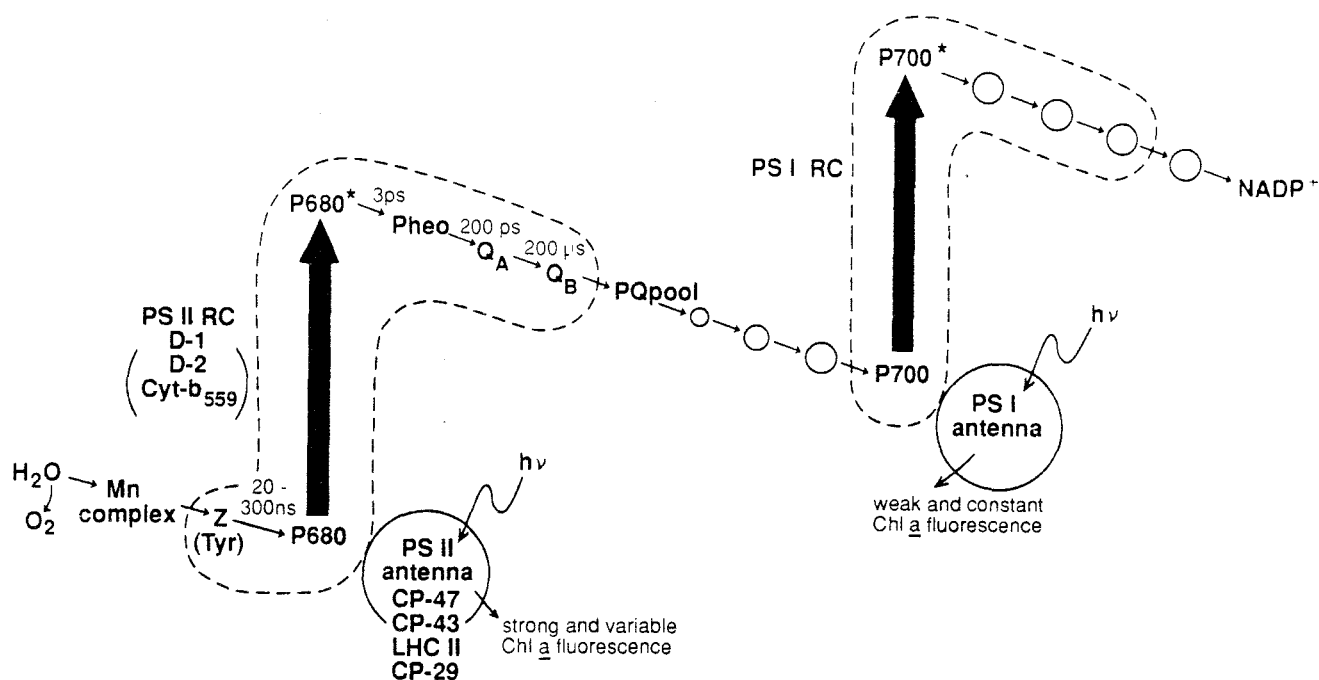


Diagram 1. A diagram showing the primary photochemical reactions and electron flow pathway in photosynthesis. Dashed lines encircle the components of the two multiprotein reaction center complexes, located in the thylakoid membrane. Electron flow is initiated when photons ($h\nu$) excite the reaction center chlorophyll a P680 (in photosystem II, PS II) and P700 (in PS I) and/or when excitons from the antenna pigments reach these centers. P680* and P700* indicate the first singlet excited states of P680 and P700. The first reaction of P680* is the conversion of excitonic energy into chemical energy (charge separation). This involves the formation of the cation P680⁺ and the anion pheophytin⁻ (Pheo⁻) within 3 ps. The P680⁺ recovers its lost electron from Z (tyrosine-160 of the D₁ polypeptide of PS II). The positive charge on Z is then transferred to an Mn-complex located on the luminal portions of the D₁ and D₂ polypeptides of PS II. The Pheo⁻ delivers the extra electron to a bound plastoquinone electron

acceptor Q_A. The reduced Q_A transfers its electron to another plastoquinone electron acceptor Q_B, strongly bound only in its semiquinone form Q_B⁻. After two turnovers of the reaction center P680, Q_BH₂ exchanges with the plastoquinone (PQ) pool. After four turnovers of the reaction center P680, the Mn complex accumulates four positive charges and oxidizes H₂O to molecular O₂ and releases 4 H⁺s. Plastoquinol formed in PS II reduces oxidized P700 (P700⁺), formed in PS I RC (reaction center), via several intermediates. The names and details of only PS II (not PS I) intermediates are included here since most of chlorophyll a (Chl a) fluorescence at room temperature originates in PS II. Furthermore, under normal conditions, only Chl a fluorescence of PS II varies with changes in photochemistry. CP-47, CP-43, LHC-II, and CP-29 stand for pigment-protein antenna complexes of PS II. The role of cytochrome $b559$ (Cyt $b559$) is unknown.

larly sensitive to the functioning of photosystem II (PSII). In PS II, the water-plastoquinone oxido-reductase, light absorption leads to charge separation at the reaction center within 3 ps: the reaction center Chl a P680 is oxidized and the reaction center pheophytin (Pheo) is reduced (12). This is followed by electron flow from the reduced pheo to a tightly bound plastoquinone Q_A within 200 ps, and then from reduced Q_A to a loosely bound plastoquinone Q_B within 200 μs. The oxidized P680 is reduced by a manganese complex viz Z (a tyrosine residue). After two turnovers of the reaction center, Q_B is double reduced and forms a plastoquinol molecule, and after four turnovers of the reaction center, H₂O is oxidized to molecular O₂ (12). The PS II is located on the thylakoid membrane and is composed of a reaction center protein complex (D1/D2/cytochrome $b559$ complex), several pigment-protein complexes that serve as antenna complexes (LHC-II, CP-29, CP-43, and CP-47), and non-pigmented protein complexes that are involved in electron flow. Most of the chlorophyll a

fluorescence at room temperature originates in the antenna complexes of PS II, primarily in CP-43 and CP-47 (11,25). Chlorophyll a fluorescence is high when Q_A in the reaction center is in the reduced state and low when Q_A is in the oxidized state (8). When present, P680⁺ and Pheo⁻ may act as quenchers of Chl a fluorescence. This Chl a fluorescence emission is in competition with many other processes in chloroplasts (7,8,11). It is expected that many analytical and separation techniques (e.g., selection of herbicide-resistant strains) may be useful in photosynthesis research based upon the analysis of Chl a fluorescence by flow cytometry.

