Photosynthesis Research **21**: 93–106, 1989.

Regular paper

Fluorescence characteristics of photoautotrophic soybean cells

C. Xu,[1] S. M. D. Rogers,[2] C. Goldstein,[2] J. M. Widholm[2] & Govindjee[1,3]
[1]*Departments of Physiology and Biophysics and* [3]*Plant Biology and* [2]*Department of Agronomy, University of Illinois at Urbana-Champaign, IL 61801, USA*

Received 3 June 1988; accepted in revised form 10 October 1988

Key words: higher plant photosynthesis, chlorophyll *a* fluorescence, plastoquinone pool, photoautotrophic soybean cell, diuron, atrazine, primary (quinone) electron acceptor, Q_B/Q_B^-, (soybean, spinach)

Abstract

We report here the first measurements on chlorophyll (Chl) *a* fluorescence characteristics of photoautotrophic soybean cells (cell lines SB-P and SBI-P). The cell fluorescence is free from severe distortion problems encountered in higher plant leaves. Chl *a* fluorescence spectra at 77 K show, after correction for the spectral sensitivity of the photomultiplier and the emission monochromator, peaks at 688, 696 and 745 nm, representing antenna systems of photosystem II-CP43 and CP47, and photosystem I, respectively. Calculations, based on the complementary area over the Chl *a* fluorescence induction curve, indicated a ratio of 6 of the mobile plastoquinone (including Q_B) to the primary stable electron acceptor, the bound plastoquinone Q_A. A ratio of one between the secondary stable electron acceptor, bound plastoquinone Q_B, and its reduced form Q_B^- was obtained by using a double flash technique. Owing to this ratio, the flash number dependence of the Chl *a* fluorescence showed a distinct period of four, implying a close relationship to the 'S' state of the oxygen evolution mechanism. Analysis of the Q_A^- reoxidation kinetics showed (1) the halftime of each of the major decay components ($\sim 300\,\mu$s fast and ~ 30 ms slow) increases with the increase of diuron and atrazine concentrations; and (2) the amplitudes of the fast and the slow components change in a complementary fashion, the fast component disappearing at high concentrations of the inhibitors. This implies that the inhibitors used are able to totally displace Q_B. In intact soybean cells, the relative amplitude of the 30 ms to 300 μs component is higher (40:60) than that in spinach chloroplasts (30:70), implying a larger contribution of the centers with unbound Q_B. SB-P and SBI-P soybean cells display a slightly different sensitivity of Q_A^- decay to inhibitors.

Abbreviations; CA - complementary area over fluorescence induction curve, Chl - chlorophyll, diuron: DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethylurea, F_m - maximum chlorophyll *a* fluorescence, F_0 - minimum chlorophyll *a* fluorescence, $F_v = F_t - F_0$, where F_v = variable chlorophyll *a* fluorescence, and F_t = chlorophyll *a* fluorescence at time *t*, PS II - photosystem II, Q_A - primary (plastoquinone) electron acceptor of PS II, Q_B - secondary (plastoquinone) electron acceptor of PS II, t_{50} - the time at which the concentration of reduced Q_A is 50% of that at its maximum value

Introduction

For several decades, Chlorophyll *a* (Chl *a*) fluorescence has been used as an intrinsic indicator of the photosynthetic reactions of isolated chloroplasts of green plants. Since Chl *a* fluorescence is in competition with photochemical reactions and other processes in chloroplasts, it has been widely used to monitor variations in photosynthetic activity (see, e.g., Duysens 1986).

Light-induced charge separation initiates the transport of reducing equivalents from the reaction center of photosystem II (PS II) to the plastoquinone (PQ) pool via a specially organized complex of two bound plastoquinones, Q_A and Q_B. Q_A is a one electron acceptor and is tightly bound, whereas Q_B is a two electron acceptor, and is loosely bound in the Q_B and Q_B^{2-} forms (Crofts and Wraight 1983). The Chl *a* fluorescence yield depends upon the redox state of Q_A, being high when the concentration of Q_A^- is high (Duysens and Sweers 1963). The size of the PQ pool can be measured by using Chl *a* fluorescence induction, i.e., fluorescence as a function of time of illumination. The area above the fluorescence induction curve (complementary area, CA) is proportional to the number of electrons accumulated in the light by the electron acceptor side of PS II and, therefore, is equivalent to the amount of PQ which has been reduced (Malkin and Kok 1966, Velthuys and Amesz 1974). Thus the measurements of CA, before and after the electron flow inhibition, have allowed estimates of the ratio of PQ pool size to Q_A. One can also measure, with a weak flash, the changes in the variable Chl *a* fluorescence yield correlated with the concentration of Q_A^-, after a strong saturating flash. From such measurements it is feasible to estimate the kinetics and equilibrium parameters for the reaction of Q_A^- with Q_B or Q_B^- when one or two electrons have accumulated from the reaction center of PS II, and then for the reactions of plastoquinone (PQ) and plastoquinol (PQH_2) at the Q_B binding site (see, e.g., Robinson and Crofts 1983, 1987, Taoka et al. 1983).

Up to now, most efforts have been put into the development of the methodology for Chl *a* fluorescence measurements with isolated chloroplasts from higher plants. Efforts are being made to use fluorescence in the study of photosynthetic phenomena in intact leaves (see Walker 1981, Sivak and Walker 1983, Walker et al. 1983, 1985). For example, state transition, energy transfer during the development of the photosynthetic apparatus, and the effects of physiological and environmental factors have been studied in intact leaves through Chl *a* fluorescence measurements (see, e.g., reviews by Briantais et al. 1986, Renger and Schreiber 1986). However, since intact leaves have higher absorbance and exhibit stronger scattering of incident light than isolated chloroplasts, their emission spectra are highly distorted due to reabsorption of fluorescence. In addition, kinetics of fluorescence are also distorted due to the light attenuation (Malkin et al. 1981); fluorescence kinetics from the interior cells are slower due to the lower light intensities, and, one measures, therefore, an average kinetics. The complexity of using intact leaves and the lack of direct biochemical data to compare with the results of fluorescence measurements make the interpretation of fluorescence in intact leaves less definitive than in isolated chloroplasts. Furthermore, the relationship of Chl *a* fluorescence to the mechanism by which plastoquinone acceptors work in intact leaves is still unresolved.

Recently, higher plant tissue culture techniques have been increasingly used for research in the areas of plant physiology, genetics and development. Now, rapidly growing photoautotrophic suspension cultures have become available for use in photosynthesis research using intact higher plant cells (Rogers et al. 1987, Xu et al. 1988). Thus Chl *a* fluorescence measurements can be used to study photosynthetic phenomena in higher plants by using suspensions of small cell clumps. Such cells are better suited for Chl *a* fluorescence measurements *in vivo* for higher plants because they can be used in dilute samples and, thus, will be relatively free of distortions encountered in leaf samples. Furthermore, it will be possible to compare their data with those from cyanobacteria, and lower plants such as red and green algae (Fork and Mohanty 1986, Govindjee and Satoh 1986).

In this paper, we present the first systematic Chl *a* fluorescence measurements of photoautotrophic soybean cells. We show that soybean cells possess a similar antenna system as that in chloroplasts of other higher plants. Furthermore, the ratio of the plastoquinone pool (including Q_B) to Q_A is 6 and the ratio between Q_B and Q_B^- after dark adaptation is one. Analyses of the kinetics of Q_A^- reoxidation suggests that the Q_B binding sites can be totally occupied by the electron transport inhibitors used. The detailed fluorescence study of soybean cells, presented here, suggests that it may be of use for the general application of fluorescence in plant physiology.