Ashcroft et al. (3) reported the first flow cytometric measurements in intact chloroplasts and thylakoid membrane preparations from spinach and maize. Intact chloroplast preparations could be subdivided into at least two subgroups based on their light scatter and fluorescence characteristics. Ashcroft et al. (3) concluded that single-laser-based flow cytometry mea-

ures the constant component of Chl *a* fluorescence, F_0 . This is, obviously, possible in a flowing system if the time of exposure is short (20). But, there was no evidence for this conclusion; furthermore, the observed dependence of Chl *a* fluorescence on the concentration of ferricyanide, an electron acceptor, questions the conclusion about the F_0 nature of fluorescence. A thorough study and a clear explanation for flow cytometry Chl *a* fluorescence data are necessary before this technique can be applied for measurements on photosynthetic samples on a wider scale. F_0 is a measure of the minimum Chl *a* fluorescence level when photochemical reaction rate is maximum and reaction centers of PS II are assumed to be open (see, e.g., ref. 11).

The relative quantum yield of Chl *a* fluorescence (ϕ_f), in the absence of a barrier to exciton migration, is best expressed (29) by the following equation:

$$\phi_f = k_f / (k_f + k_t + k_h + k[Q]) \quad (1)$$

where k_f , k_t , and k_h are the rate constants for fluorescence, excitation energy transfer, and heat loss, and $k[Q]$ is the term representing the quenching process by intrinsic or extrinsic quencher. When only intrinsic quencher exists, this term is written as $k[Q_A]$. In the absence of electron flow inhibitors and in weak light, all Q_A exists in the oxidized form. Thus $k[Q_A]$ becomes maximum and a minimum fluorescence quantum yield level, F_0 , is reached. This fluorescence level is affected mostly by changes in excitation energy transfer.

At high light intensities, when all Q_A molecules are Q_A^- , $k[Q_A]$ approaches zero and ϕ_f approaches a maximum value (F_{max}). Furthermore, addition of electron transport inhibitors, which inhibit beyond Q_A , moves the equilibrium to favor the formation of Q_A^- . Then $k[Q_A]$ decreases and the quantum yield of Chl *a* fluorescence (F) increases. In the presence of a saturating concentration of an inhibitor diuron, the maximum fluorescence quantum yield, F_{max} , is reached; here also, $k[Q]$ in equation (1) approaches zero. The variable fluorescence, i.e., the difference between F (or F_{max}) and F_0 , is affected mostly by changes in photochemistry.

Since fluorescence intensity is one of the major parameters measured by a flow cytometer, it is necessary to reexamine the question of whether the instrument measures F_0 in photosynthetic samples. We show in this paper that the Chl *a* fluorescence being monitored by the single-laser-based flow cytometer is the maximum fluorescence when the PS II reaction centers are locked in the Q_A^- state. In addition, comparison of measurements on the quenching of Chl *a* fluorescence by various quinones (p-benzoquinone, phenyl-benzoquinone, and 2,5-dibromo-3-methyl-6-isopropyl-benzoquinone, DBMIB) with those obtained by Karukstis et al. (15-17), using conventional methods, suggests that flow cytometry gives different and unique results due to the high intensity used; it is suggested here that this is due to higher rate of reduction of quinones than equilibration with the bulk quinones.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea*) thylakoids were prepared as previously described by Eaton-Rye and Govindjee (10). Thylakoids were stored in liquid nitrogen and thawed immediately prior to experiments. Chlorophyll *a* concentrations were determined according to ref. 2. The chlorophyll concentration for fluorescence measurements was about 20 μM in flow cytometry, 10 μM in the double flash fluorescence instrument (which measures the decay of Q_A^- to Q_A), and 40 μM in the fluorescence transient experiments (which measure F_0 and F_{max}).

A Coulter EPICS™ 751 single-laser-based flow cytometer was used in this work. This system is composed of the EPICS™ V Multiparameter Sensor and the MDADS (Multiparameter Data Acquisition and Display System). The sensor unit consists of hardware associated with sample flow, laser excitation, and fluorescence detection of the single-subpopulation suspensions. The single-laser excitation was achieved with a 5 W argon ion laser (Coherent, Palo Alto, CA) tuned to 488 nm with power output at 100 mW. Right-angle fluorescence, passed through a red cutoff Corning glass CS 2-58, was collected by an RCA 4526 photomultiplier tube. Forward and 90° light-scattering signals were collected by a photodiode and a Hamamatsu 72271-01 photomultiplier, respectively. Fluorescence pulse signals from each particle were integrated and processed through either a linear amplifier (IRFL) or a logarithmic amplifier (LIRFL) and then passed to the computer for digitization, display and storage. The relevant 256 channel frequency distribution histogram spans a three decade log scale when the log amplifier is used. Alignment and calibration were achieved by using fluorescent microspheres. The light intensity of the laser beam was calculated to be $4 \times 10^7 \text{ W m}^{-2}$ with a Coherent radiometer. The number of absorbed photons in the sample was about 10^6 and the light was polarized. The flow rate for the sample was 10 m/s.

The Chl *a* fluorescence of the sample was also measured, after a weak xenon flash, by a conventional double-flash fluorometer (10). Chl *a* fluorescence transients, i.e., changes in Chl *a* fluorescence from F_0 to the F_{max} in continuous light, were measured by using a laboratory-built spectrofluorometer (5). The exciting light was provided by a Kodak 4200 projector with the light filtered by two Corning blue filters (CS 4-71 and CS 5-56). Fluorescence emission was detected by a S-20 photomultiplier (EMI 9558B) through a Bausch and Lomb monochromator and a Corning red filter CS 2-61. The light intensity of the exciting light, measured with a YSI-Kettering Model 65 Radiometer (Yellow Springs, Ohio), was $2 \times 10^2 \text{ W m}^{-2}$.

RESULTS

Scattering and Fluorescence Measurements

Figure 1 displays the typical flow cytometry data obtained from measurements of spinach thylakoids.