Materials and methods

The intact soybean (*Glycine max* Merr.) cell lines SB-P (*Corsoy*) and SBI-P (PI-437 833) were established by Horn et al. (1983) and Rogers and Widholm (1988), respectively. The cells were cultured in a KN^0 medium (Rogers et al. 1987), which contained in addition to inorganic salts (Murashige and Skoog 1962), Thiamine–HCl, kinetin and naphthalene acetic acid as the sole organic components (see Rogers and Widholm 1988). The cells were photoautotrophically cultured for 14 days at 28°C in a 5% CO_2 atmosphere under continuous light of 300 μmol photon $m^{-2} s^{-1}$, and were shaken at 130 rpm on a gyratory shaker. The pH of the KN^0 medium before cell growth was about 5.2 (or 7.0 with 5 mM Hepes buffer), but within 48 h the medium pH dropped to around 4.2, and then rose to 5.0 after 14 days (Horn et al. 1983).

After growth for three weeks under the illumination of a metal-halide lamp for 16 hours every day, the mature soybean (*Corsoy*) leaves were collected from the green house for fluorescence measurement.

The isolation of spinach (*Spinacia oleracia*) chloroplasts were performed as previously described by Eaton-Rye and Govindjee (1988).

Chlorophyll was extracted in 80% acetone (v/v) and its concentration was determined spectrophotometrically using the method of Arnon (1949). The cells were diluted in KN^0 medium to give a chlorophyll concentration of about 10 μg/ml in the double flash fluorescence experiment, and about 40 μg/ml in the fluorescence transient experiment.

Chlorophyll *a* fluorescence spectra and fluorescence transient measurements were made using a laboratory-built spectrofluorometer. 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, with a slitwidth of 3.3 nm, by a S-20 photomultiplier (EMI 9558B) through a Bausch and Lomb monochromator, protected from the exciting light by a red Corning filter (CS 2-61). Signals were stored and analyzed by Biomation 805 waveform recorder and a LSI-11 computer (see Blubaugh 1987). Other details are in the legend of Fig. 1.

Chlorophyll *a* fluorescence kinetics after single flashes of light were measured by an instrument

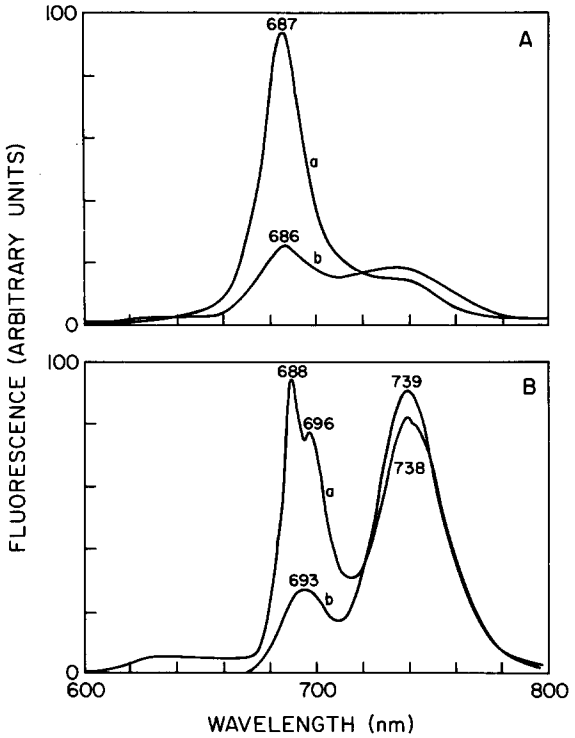

Fig. 1. Fluorescence emission spectra (from 600 to 800 nm) of SB-P cells (subfig. a) and soybean leaves (subfig. b) at (A) room temperature and (B) 77 K. Monochromator, Bausch and Lomb (focal length, 500 nm, f/5, grating size, 100 × 100 mm, blazed at 750 nm, linear dispersion, 3.3 nm/mm); photomultiplier, S-20, EMI 9558B. Spectra were normalized at 724 nm. Spectra, presented here, are not corrected for the spectral response of the monochromator and the photomultiplier. Wavelengths shown on the graph are for the peaks of emission.

described by Eaton-Rye (1987). Fresh soybean cell suspensions were diluted in a dark reaction vessel and dark-adapted at room temperature for 10 min. A flow cuvette was filled from this vat. Because the size of cells is larger than that of thylakoid membrane fragments, the gas pressure in the flow system had to be changed from that used for thylakoids (see Eaton-Rye 1987). The illumination volume of the sample was 0.6 ml. At the end of each measurement, the cuvette was emptied by reversing the pressure. Using a weak measuring flash, the level of Chl *a* fluorescence yield of the soybean cells was measured at 685 nm (10 nm bandwidth) by a EMI 9558A photomultiplier tube. An FX-124 flash lamp (EG and G, 2.5 μs duration at half-maximal peak height) was used as the saturating flash, where the measuring flash (2.5 μs duration, CS 4-96 filter) was provided by the 1539A Stroboslave (General

Radio) flash system. After a series of saturating flashes, the measuring flash could be fired at variable times under computer control. For further details, see Eaton-Rye (1987) and Eaton-Rye and Govindjee (1988).

The concentration of reduced Q_A was estimated from the above Chl *a* fluorescence data by using the following equation (Joliot and Joliot 1964; see Mathis and Paillotin 1981):

$$\frac{F(t) - F_0}{F_{max} - F_0} = \frac{(1 - p)q}{1 - pq} \quad (1)$$

where $F(t)$ is the Chl *a* fluorescence yield at time t, F_0 is the fluorescence yield when all Q_A is in the oxidized state, F_{max} is the maximum fluorescence yield when all Q_A is in the reduced state, p is the connection parameter or the probability of the intersystem energy transfer and q is the fraction of the closed reaction centers (i.e., $q = 1$, when Q_A^- is maximum). Here p was taken as 0.5 (Joliot and Joliot 1964) for calculations in our paper. Therefore, q can be represented by the following formula:

$$q = \frac{F(t) - F_0}{(F_{max} - F_0) - 0.5[F_{max} - F(t)]} \quad (2)$$

Apparent halftimes of $[Q_A^-]$ decay, labeled as t_{50}, are times at which $[Q_A^-]$ is 50% of maximum $[Q_A^-]$ (at $t = 0$), whereas all other $t_{\frac{1}{2}}$s, given together with their amplitudes, are obtained from plots of log of $[Q_A^-]$ as a function of time after evaluation into a fast and a slow component.

Results and discussion

Fluorescence spectra

Fluorescence spectroscopy is a powerful means to analyze the components of light harvesting systems as well as the energy transfer between them in higher plants and algae (see, e.g., Briantais et al. 1986, Fork and Mohanty 1986, Govindjee and Satoh 1986). In order to minimize the influence of scattering and reabsorption of fluorescence in clumps of soybean cells, the cell suspension was filtered with cheese cloth to remove all the larger clumps. Figure 1 displays the fluorescence emission spectra of SB-P soybean cells at room temperature (A) and 77 K (B). At room temperature, reabsorption of fluorescence at 687 nm is dramatically reduced here (curve a) as compared to the soybean leaf (curve b). This is evident from the relatively high ratio (5.5) of fluorescence at 687 nm to that at 740 nm in cell suspensions in comparison to a low ratio (1.7) in leaves. At 77 K, the ratio of fluorescence at 688 nm to that at 740 nm changed from a value of 1.0 in the cell suspension to 0.3 in leaves. Furthermore, the separation of the emission bands at 688 and 696 nm was obliterated in the leaves at the 3.3 nm slitwidths used here. The 688 nm band is reabsorbed more than the 696 nm band. A single band at 693 nm was observed.

The fluorescence emission spectra, shown in Fig. 1, have not been corrected for the spectral sensitivity of the photomultiplier and the monochromator. This fluorescence emission spectrum of the SB-P cells obtained at room temperature has, however, the usual peak at 687 nm (F686), because the instrumental spectral response is almost flat in this region. F686 is universally found in all oxygenic photosynthetic samples (Murata and Satoh 1986); and, it has been suggested to originate in the antenna Chl *a* molecules of PS II. At the lower temperature (77 K), the uncorrected fluorescence emission spectrum of the SB-P cells showed three bands near 688 (F686), 696 (F695) and 739 (F740) nm. Corrections for the instrumental spectral response gave almost the same locations for these three peaks: at 688, 698 and 745 nm. Both F686 and F695 bands originate in the antenna Chl *a* molecules of PS II, the former in the so-called CP-43 (Chl *a*-protein complex of 43 kilodalton mass), and the latter in the so-called CP-47 (Chl *a*-protein complex of 47 kilodalton mass), the antenna complexes that feed energy to the reaction center II complex (Murata and Satoh 1986; for a review on pigment-protein complexes, see Green 1988). It is now generally agreed that F740 at 77 K belongs to photosystem I (PS I) complexes. The emission spectrum obtained for soybean cells is very similar to that found in chloroplasts from higher plants (Govindjee and Yang 1966). In green algae, the F740 band has two maxima at 717 and 725 nm (Cho and Govindjee 1970); soybean cells also show a double band (see Fig. 1B), but the bands are shifted to higher wavelengths, as expected for higher plants. This complex long-wavelength band is at still shorter wavelengths (712–720 nm) in cyanobacteria and red algae (Fork

and Mohanty 1986). The above result with the photoautotrophic soybean cells suggests that its antenna system is similar, as expected, to those of chloroplasts from other higher plants. Similar results have now been found by the authors in other photoautotrophic cell lines from intact tobacco, Datura and cotton cells (see Xu et al. 1988).

Fluorescence induction

The characteristics of PS II and of the plastoquinone electron acceptors have been studied in higher plants and algae by monitoring Chl *a* fluorescence induction curves (see, e.g., Govindjee and Papageorgiou 1971, Papageorgiou 1975). The initial ('origine') fluorescence (O level) is thought to be emitted from the antenna Chl *a* molecules prior to photochemistry and is, thus, independent of photochemical reaction. The true quantum yield of F_0 (fluorescence at the 'O' level) can be measured when the first plastoquinone electron acceptor of PS II, Q_A, is fully oxidized before the onset of illumination (see, e.g., Govindjee and Papageorgiou 1971, Govindjee and Satoh 1986). The fluorescence rise, following the 'O' level, is generally considered to reflect the reduction of Q_A. Therefore the rapid transient at 685 nm depends mainly on the rate of the reduction of Q_A by PS II and the redox state of the PQ pool. At the end of the rapid transient (OIDP, where 'I' stands for an intermediate peak, D, a plateau and P, a peak), the fluorescence intensity reaches the maximum (F_{max}). Figure 2 (curve B) shows the Chl *a* fluorescence induction curve of SB-P soybean cells which had been dark-adapted for five minutes before the measurement. The ratio between the fluorescence of the 'P' (F_{max}) and the 'O' level (F_0) is 4.4, which is quite close to the value reported in spinach chloroplasts and some algal cells (see, e.g., Yamagishi et al. 1978, Briantais et al. 1986). In some experiments with soybean cells, we were able to even reach a value of 5.0 for the ratio of 'P' to the 'O' level (Table 1). At the light intensity used, the approximate half time for the O–P rise is about 390 ms in the absence of any herbicide and 13 ms in the presence of 1 μM diuron (DCMU,3-(3,4)-dichloro-1,1-dimethyl urea; curve A; see Table 1). Diuron accelerates the Chl *a* fluorescence rise by occupying the Q_B binding site and, thus, preventing the reoxidation of Q_A^- (see, e.g., Velthuys 1981).

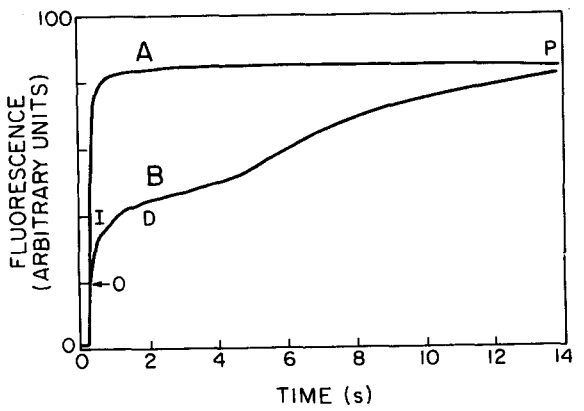

Fig. 2. Chlorophyll *a* fluorescence induction curves of SB-P cells in the presence (A) or absence (B) of 1 μM DCMU. 'O' level is the initial level, and 'P' level is the maximal fluorescence level. At 'P' level all Q_A is reduced and at 'O' level all Q_A is in the oxidized form. (For I and D, see text.)

Table 1. The ratio between the Chl *a* fluorescence at F_{max} and F_0 levels, the complementary area, derived from the Chl *a* fluorescence induction curve of the SB-P soybean cells with or without DCMU in Fig. 1, and the approximate halftime for the F_0 to F_{max} rise

	$\frac{F_{max}}{F_0}$	Complementary area (relative units)	Approximate half-time for F_0 to F_{max} rise, ms
−DCMU	5.1	49.3	390
+DCMU (1 μM)	4.8	3.8	13

The complementary area above the fluorescence transient curve is proportional to the number of electrons accumulated on the electron acceptor side of PS II (Malkin and Kok 1966, Velthuys and Amesz 1974). In the absence of any inhibitor, it is proportional to the number of electron equivalents on the plastoquinone (PQ) pool, Q_A and Q_B, which have been reduced during illumination. However, in the presence of saturating concentration of diuron, the area is solely proportional to the number of electron equivalents on Q_A. In our experiment, the ratio of the complementary area without and with 1 μM diuron is about 13. Since PQ accumulates two electrons to be reduced to plastoquinol, this shows that the ratio of the plastoquinone pool (including Q_B) to Q_A is 6 in SB-P soybean cells. This value is of the same order of magnitude as reported

for isolated spinach chloroplasts (Malkin and Kok 1966, Robinson and Crofts 1983).