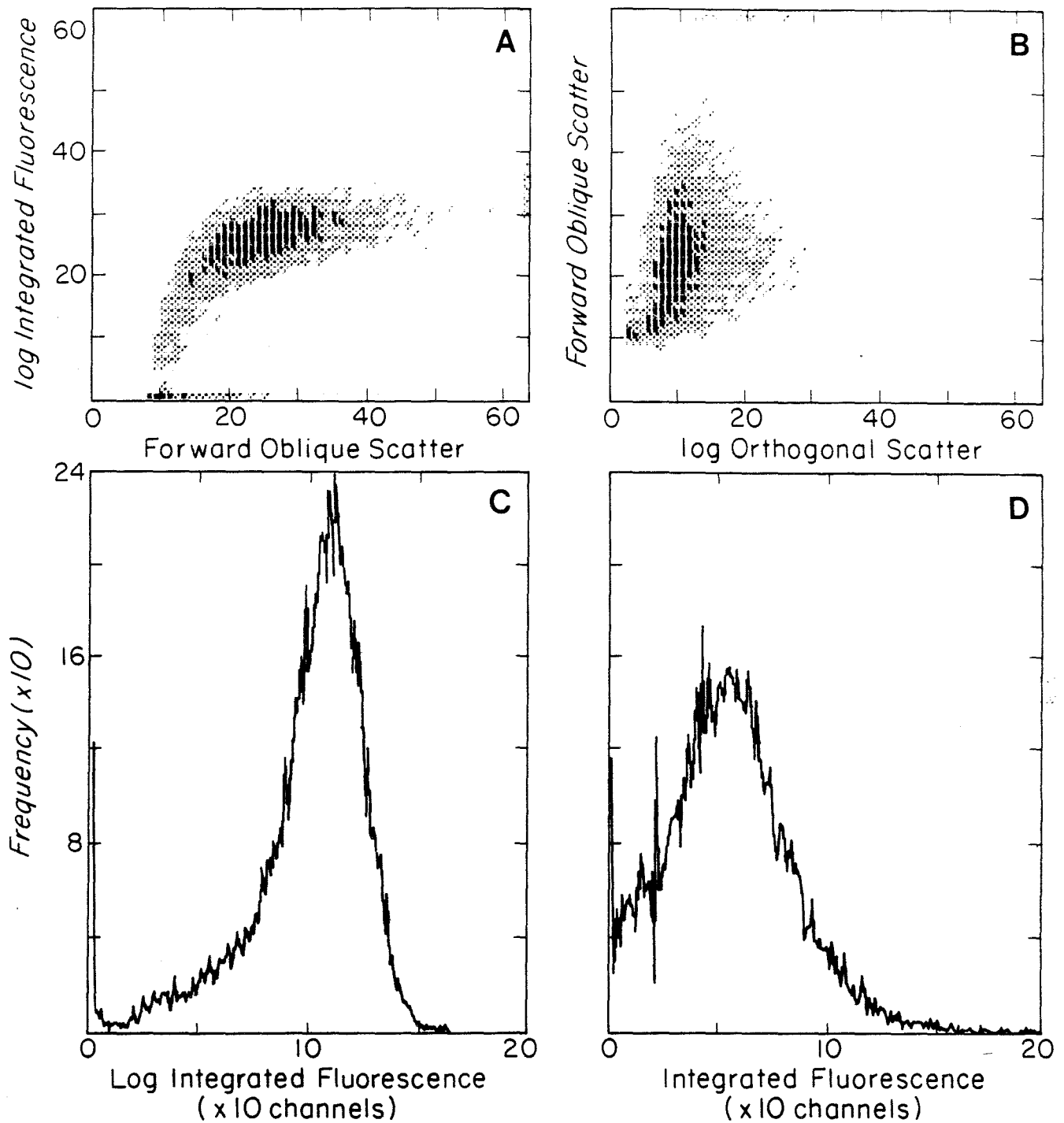


FIG. 1. Two-dimensional contour frequency distributions (histograms) for spinach thylakoids. **Panel A:** Logarithmic integrated Chl *a* fluorescence as a function of forward oblique scatter (related to the size of the object). **Panel B:** Forward oblique scatter as a function of logarithmic orthogonal scatter (related to internal structure of the

object). **Panels C and D:** Subpopulation frequency as a function of logarithmic integrated fluorescence and integrated fluorescence, respectively. Histograms show only one major population for spinach thylakoid membrane preparations.

Data were plotted as two-dimensional frequency distributions (histograms). Panel A shows logarithmic integrated fluorescence as a function of forward oblique

scatter. Forward scatter measurements are somewhat related to the size and the refractive index of the organelle. Panel B shows the forward oblique scatter as a

function of logarithmic orthogonal scatter. Orthogonal scatter is related to the internal structure of the organelle. Panels C and D display the population frequency data as functions of logarithmic integrated fluorescence and integrated fluorescence, respectively. Only one major population was shown on these histograms for spinach thylakoid membrane preparations. These data are consistent with those of Ashcroft et al. (3).

Fluorescence is linearly related to the concentration of the chromophore in fluorescence measurements (22). In the flow cytometer, the same level of fluorescence yield was maintained when the thylakoid concentration, in terms of Chl *a* concentration (5 to 500 $\mu\text{g/ml}$), was changed. This was as expected since by design the flow cytometry measurements indicate the fluorescence signal emitted by each particle, and the final fluorescence histogram represents a distribution plot from all the particles analyzed. Thus, this value is independent of thylakoid concentration within limits, as observed.

Source of fluorescence. The argon ion laser, used in flow cytometry experiments as the exciting source, was tuned to 488 nm. To ensure that there was no interference of scattered laser light, a 475 nm-long wavelength pass filter and a 530 nm short wavelength pass filter were placed in the front of the photomultiplier. If there was any excitation light scattered off the thylakoid suspension into the photomultiplier, a signal was expected. Since no signal was detected, this implied that scattering of the laser beam from the sample would not contribute to our measurements. Experiments were performed to determine whether the source of measured fluorescence was indeed Chl *a*. First, a partially bleached chloroplast suspension was prepared for comparison. In thylakoids in which 75–80% of the chlorophyll was extracted by 80% acetone, the fluorescence signal was one fourth of that of the control, showing a qualitative relationship between the chlorophyll content and the fluorescence signal. This indicates that the measured fluorescence signal in flow cytometry is chlorophyll fluorescence. Second, a red filter (Corning CS 2-58) was placed in front of the photomultiplier to cut off all the spectral signals shorter than 640 nm. Chlorophyll *a* fluorescence *in vivo* peaks at 685 nm (see, e.g., ref. 11,25). The observed signal with and without CS 2-58 was of the same amplitude, showing consistency with it being Chl *a* fluorescence. In all further experiments, the CS 2-58 filter was used.

Effects of heat treatment. Heat treatment influences the initial fluorescence F_i and the maximum fluorescence F_{max} of Chl *a* differently (28). Lavorel (20) had earlier discovered the heat-induced rise in initial Chl *a* fluorescence with a fluorometer. This heat-induced fluorescence increase was explained to reflect changes at the pigment level. Schreiber and Armond (28), who had also used a conventional fluorometer, showed that increasing the incubating temperature from 25 to 55°C (time, 5 min) produced a threefold in-