As noted earlier, the pH of the growth medium (pH 7.0) decreased rapidly to near 4.2 and gradually rose to near 5.0 by day 14 after inoculation. In order to correlate the photosystem II activity of the cell with the medium pH, the Chl *a* fluorescence transient was measured in different pH media. The cell suspensions were incubated in different pHs for, at least, 10 minutes before measurement. Varying the pH from 4.0 to 8.0 led to no significant changes in the kinetic characteristic of the fluorescence induction curve and in the intensities of fluorescence levels at 'O' and at 'P' (data not shown). The insensitivity of fluorescence of soybean cells to the medium pH may indicate that the chloroplast pH remains unchanged, since strong effects of pH have been reported on the 'P' level fluorescence of the oxygen evolving PS II enriched preparations (see Hodges and Barber 1986).

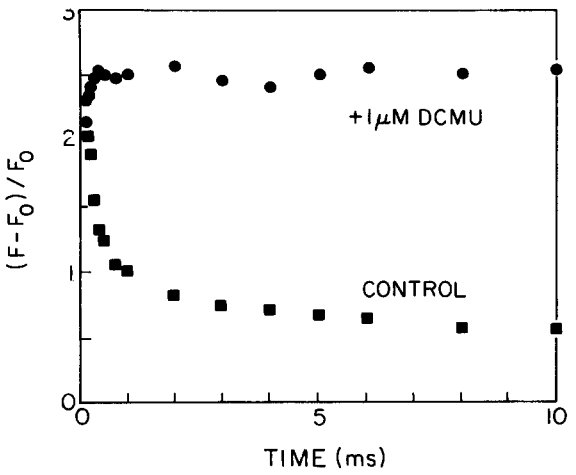

Fig. 3. The effect of electron transport inhibitor (DCMU) on the decay of Chl *a* fluorescence yield after the first actinic flash in SB-P cells. ■ and ● were without or with 1 μM DCMU. *F* is for fluorescence yield at time *t* after the flash and F_0 is the fluorescence yield before the actinic flash, i.e., when $[Q_A]$ is maximum.

Decay of Chl a fluorescence yield or $[Q_A^-]$ after light flash 1 or 2

Using a double flash technique for Chl *a* fluorescence measurement, the kinetics of Q_A^- oxidation by Q_B and Q_B^- have been measured and the equilibrium binding constant for plastoquinone at Q_B site has been estimated in chloroplasts (see, e.g., Robinson and Crofts 1983, Taoka et al. 1983). Figure 3 shows the decay of chlorophyll *a* fluorescence yield measured by this technique in soybean cells and the effect of 1 μM DCMU (diuron) on it; 10 μM atrazine had a similar influence. A fluorescence rise component (in 250 μs range) is explained to be due to a slow re-reduction of $P\text{-}680^+$ on the donor side of PS II, as already suggested for isolated chloroplasts (Robinson and Crofts 1987). The quencher $P680^+$ is reduced to P680 by the electron donor Z (see Butler 1973, for the idea that $P680^+$ is a quencher of Chl *a* fluorescence and Jursinic and Govindjee 1977, for the electron donation by Z in the μs range). The Chl *a* fluorescence decay in the micro- to millisecond range represents electron flow from the reduced Q_A, Q_A^-, to Q_B; diuron, however, blocks the decay by competing with plastoquinone and plastoquinol for the Q_B binding side (Velthuys 1981).

In isolated chloroplasts, the decay of Q_A^- to Q_A is faster after the first flash than after the second flash, because the electron transfer from Q_A^- to Q_B is faster than to Q_B^- and there is a preponderance of Q_B in dark adapted chloroplasts (see, e.g., Wollman 1978, Robinson and Crofts, 1983). In intact soybean cells, the time course of Chl *a* fluorescence emission after the first or the second flash was virtually identical, implying a ratio of one between Q_B and Q_B^- in dark-adapted cells (Fig. 4A). This is consistent with the result in spinach leaves, obtained by the thermoluminescence method (Rutherford et al. 1984). After the first flash, the initial 50% Q_B is reduced to 50% Q_B^- form and the initial 50% Q_B^- becomes 50% Q_B^{2-}, which then exchanges with the plastoquinone (PQ) pool to become 50% Q_B. Thus, the ratio of Q_B to Q_B^- is maintained as one, and the kinetics of Chl *a* fluorescence decay after the second flash is the same as after the first flash. A replot of Chl *a* fluorescence change on a log scale, as a function of time, shows two decaying components, a fast and a slow one (see Fig. 4B); both components have about the same kinetics and are of about the same magnitude after flash 1 and 2. This confirms the conclusion made above that $[Q_B] = [Q_B^-]$ in dark-adapted cells.

Using equations (1) and (2), Chl *a* fluorescence yield was converted into the relative concentration of reduced Q_A. In SB-P and SBI-P soybean cells,

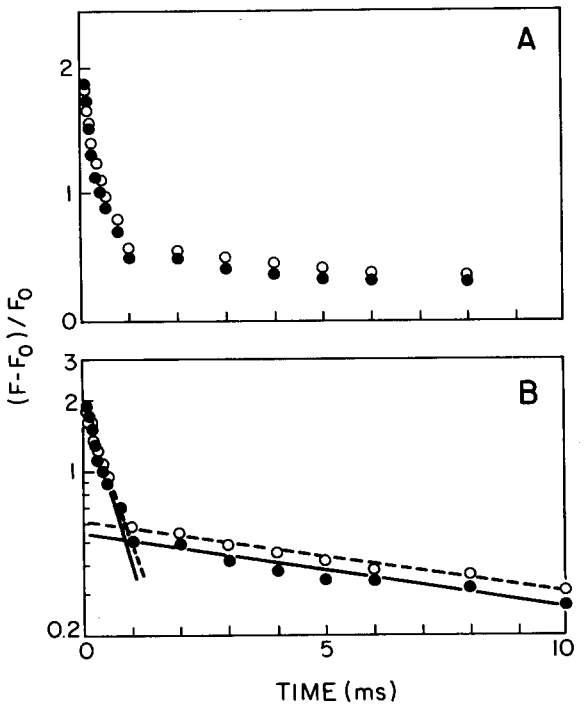

Fig. 4. (A) Decay of variable Chl *a* fluorescence $[(F - F_0)/F_0]$ after first (●) and second (○) actinic flashes, spaced 1 second apart, in SB-P cells. (B) same data, plotted on a semilogarithmic scale. F_0 is the fluorescence yield taken with the measuring light when Q_A is oxidized and F ($F(t)$ in equations (1) and (2)) is the fluorescence yield after the actinic flash. From these data, a fast and a slow component can be separated. The amplitudes and the halftimes of these components can be obtained from the intersections of the best-fit lines with the ordinate.

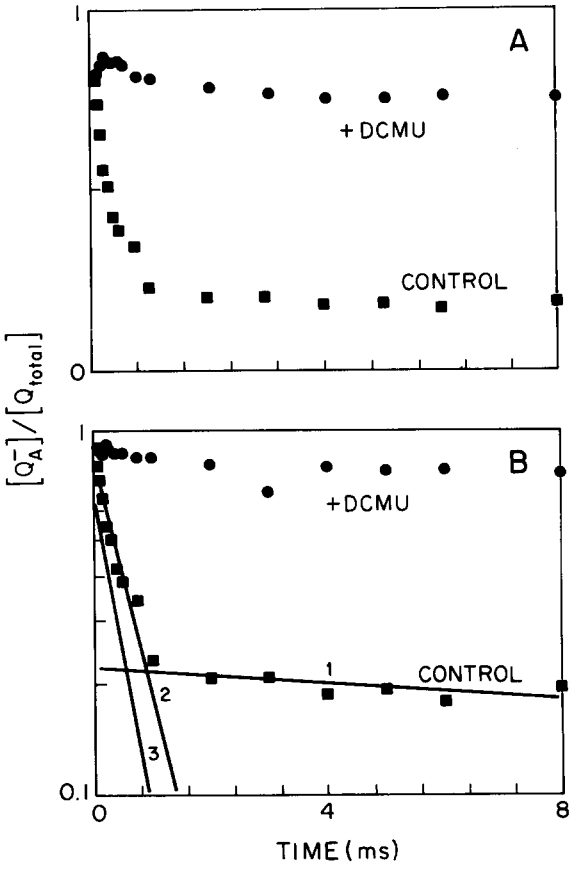

Fig. 5. (A) normal and (B) semi-logarithmic plots of the decay of q ($=[Q_A^-]/Q_{total}$] after a single actinic flash from Chl *a* fluorescence decays in SB-P cells. ■, obtained in the absence of DCMU, while, ● was in the presence of 0.8 μM DCMU. For details of curves 1, 2 and 3, see text.

the F_{max} is often lower than that in isolated spinach thylakoid fragments. As mentioned under Methods, the value of p, the connection parameter, was taken as 0.5. Figure 5A shows the relative concentration of reduced Q_A, $[Q_A^-]/[Q_{total}]$(equivalent to q in equations 1 and 2) as a function of time, whereas Fig. 5B shows a log plot of $[Q_A^-]/[Q_{total}]$ as a function of time. Two distinct components (fast and slow) were clearly observed in this semi-logarithmic plot. In Fig. 5B, line 1 mainly represents the decay of the slow component, line 2 displays effects of both the fast and the slow components. By subtracting line 1 from line 2, line 3, indicating the decay of the fast component, is obtained. Both the amplitude and the half times of decay for each component can be extracted from this plot.

Figure 6 shows the plot for t_{50}, the time at which $[Q_A^-]$ is 50% of that at time zero, against the concentrations of diuron (DCMU) and atrazine in soybean cells. Since the inhibitory effect of diuron is stronger than that of atrazine at the same concentration, the curve in Fig. 6A shows a shift to the lower side of the concentration range. Thus the midpoint of the t_{50} value in the presence of atrazine is at a much higher concentration than that in the presence of diuron.

Both the amplitudes and the halftimes of the slow and the fast components of $[Q_A^-]$ decay were obtained as shown in Fig. 5(B). From the semi-logarithmic plot, two different exponential decaying components are separated, the two best fit straight lines drawn, the half-times of the decaying components calculated and the amplitudes of the

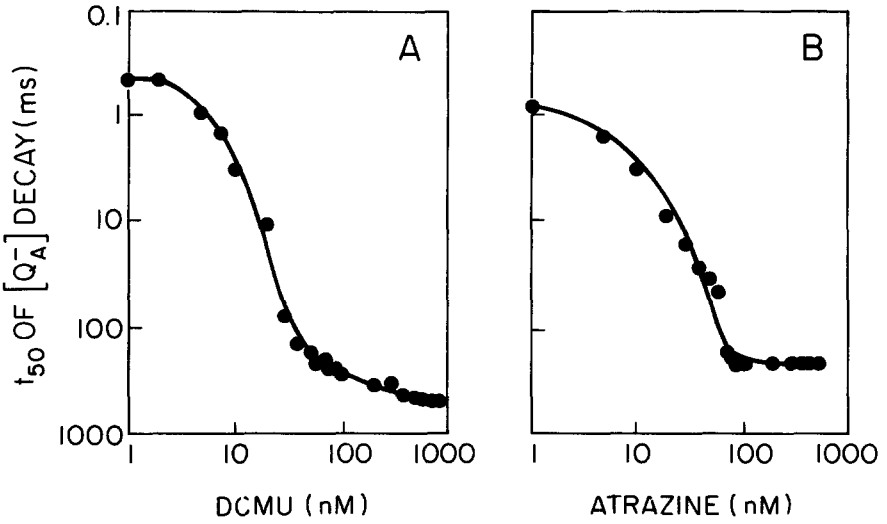

Fig. 6. Plots of t_{50}, i.e., time at which $[Q_A^-]$ is 50% of that at F_{max}, versus concentration of (A) DCMU and (B) atrazine in SB-P cells.

different components obtained from the intersections of the two decay components on the ordinate. The amplitudes of both the fast and the slow decay components of $[Q_A^-]$ as a function of [DCMU] and [atrazine] are plotted, respectively, in the left or right panels of Fig. 7. Results obtained with the cells (curves A–D) can be compared with those on isolated chloroplasts (curves E and F). The oxidation of Q_A^-, after one flash, is biphasic and it has been suggested (see Robinson and Crofts 1983) that the amplitude of the fast component is related to centers that have Q_B bound before the actinic flash, whereas, the slow component represents a second order process involving the binding of Q_B from the PQ pool. Electron transport inhibitors such as DCMU or atrazine induce an enhancement of Chl *a* fluorescence since they displace Q_B from its binding site and shift the equilibrium of the reaction $Q_A^- + Q_B \rightleftharpoons Q_B^- + Q_A$ to the left (Velthuys 1981). We expect that an increase in the concentration of these inhibitors would decrease the amount of Q_B bound, and thus the amplitude of the fast component is expected to decrease. Indeed, we found the amplitude to decrease from a value of 70% to near zero (see Fig. 7). If the above expectation is correct, the results of Fig. 7 would imply that diuron and atrazine can totally displace Q_B. It appears from the data on halftimes of the decay of fast (Fig. 8) and slow (Fig. 9) components that high concentrations of inhibitors cause a slowing down of both the components. We interpret these results to mean that the electron flow inhibitors induce the decrease of Q_B binding as well as the decrease of the exchange of PQ pool at the Q_B binding site.

A further comparison between the intact soybean cells and spinach chloroplasts was made from the data listed in Table 2. The halftime of the fast component is approximately 300 μs and that of the slow component is approximately 30 ms. In spinach chloroplasts, the percent of the amplitude of the slow component usually is near 30. This value is almost the same as reported elsewhere (Eaton-Rye and Govindjee 1988). In intact soybean cells, the percent of the amplitude of the slow component, however, reaches 40, implying that the contribution of the centers with unbound Q_B is larger in these cells than in the spinach chloroplasts. As for the physiological meaning, it deserves further study. Although inhibitors increase the halftimes of both components, atrazine is less effective than diuron in the same concentration range. 0.5 μM DCMU is much more effective at increasing the halftime of both components in spinach chloroplasts than in SB-P and SBI-P cells, but atrazine does not show a significant difference between each system. Data in Table 2 also show differences in the responses to the electron transport inhibitors between these two soybean cell lines. At a certain concentration (0.5 μM DCMU or 5 μM atrazine), each inhibitor produces a larger increase in the