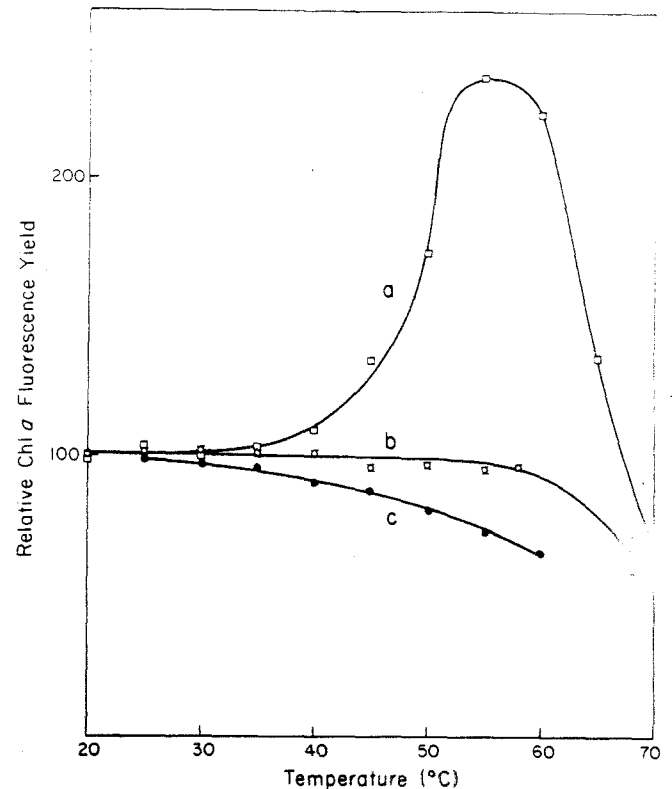


FIG. 2. Temperature dependence of Chl *a* fluorescence of spinach thylakoids. **Curve a:** Measured at low light intensity (sampling 1% of the centers) for initial Chl *a* fluorescence (F_i) by a double-flash fluorometer. **Curve b:** Measured by Coulter EPICS™ 751 single-laser-based flow cytometer. **Curve c:** Measured by Schreiber and Armond (28) for F_{max} , excited at 480 nm. All fluorescence data have been normalized to 100 at 20°C. Flow cytometer data are similar to those of F_{max} , not F_i .

crease in the initial Chl *a* fluorescence yield (F_i). Above 60°C, fluorescence decreased below the level seen at room temperature. The increase in fluorescence was explained (28) by a block of excitation energy migration in the pathway from the antenna pigments to the PS II reaction centers, i.e., a decrease in k_t in equation (1). This block was suggested to be related to a heat-induced structural change of pigments and thylakoid organization. On the other hand, a decrease in fluorescence was explained by Sane et al. (27) to be due to a change from the fluorescent *state I* to a weakly fluorescent *state II*. Recently, J. Cao and Govindjee (6) have suggested that most of the heat-induced fluorescence rise is not at the true F_0 level but at a fluorescence level above F_0 , perhaps at the so-called "I" level, the intermediate level of Chl *a* fluorescence (F_I) (11). The increase of fluorescence found at 55°C was suggested to be due to an increase in the concentration of "inactive" PS II centers where reoxidation of Q_A^- is slowed. In contrast to results on F_i , heat treatment caused only a decrease in fluorescence intensity on F_{max} (28) (see trace c in Fig. 2). When we measured Chl *a* fluores-

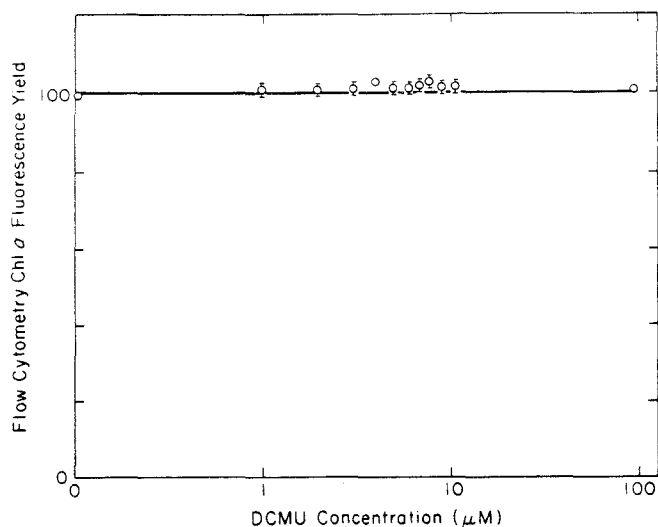


FIG. 3. Chl *a* fluorescence intensity of spinach thylakoids, measured by flow cytometry, as a function of DCMU concentration. Cytometry Chl *a* fluorescence data show that it is independent of DCMU concentration up to 100 μM .

cence yield in heat-incubated chloroplasts by using single-laser-based flow cytometry, the fluorescence remained unchanged until 60°C and then decreased (trace b in Fig. 2). However, when the initial Chl *a* fluorescence (F_i) of heat-treated chloroplasts was measured by weak light in the double-flash instrument, the rise was found in the 40 to 55°C range (trace a in Fig. 2). The temperature dependence of the flow cytometry result (trace b) is totally different from that for the initial fluorescence (trace a), but similar to that for F_{max} (trace c). Thus, the flow-cytometer-induced Chl *a* fluorescence is consistent with it being F_{max} . The quantitative difference between our observed results (trace b) and that of Schreiber and Armond (trace c) may be due to differences in samples used as well as to differences in light intensity, the latter being much higher for trace b.

Effect of diuron concentration. DCMU (3-(3,4)-dichlorophenyl-1,1, dimethyl urea), or diuron, inhibits photosynthetic electron transport by occupying the binding site of Q_B . Since Q_B is the secondary quinone electron acceptor of PS II, the reoxidation of Q_A^- is prevented (33). If the measuring light is very weak, the addition of herbicide should not influence the fluorescence yield at true F_0 . Similarly, in bright actinic light, when fluorescence reaches F_{max} due to the blockage in reoxidation of Q_A^- , the addition of herbicide should not influence the fluorescence yield either (see equation 1; cf. ref. 8). However, at weak to moderate light intensities an increase of [DCMU] from 1 to 10 μM should lead to a severalfold increase in Chl *a* fluorescence intensity. Since in our flow cytometric Chl *a* fluorescence experiment, the light intensity is not weak and since the addition of 1 to 100 μM DCMU induced no change in Chl *a* fluorescence (Fig. 3), the single-laser-based

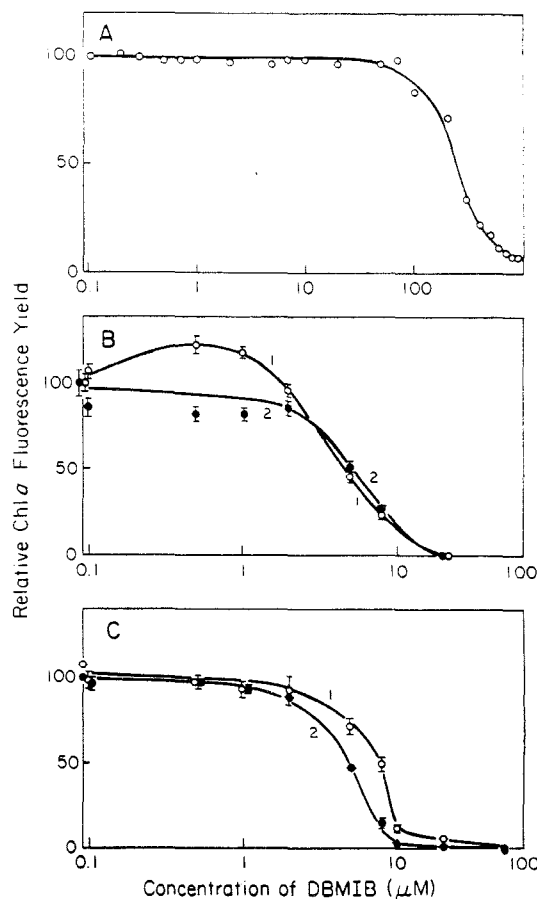


FIG. 4. DBMIB titration graphs of Chl *a* fluorescence intensity under different conditions for spinach thylakoids. The curve in panel A was measured with flow cytometer (light intensity $4 \times 10^7 \text{ W m}^{-2}$). Curves 1 in panel B (initial Chl *a* fluorescence, close to F_0) and in panel C (Chl *a* fluorescence measured at 17 sec, close to F_{max}) were measured with $2 \times 10^2 \text{ W m}^{-2}$ light intensity; and curves 2 were measured with 12 W m^{-2} . Note the difference in the abscissa scale between panel A and the other two.