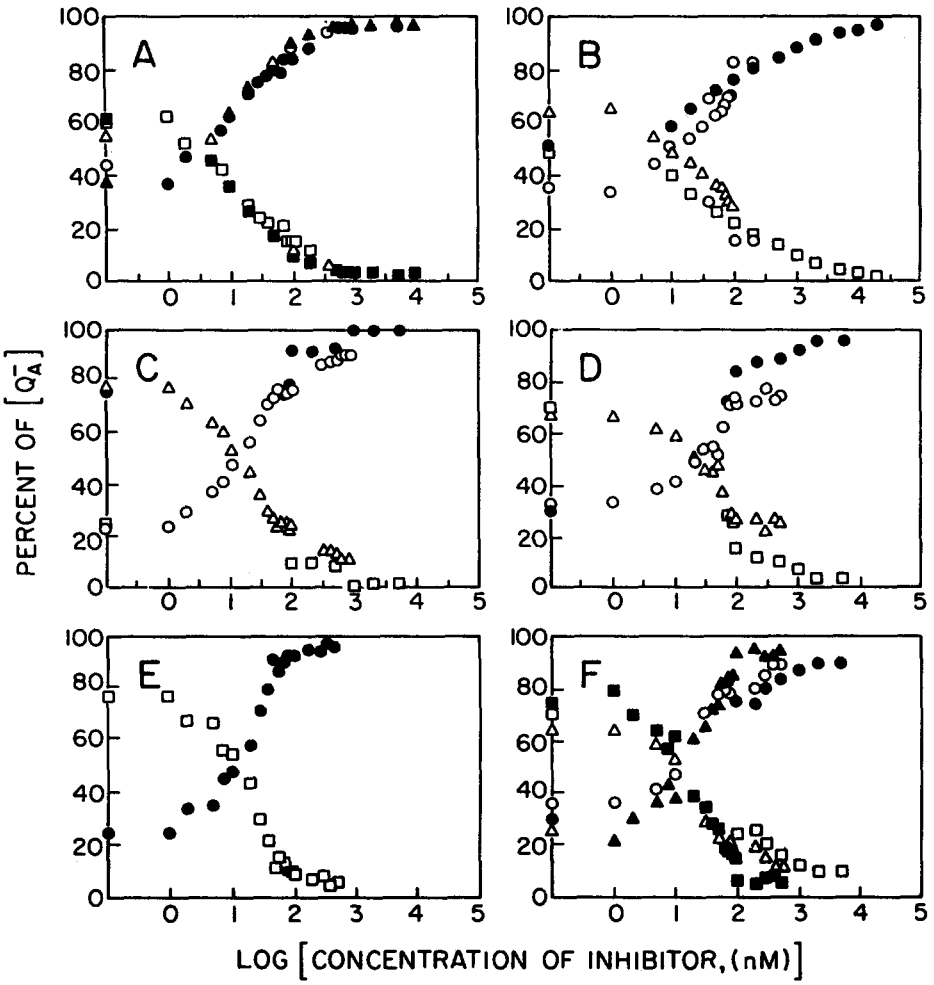

Fig. 7. The inhibitor concentration dependence of the amplitude of the fast and slow [Q_A^-] decay components in SB-P (A, B) and SBI-P (C, D) cells, and in spinach chloroplasts (E, F). Left panels: DCMU; right panels: atrazine. Symbols at the ordinate are control values. Different symbols represent different experiments. ●, ○ and ▲ represent data for the slow component, and ■, □ and △ for the fast component.

halftimes of both the components of Q_A^- decay in SB-P cells than in SBI-P cells. Thus, this method may be successfully used in selecting herbicide resistance and other mutation among the various cell lines.

Oscillations in flash dependence of Chl a fluorescence

Delsome (1971) discovered a period-of-four oscillation in Chl *a* fluorescence emission in the green alga *Chlorella* and spinach chloroplasts, with maxima at flash 1, 5 and 9, and minima at flash 3, 7 and 11. By comparing the fluorescence yield with the sum of the concentrations of S_2 and S_3 states, calculated from oxygen evolution in spinach chloroplasts, he ascribed this oscillation to the cycling of these states associated with the water oxidation process. The reduction of Z^+ (Z being the electron donor to $P680^+$) by different S-states proceeds at different rates and different equilibria are involved (Babcock et al. 1976). In turn, this affects the rereduction rate of $P680^+$ by Z after an actinic flash and the associated equilibria between these two species (Robinson and Crofts 1987). The resulting

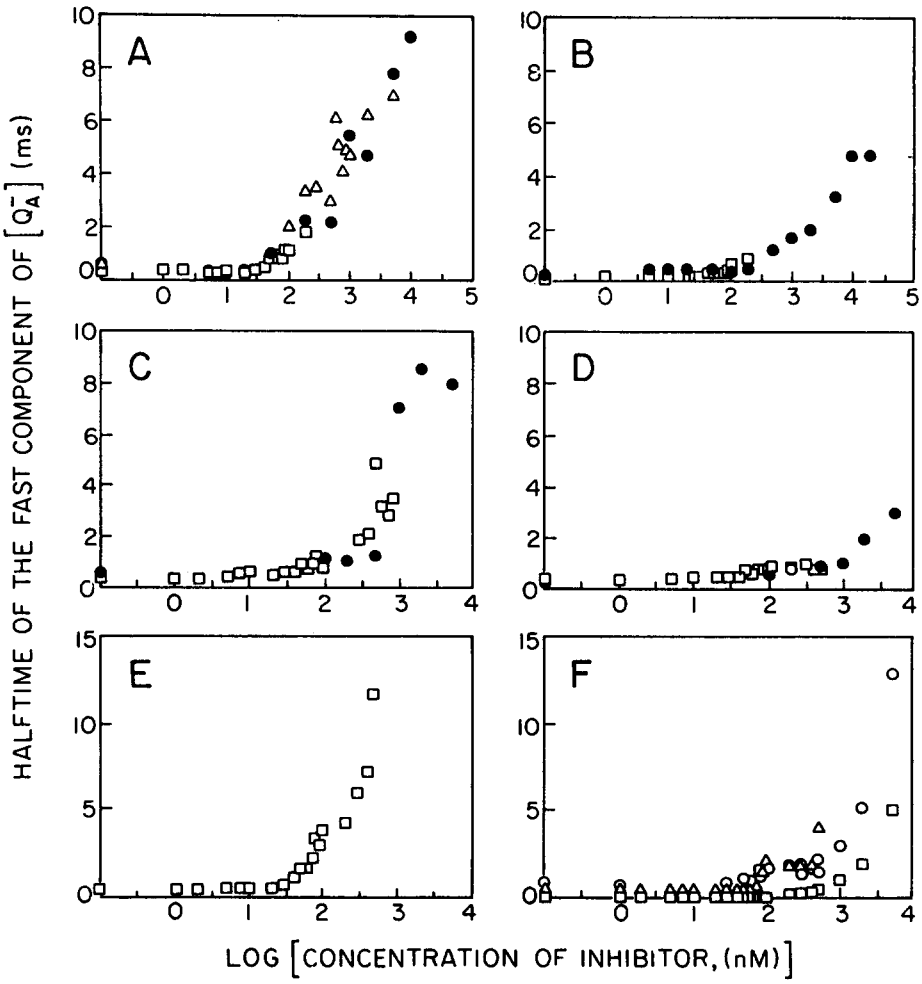

Fig. 8. The inhibitor concentration dependence of the half time of the fast [Q_A^-] decay component in SB-P cells (A, B), SBI-P cells (C, D) and spinach chloroplasts (E, F) obtained in the presence of DCMU (left panels) and atrazine (right panels). Symbols at the ordinate are control values. Different symbols represent different normalized experiments.

changes in the $P680^+$ population produce the period-of-four oscillation (see Sonneveld et al. 1979). Maxima are at flashes 1 and 5 because electron donation to $P680^+$ is fastest and most efficient when the system starts in the S_1 state (see, e.g., Witt et al. 1986). Superimposed upon this oscillation, and seen at 70 μs after the actinic flash, is a binary oscillation arising from the differential rates of Q_A^- oxidation by either Q_B after an odd number or Q_B^- after an even number of flashes (Robinson and Crofts 1983).