flow cytometer must measure F_{max} , not a fluorescence level elicited by weak-to-moderate light.

Quenching by Extrinsic Quenchers

Fluorescence quenching is a general property of oxidized quinones (1). In the present study, three different fluorescence quenchers, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), p-benzoquinone, and phenyl-benzoquinone, were selected to further characterize the properties of Chl *a* fluorescence measured by flow cytometry. DBMIB is an inhibitor that usually acts at the cytochrome b_6/f complex in a photosynthetic electron pathway (32). The oxidized form of DBMIB is more effective in fluorescence quenching than that of the other two quinones. A DBMIB titration graph obtained from flow cytometry Chl *a* fluorescence measurements is shown in Figure 4A; this can be compared with Kitajima and Butler's data (14) on F_0 and F_{max} obtained by conventional fluorometry. Although

DBMIB displayed quenching in our flow cytometry measurements, the quenching effect of the same concentration of DBMIB was weaker than or absent relative to that observed by Kitajima and Butler for F_0 and F_{max} . DBMIB concentrations for 50% quenching of Chl *a* fluorescence are 2×10^{-4} M for flow cytometry (this paper), 2×10^{-5} for F_0 , and 1.1×10^{-5} M for F_{max} (14). The DBMIB concentration at 50% quenching differs between the two types of experiments by more than one order of magnitude. We repeated these experiment with a double-flash fluorometer (10); our results (data not shown) were similar to that obtained in ref. 14. One possible explanation for the apparently contradictory data is the extremely high incident intensity (4×10^7 W m^{-2}) of the laser beam, which is five orders of magnitude stronger than that of the lamp used in the conventional fluorometer (2×10^2 W m^{-2}). In strong light, the photoreduction rate of DBMIB must be much larger than the rate of equilibration between the few reduced molecules of DBMIB and the large number of oxidized DBMIB molecules in the medium. Thus the effective amount of oxidized DBMIB in the vicinity of Chl *a* molecules becomes less in the laser beam of the cytometry instrument than in the conventional fluorometers. The decrease in the amount of DBMIB, however, must be a local effect within the thylakoid since plenty of DBMIB is present in the suspension medium. The decrease in the effective amount of localized oxidized form of quinone analogues in bright light induces the shift of its titration graph. Experiments on Chl *a* fluorescence transient were done to test the light-intensity dependence idea. By measuring Chl *a* fluorescence transient, the initial fluorescence, (F_i , Fig. 4B), and the Chl *a* fluorescence at 17 s after the commencement of illumination, F_{17s} , close to F_{max} (Fig. 4C), were obtained. DBMIB titration graphs were plotted at two different light intensities (traces 1 at 2×10^2 W m^{-2} and 2 at 12 W m^{-2} , respectively). By decreasing the intensity of illuminating light, a shift of the DBMIB titration graph towards the low concentration side was found for F_{17s} (Fig. 4C). In contrast, the titration graph for F_i did not show any light-intensity-dependent shift (Fig. 4B). This indicates that the light-intensity dependence of the quinone quenching is related to the property of F_{max} , not F_i . This supports our conclusion that the fluorescence we measure with cytometry is likely to have F_{max} character, but not that of initial fluorescence. Although the explanation of the difference in the fluorescence intensity between the samples excited by high and low intensity (curves 1 and 2 in Fig. 4B) requires further study, it may be due to a greater reduction rate of Q_A to Q_A^- in curve 1.

Figure 5 displays the p-benzoquinone titration graphs of the Chl *a* fluorescence measured by flow cytometry (panel A), by another fluorometer for fluorescence close to F_0 (panel B), and for F_{17s} (panel C) respectively. Similar titration graphs are displayed in Figure 6 for phenyl-benzoquinone. When the intensity of the illuminating light was reduced from 4×10^7

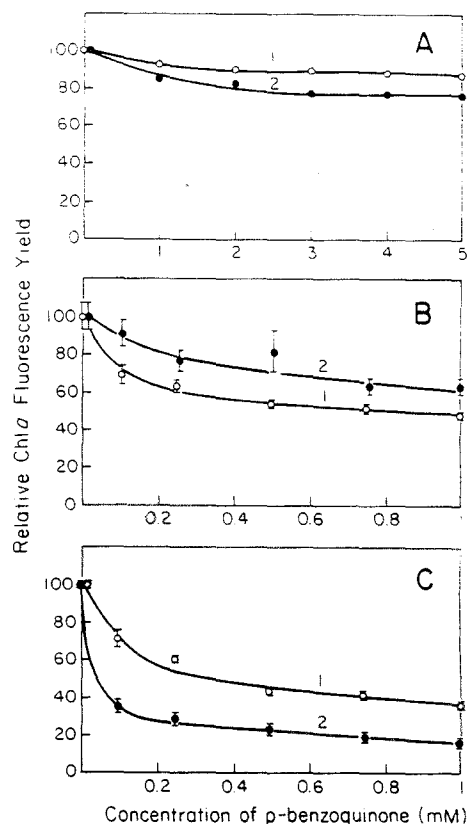


FIG. 5. Para-benzoquinone titration graphs of Chl *a* fluorescence intensity under different conditions for spinach thylakoids. Curves 1 and 2 in panel A (flow cytometry) were measured with incident light intensity of 4×10^7 or 4×10^6 W m^{-2} , respectively. Curves 1 in panels B (initial Chl *a* fluorescence, close to F_0) and C (Chl *a* fluorescence measured at 17 s, close to F_{max}) were measured with 2×10^2 W m^{-2} light intensity, but curves 2 were measured with 12 W m^{-2} . Note the difference in the abscissa scale between panel A and the other two.

(trace 1) to 4×10^6 W m^{-2} (trace 2) in panels A and from 2×10^2 (trace 1) to 12 W m^{-2} (trace 2) in panels B and C, the quenching by the same concentration of quinone on the Chl *a* fluorescence increased (see panels A and C). These changes brought about shifts in the quencher titration graphs. In agreement with data in Figure 4B, no shift was found for the titration graphs for the initial fluorescence (F_i) in panel B (Fig. 6). The above results also support the conclusion that the fluorescence measured with the flow cytometer is likely to have F_{max} character. The opposite result on the intensity dependence on F_i in Figure 5B remains unexplained. Trace 3 in Figure 6A is plotted from Ashcroft et al.'s data for phenylbenzoquinone. The light intensity used in that experiment was 10 m mol $m^{-2}s^{-1}$, at 476 nm.

To confirm the existence of the diffusion equilibrium (29) between the oxidized and the reduced forms of quinone molecules in our experimental system, an experiment changing the viscosity of the suspension medium was designed. Figure 7 displays the p-benzoquinone titration graphs of Chl *a* fluorescence measured with

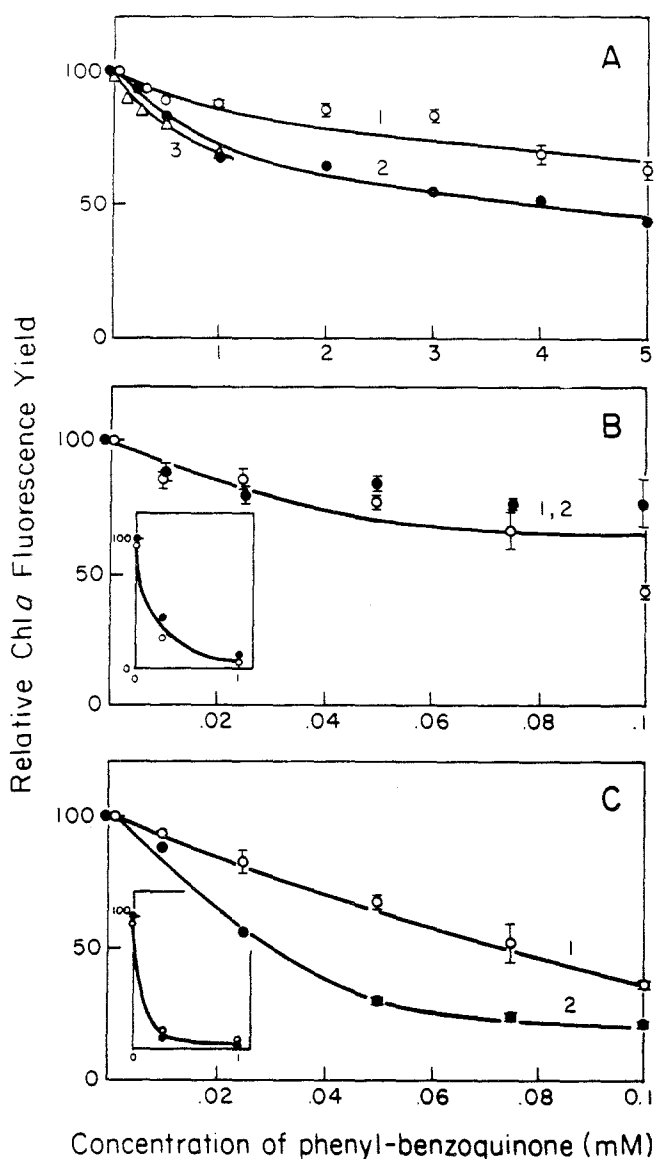


FIG. 6. Phenyl-benzoquinone titration graphs of Chl *a* fluorescence intensity under different conditions for spinach thylakoids. Experimental details are as in Figure 5. Note the difference in the abscissa scale between **panel A** and the other two. For convenience of comparison with Figure 5, **insets in B and C** have the same concentration scale as the scales in Figure 5B and 5C.

conventional fluorometry when the viscosity was changed. Panel A is for F_i and panel B is for F_{17s} . Increasing the viscosity of the suspension medium (replacing 50% of the suspension medium by glycerol, traces 2) did not affect the trace for F_i but decreased the quenching activity of the same concentration of the quinone on F_{17s} . This implies that F_{max} may be influenced by the diffusion rate of quinones across the thylakoid membranes.

DISCUSSION

In conventional fluorescence measurements (21) decreasing the illumination time (t) instead of decreasing

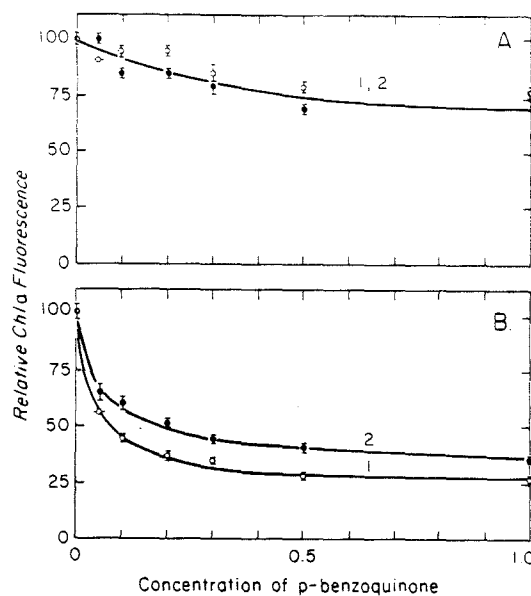


FIG. 7. Effect of changing viscosity on p-benzoquinone titration graphs for Chl *a* fluorescence from spinach thylakoids, measured with conventional fluorometry. Traces 1: control; traces 2: viscosity was increased by the addition of 50% glycerol. **Panel A** is for fluorescence close to F_0 and **panel B** is for F_{17s} . The fluorescence yield has been normalized to 100 when the quinone concentration is zero.

light intensity in the flowing suspension system reduces the photochemical activation in the reaction center. Thus, when the flow is fast and the illumination time short, one measures F_0 in the flow system. Stopping the flow, that is, increasing the time t , allows one to measure F_{max} . Since the flow cytometer is a flowing system, it is reasonable to expect that the Chl *a* fluorescence measured by flow cytometer may be F_0 . Mauzerall (24) reported that, in a dark-adapted sample, the fluorescence rise time is 25 ns. This rise was explained later to be due to the reduction of the oxidized form of the reaction center Chl *a* of PS II, P_{680}^+ , another Chl *a* fluorescence quencher of PS II (see, e.g., ref. 30). This rise time is, however, increased to approximately 0.3 μ s after a second illumination (30). The current picture is that this fast fluorescence rise measures electron flow from the electron donor Z to P_{680}^+ . In our flow cytometer, the time for a 1μ m particle to cross a length of the laser spot (16 μ m) is 1.7 μ s, which is much longer than 20 ns to 0.3 μ s for the reduction of P_{680}^+ and for the reduction of Q_A to Q_A^- (510 ps) (12,13). Thus, it is reasonable to expect that in a flow cytometer, we will be measuring F_{max} , not F_0 , especially because the laser light intensity is strong. Estimates based on several assumptions and considerations of energy losses lead us to conclude that about 10^6 photons were absorbed per 10^7 – 10^8 chlorophyll molecules present, i.e., about one photon per 10–100 chlorophyll molecules present. Thus, all reaction centers must undergo at least one turnover and the system must reach the F_{max} state.