Since in intact soybean cells, the ratio of Q_B to Q_B^- is one (see Fig. 4), the influence of the binary oscillation is absent; therefore, the period-of-four oscillation pattern predominates (Fig. 10A). This is most clearly observed in fluorescence 70 μs after the flash. With increasing time (> 500 μs) after the flash (Fig. 10A) and after the addition of DCMU (0.8 μM)(Fig. 10B), this oscillation disappears and a drastically altered flash pattern is observed. At increasing times (> 500 μs) after the flash, the influence of the donation of electrons by S-states is over; thus, a clear oscillation cannot be distinguished. Since the inhibitors, such as DCMU, inhibit PS II photoreaction by competing with the plastoquinone at the secondary quinone electron acceptor (Q_B) site, the oscillation is abolished as the system is blocked in the $S_2Q_A^-$ state, and is indepen-

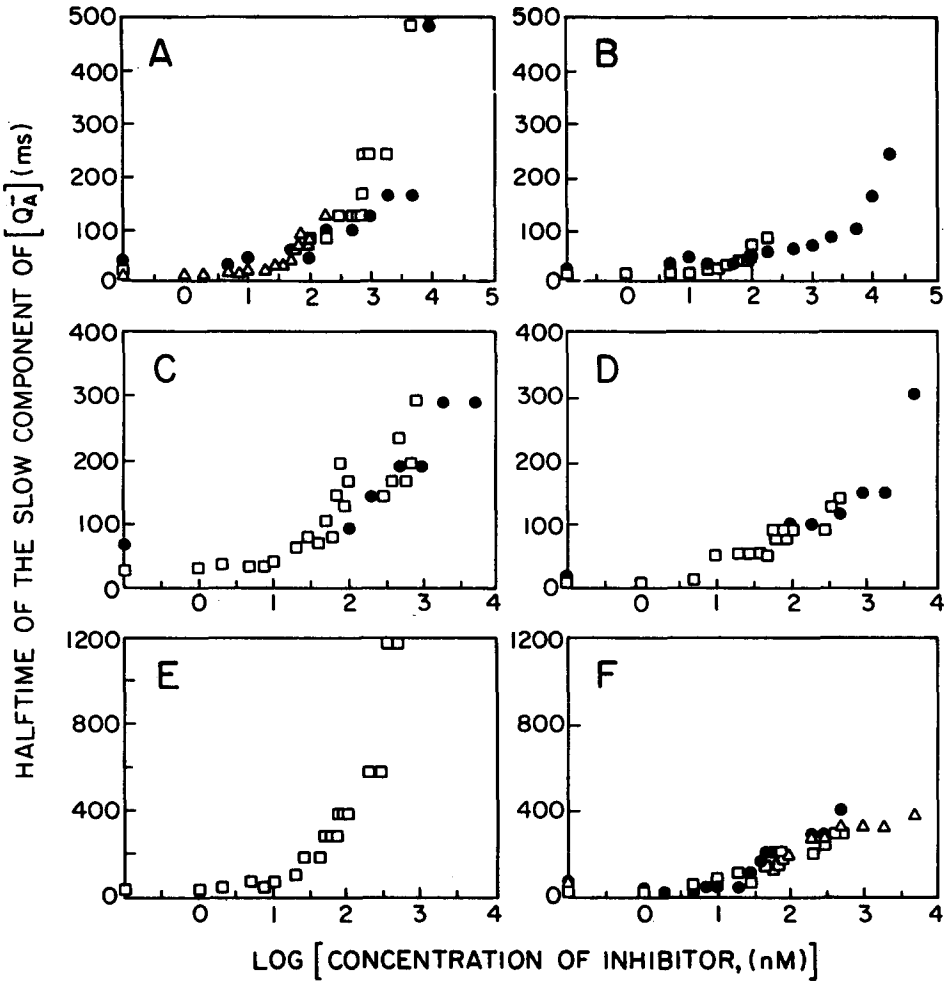

Fig. 9. The inhibitor concentration dependence of the half time of the slow [Q_A^-] decay components in SB-P cells (A, B), SBI-P (C, D) and in spinach chloroplasts (E, F). Left panels: DCMU; right panels: atrazine. Symbols at the ordinate are control values. Different symbols represent different normalized experiments.

Table 2. The amplitude (α) and the halftime (τ) of the fast (f) and slow (s) Q_A^- decay components, after the first flash, in SB-P and SBI-P cells (pH 5.5) and in spinach chloroplasts (pH 7.8)*

	SB-P cells				SBI-P cells				Spinach chloroplasts			
	α_f %	α_s %	τ_f	τ_s	α_f %	α_s %	τ_f	τ_s	α_f %	α_s %	τ_f	τ_s
			μs	ms			μs	ms			μs	ms
Control	60 ± 10	40 ± 10	297 ± 24	34 ± 9	59 ± 2	41 ± 2	286 ± 10	23 ± 4	73 ± 3	27 ± 3	368 ± 55	33 ± 5
			ms	ms			ms	ms			ms	ms
+0.5 μM DCMU	6 ± 4	94 ± 4	2 ± 1	199 ± 29	5 ± 1	95 ± 1	2 ± 1	108 ± 9	5	95	12	1200
			ms	ms			ms	ms			ms	ms
+5 μM Atrazine	14 ± 8	86 ± 8	5 ± 3	195 ± 32	5	95	3	96	8 ± 1	92 ± 1	3 ± 1	264 ± 74

* All values presented here should be rounded to the nearest whole number.

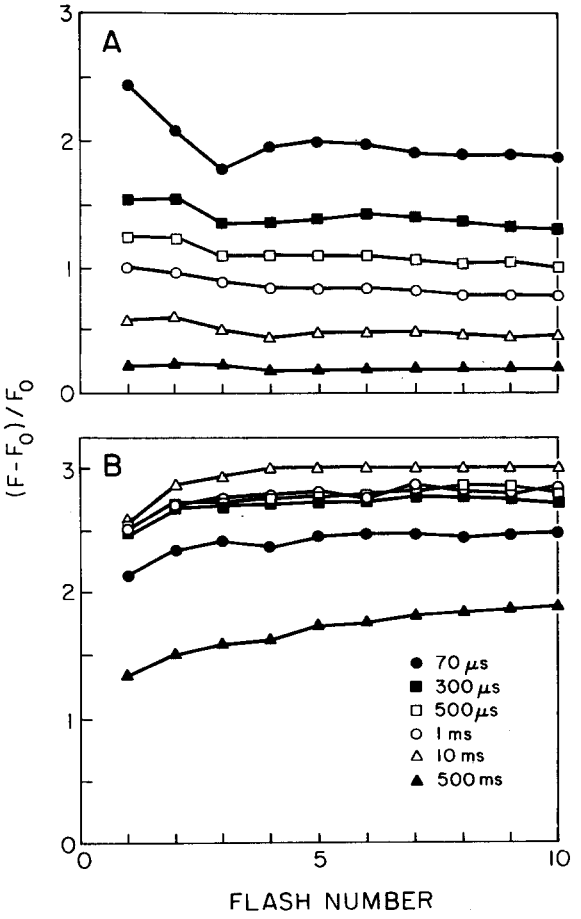

Fig. 10. Chlorophyll *a* fluorescence, $(F - F_0)/F_0$, measured at different times after an actinic flash as a function of flash number. The times indicated inside B are when the measuring flash was fired. A and B represent results with control and with 0.8 μM DCMU treated SB-P cells.