At very high intensities, however, multiple hits in a single photosystem unit are commonly observed (see, e.g., ref. 18), leading to exciton-exciton annihilation. Therefore, increasing the intensity of the light source will decrease the relative quantum efficiency for sample fluorescence. Although high light intensity was applied in flow cytometry experiments, our data on light-intensity dependence (Figs. 5A, 6A) display an increase in Chl *a* fluorescence quantum yield when the laser light intensity is increased from 4×10^6 to 4×10^7 W m⁻², implying the absence of exciton-exciton annihilation process in spinach thylakoids flowing in the cytometer under our experimental conditions. Furthermore, the same experiment implies the absence of photoinhibitory phenomenon since photoinhibition 1) leads to reduction of variable Chl *a* fluorescence with increasing light intensity and 2) involves long-term (not 2.5 μ s exposure) chemical and physical changes (see, e.g., a review by Kyle and Ohad (19)).

The conclusion of Ashcroft et al. (3) that the flow cytometry Chl *a* fluorescence is F_0 was not supported. In hypotonically shocked chloroplasts, ferricyanide decreased (3) Chl *a* fluorescence significantly (by 50 to 75%); at F_0 , all Q_A should have been in the oxidized form and ferricyanide could not have further increased the concentration of Q_A and, thus, decreased the quantum yield of Chl *a* fluorescence. The absence of the effect of varying [DCMU] on fluorescence intensity cannot prove its F_0 character either (Fig. 3) (3). An independence of the effect of [DCMU] on fluorescence is also expected for F_{max} . Only when Chl *a* fluorescence is between F_0 and F_{max} should increasing [DCMU] cause an increase in its yield. High light intensity used in this flow cytometer is not expected to keep the fluorescence at the F_0 level. Thus, the independence of fluorescence yield on [DCMU] is considered consistent with the F_{max} character of fluorescence. Chl *a* fluorescence in weak light increases with temperature up to 55°C and then decreases (Fig. 2). This character was not observed in the Chl *a* fluorescence with a flow cytometer. Furthermore, a comparison of flow cytometry data with those obtained on F_{max} showed a similarity between the latter two, suggesting that the flow cytometer measures F_{max} , and not some level close to F_0 (Fig. 2).

All other experimental results can also be explained by the hypothesis that flow cytometry measures F_{max} . In our experimental system, there were two sources to quench Chl *a* fluorescence: 1) the oxidized form of Q_A and 2) the oxidized form of externally added quinone, which may come close to Chl *a* molecules. Due to the high light intensity used in flow cytometry, the rate constants of Q_A reduction and even that of the reduction of extrinsic quinones increase. High concentrations of Q_A^- and even reduced quinone analogues must have been created in samples illuminated with such a high-intensity beam. The equilibrium between Q_A and Q_A^- is determined by the [Q_B] and the equilibrium between the local oxidized form of quinone and the

Table 1
Quinone Concentrations Causing 50% Quenching of Chl *a* Fluorescence, in Spinach Thylakoids^a

	Cytometry fluorescence	F_{17s} (I_1)	F_{17s} (I_2)
p-benzoquinone	>>5mM	0.37mM	60 μ M
Phenyl-benzoquinone	>5mM	72 μ M	31 μ M
DBMIB	0.24mM	8 μ M	5 μ M

^aData were obtained by flow cytometry (light intensity 4×10^7 W m⁻²) and by a home-built fluorometer (5): F_{17s} at 2×10^2 W m⁻² (I_1) and F_{17s} at 12 W m⁻² (I_2).

bulk quinone. The viscosity experiment in Figure 7 confirmed that the diffusion rate of the quinone molecules has a significant effect on the fluorescence yield. Compared to the reduction rate of Q_A and quinone in strong light, the exchange rate of the reduced quinones with bulk quinone is expected to be rather slow. Therefore, in the flow cytometer used, almost all the Q_A and the "local" externally added quinone can be considered to exist in the reduced form. Thus, Chl *a* fluorescence yield reaches maximum even at high concentrations of externally added quinone quenchers when measured with a flow cytometer. However, when conventional fluorometers with lower light intensity were used, the equilibration of reduced quinones with bulk quinones allows more oxidized quinones to exist in the vicinity of Chl *a* and to quench fluorescence. Our results, in which decreasing light intensity increases the quenching effect of the same concentration of quinones in flow cytometry (Figs. 5A, 6A) and in conventional fluorometers (Figs. 4C, 5C, and 6C), confirm the above hypothesis. This change only occurs in the fluorescence close to F_{max} . Therefore, in our cytometry experiment the fluorescence measures F_{max} , but not F_0 character.

There are two modes of action proposed to describe the quinone-induced fluorescence quenching. One (1,4,15) suggests that the added quinones function to dissipate excitation energy by interaction with either PS II light-harvesting chlorophyll-protein complexes or the PS II reaction center chlorophyll. An alternative mechanism (see, e.g., ref. 17) suggests that the added quinones compete with the intrinsic plastoquinone electron acceptor, Q_B , for the same or overlapping binding sites in a common binding domain on the D-1 protein, since many substituted quinones act as Q_B analogs. Data in Table 1 show the quenching capabilities of three quinones. In three different experiments, DBMIB quenched more Chl *a* fluorescence than the other two quinones at the same concentration (data not shown). The concentration of DBMIB, at which 50% quenching of Chl *a* fluorescence occurs, is much lower than that of the other two in all three cases (Table 1), implying that DBMIB is more accessible to Chl *a* or Q_B than the other two. Although qualitatively similar, quantitatively different results were obtained by Karukstis et al. (15). These differences are due to the unique conditions of flow cytometer data discussed ear-

lier: a larger rate of reduction of quinones as compared to the rate of exchange of these quinones with the bulk quinones.

The flow cytometry experiments are useful for photosynthesis research. Progress in this area potentially depends on the correct analysis of intrinsic Chl *a* fluorescence. Therefore, information in this paper is valuable for further flow cytometry studies on photosynthetic samples related to plant biology or marine biology research. Dual-laser-based flow cytometry may also be useful (see ref. 3 for preliminary observation) since one can change the time interval between two flashes (actinic and measuring) and then measure changes in the fluorescence yield with time, thereby providing information on the time-dependent changes in the photochemical events of photosystem II.

ACKNOWLEDGMENTS

The authors thank Drs. Gregorio Weber and Jack Widholm for their useful suggestions.

LITERATURE CITED

1. Amesz J, Fork DC: Quenching of chlorophyll fluorescence by quinones in algae and chloroplasts. *Biochim Biophys Acta* 143:97-107, 1967.
2. Arnon DI: Copper enzyme in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol* 24:1-15, 1949.
3. Ashcroft RG, Preston C, Cleland RE, Critchley C: Flow cytometry of isolated chloroplasts and thylakoids. *Photobiochem Photobiophys* 13:1-14, 1986.
4. Berens SJ, Scheele J, Butler WJ, Magde D: Time-resolved fluorescence studies of spinach chloroplasts—evidence for the heterogeneous bipartite model. *Photochem Photobiol* 42:51-57, 1985.
5. Blubaugh DJ: The Mechanism of Bicarbonate Activation of Plastoquinone Reduction in Photosystem II of Photosynthesis. Ph.D. Thesis, Biology, University of Illinois at Urbana-Champaign, 1987.
6. Cao J, Govindjee: Chlorophyll *a* fluorescence transient as an indicator of active and inactive photosystem II in thylakoid membranes. *Biochim Biophys Acta*.
7. Duysens LNM: Introduction to (bacterio)chlorophyll emission: A historical perspective. In: *Light Emission by Plants and Bacteria*, Govindjee, Amesz J, Fork DC (eds). Academic Press, Orlando, 1986, pp 3-28.
8. Duysens LMN, Sweers HE: Mechanism of two photo-chemical reactions in algae as studied by means of fluorescence. In: *Studies on Microalgae and Photosynthetic Bacteria*, Japanese Society of Plant Physiologists (eds). University of Tokyo Press, Tokyo, 1963, pp 353-372.
9. Dive C, Workman P, Watson JV: Improved methodology for intracellular enzyme reaction and inhibition kinetics by flow cytometry. *Cytometry* 8:552-561, 1987.
10. Eaton-Rye JJ, Govindjee: Electron transfer through the quinone acceptor complex of photosystem II in bicarbonate depleted spinach thylakoid membranes as a function of actinic flash number and frequency. *Biochim Biophys Acta* 935:237-247, 1988.
11. Govindjee, Satoh K: Fluorescence properties of chlorophyll *b*- and chlorophyll *c*-containing algae. In: *Light Emission by Plants and Bacteria*, Govindjee, Amesz J, Fork DC (eds). Academic Press, Orlando, 1986, pp 497-538.
12. Govindjee, Wasielewski M: Photosystem II: From a femtosecond to a millisecond. In: *Photosynthesis*, Briggs WR (ed). Alan R. Liss, Inc., New York, 1989, pp 71-103.
13. Schatz GH, Brock H, Holzwarth AR: Picosecond kinetics of fluorescence and absorbance changes in photosystem II excited at low photon density. *Proc Natl Acad Sci USA* 84:8414-8418, 1987.
14. Kitajima M, Butler WL: Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. *Biochim Biophys Acta* 376:105-115, 1975.
15. Karukstis KK, Boegeman SC, Fruetel JA, Gruber SM, Terris MH: Multivariate analysis of photosystem II quenching by substituted benzoquinones and naphthoquinones. *Biochim Biophys Acta* 891:256-264, 1987.
16. Karukstis KK, Gruber SM, Fruetel JA, Boegeman SC: Quenching of chlorophyll fluorescence by substituted anthraquinones. *Biochim Biophys Acta* 932:84-90, 1988.
17. Karukstis KK, Monell CR: Reversal of quinone-induced chlorophyll fluorescence quenching. *Biochim Biophys Acta* 973:124-130, 1989.
18. Knox RS: Photosynthetic efficiency and exciton transfer and trapping. In: *Primary Processes of Photosynthesis*, Barber J (ed). Elsevier, New York, 1977, pp 55-97.
19. Kyle DJ, Ohad I: The mechanism of photoinhibition in higher plants and algae. *Encyclopedia Plant Physiol New Ser* 19:468-475, 1986.
20. Lavorel J: On a relation between fluorescence and luminescence in photosynthetic systems. In: *Progress in Photosynthesis Research*, Vol. 2, Metzner H (ed). H. Laupp, Tübingen, 1969, pp 883-898.
21. Lavorel J: Heterogenèité de la chlorophylle in vivo. I. Spectres d'émission de fluorescence. *Biochim Biophys Acta* 60:510-523, 1962.
22. Lavorel J, Breton J, Lutz M: Methodological principles of measurement of light by photosynthetic systems. In: *Light Emission by Plants and Bacteria*, Govindjee, Amesz J, Fork DC (eds). Academic Press, Orlando, 1986, pp 57-98.
23. Marti GE, Magruder L, Schuette WE, Gralnick HR: Flow cytometry analysis of platelet surface antigens. *Cytometry* 9:448-455, 1988.
24. Mauzerall D: Light-induced fluorescence changes in *Chlorella*, and the primary photoreactions for the production of oxygen. *Proc Natl Acad Sci USA* 69:1358-1362, 1972.
25. Murata N, Satoh K: Absorption and fluorescence emission by intact cells, chloroplasts, and chlorophyll-protein complexes. In: *Light Emission by Plants and Bacteria*, Govindjee, Amesz J, Fork DC (eds). Academic Press, Orlando, 1986, pp 137-160.
26. Papa S, Capitani S, Matteucci A, Vitale M, Santi P, Martelli AM, Maraldi NM, Manzoli FA: Flow cytometric analysis of isolated rat liver nuclei during growth. *Cytometry* 8:595-601, 1987.
27. Sane PV, Desai TS, Tataka VG, Govindjee: Heat induced reversible increase in photosystem I emission in algae, leaves and chloroplasts: Spectra, activity and relation to state changes. *Photosynthetica* 18:439-444, 1984.
28. Schreiber U, Armond PA: Heat-induced changes of chlorophyll fluorescence in isolated chloroplasts and related heat-damage at the pigment level. *Biochim Biophys Acta* 502:138-151, 1978.
29. Seely GR, Connolly JS: Fluorescence of photosynthetic pigments *in vitro*. In: *Light Emission by Plants and Bacteria*, Govindjee, Amesz J, Fork DC (eds). Academic Press, Orlando, 1986, pp 99-133.
30. Sonneveld A, Rademaker H, Duysens LNM: Chlorophyll *a* fluorescence as a monitor of nanosecond reduction of the photooxidized primary donor P-680⁺ of photosystem II. *Biochim Biophys Acta* 548:536-551, 1979.
31. Toppari J, Bishop PC, Parker JW, diZerega GS: DNA flow cytometric analysis of mouse seminiferous epithelium. *Cytometry* 9:456-462, 1988.
32. Trebst A, Harth E, Draber W: On a new inhibitor of photosynthetic electron-transport in isolated chloroplasts. *Z Naturforsch* 25:1157-1159, 1970.
33. Velthuys BR: Electron-dependent competition between plastoquinone and inhibitors for binding to photosystem II. *FEBS Lett* 126:277-281, 1981.