dent of the flash number. In soybean cells, the Chl *a* fluorescence intensity at 70 μs after the first flash is higher than that after the second flash. This is different from that in isolated spinach chloroplasts, indicating a somewhat higher percentage of Q_B^- existing in the intact soybean cells than in the isolated chloroplasts. We have already indicated on the basis of almost similar decay of Q_A^- after flash 1 and 2, that Q_B:Q_B^- ratio is 1.0 in soybean cells as opposed to 0.7:0.3 in isolated spinach chloroplasts (Wollman 1978). At early times (70 μs), the decrease of the reoxidation rate of Q_A^- enhances the fluorescence intensity after the first flash. It appears that the minimum is after the third flash from 70 μs to 500 μs after the actinic flash, and after 1 ms, this minimum shifts to flash 4. Detailed analysis of this data requires further study.

Concluding remarks

During the past decade, interest has been growing in the practical application of Chl *a* fluorescence measurements as a rapid, sensitive, and nondestructive method for the determination of photosynthetic activity in higher plants. But in the practical use of Chl *a* fluorescence, many real problems have hampered the growth of this study (Briantais et al. 1986, Renger and Schreiber 1986). A photoautotrophic higher plant cell suspension is an easily manipulated system, not only for studying physiological and biochemical aspects of photosynthesis but also for studying the primary process of photosynthesis. Such cell lines have only been recently established (Rogers et al. 1987, Xu et al. 1988). Since problems such as reabsorption of fluorescence can be much more easily minimized in cells than in leaves (see Fig. 1), these cells can be used as reliable models for investigations on higher plant leaves.

We have shown here measurements on the fluorescence spectra of soybean cells (cell lines SB-P and SBI-P) at room temperature and 77 K; we found that these intact higher plant cells have identical antenna system as that of chloroplasts from other higher plants. Here, the long wavelength fluorescence band occurs at approximately 740 nm (F740). However, this band is shifted to about 725 nm in green algae and to still shorter wavelengths (e.g., 712–718 nm) in red and blue-green algae (Govindjee and Satoh 1986, Fork and Mohanty 1986). From the fluorescence induction curves, a ratio of the PQ pool (including Q_B) to Q_A was calculated to be 6 (Fig. 2; Table 1). By using the double flash technique of Chl *a* fluorescence measurement, the decay kinetics after the first flash was found to be identical to that after the second flash, suggesting that the ratio of Q_B to Q_B^- is 1 (Fig. 4). The flash-dependence pattern also confirms this relation, indicating a higher relative concentration of bound Q_B^- in physiological conditions than in isolated thylakoids (Fig. 10).

Using equations (1) and (2), the concentration of Q_A^- was evaluated and then its decay. The decay of Q_A^- can be divided into two components (generally

represented in the μs and the ms time range, Fig. 5). The half times of these components in soybean cells were found to be similar to those in spinach chloroplasts in the range of 300 μs or 30 ms. The relative proportion of the fast and slow component was approximately 60 to 40 in cells as opposed to 70 to 30 in chloroplasts, but the reason for these differences need to be studied further.

It is well known that the mechanism of diuron and atrazine action is to displace Q_B at its binding site. We show here that they not only influence the fast decay components of Q_A^- reoxidation but also the slow components. This implies that they affect not only the electron flow from Q_A^- to Q_B, but also the exchange between Q_B and the PQ pool (Table 2). Since the fast component, that is believed to be due to the bound Q_B, was totally replaced by the slow component upon inhibitor treatment (Fig. 7), we suggest that the inhibitors can fully replace Q_B at the Q_B binding site.

In this paper we have found that the two different lines of soybean cells display rather different sensitivities to the inhibitors. Although inhibitors used (DCMU and atrazine) replace Q_B at its binding site, the increases of the halftime of both components are not the same in different cell lines (Table 2). Thus, this method can be used as a means to screen inhibitor resistance cell lines.

Since the physiological condition of the intact (soybean) cells is different from that of the isolated (spinach) thylakoids, the diffusion of inhibitors across the membrane is expected to be different. In the plots showing the inhibitor concentration dependence of t_{50} of Q_A^- in intact cells (Fig. 6), the range covering the change in decay time over inhibitor concentration is broader than that obtained from chloroplasts (data not shown). The concentration of electron transfer inhibitors (such as DCMU and atrazine) needed to give the same effect is higher for cells than that observed for isolated chloroplasts (data not shown) simply because the data are plotted in terms of the concentration of the inhibitors in the outside medium, and that their actual concentration in the chloroplast *in vivo* may be smaller than that in isolated chloroplasts. Such problems deserve further study.

Acknowledgements

We are grateful to Drs J. J. Eaton-Rye and D. J. Blubaugh for their help. This work was supported by the funds from the McKnight Interdisciplinary Photosynthesis Research Program.

References

Arnon D I (1949) Copper enzyme in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15

Babcock G T, Blankenship R E and Sauer K (1976) Reaction kinetics for positive charge accumulation on the water side of chloroplast Photosystem II. FEBS Lett 61: 286–289

Blubaugh D J (1987) The mechanism of bicarbonate activation of plastoquinone reduction in Photosystem II of photosynthesis. PhD Thesis, Biology, University of Illinois at Urbana-Champaign

Briantais J, Vernotte C, Krause G H and Weis E (1986) Chlorophyll *a* fluorescence of higher plants: chloroplasts and leaves. In Govindjee, Amesz J and Fork D C (eds) Light Emission by Plants and Bacteria, pp 539–583. Orlando: Academic Press

Butler W L (1972) Primary photochemistry of Photosystem II of photosynthesis. Accnts Chem Res 6: 177–184

Crofts A R and Wraight C A (1983) The electrochemical domain of photosynthesis. Biochim Biophys Acta 726: 149–185

Delosme R (1971) New results about chlorophyll fluorescence '*in vivo*'. In Forti G, Avron M and Melandri A (eds) 2nd Int Congr Photosynthesis Res, pp 187–195. The Hague: Dr W Junk Publishers

Duysens L N M (1986) Introduction to (bacterio) chlorophyll emission: a historical perspective. In Govindjee, Amesz J and Ford D C (eds) Light Emission by Plants and Bacteria, pp 4–28. Orlando: Academic Press

Duysens L N M and Sweers H E (1963) Mechanism of two photochemical reactions in algae as studied by means of fluorescence. In Japanese Society of Plant Physiologists (eds) Studies of Microalgae and Photosynthetic Bacteria, pp 353–372. Tokyo: University of Tokyo Press

Eaton-Rye J J (1987) Bicarbonate reversible anionic inhibition of the quinone reductase in Photosystem II. PhD Thesis, Biology, University of Illinois at Urbana-Champaign

Eaton-Rye J J and Govindjee (1988) 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

Fork D C and Mohanty P (1986) Fluorescence and other characteristics of bluegreen algae (cyanobacteria), red algae, and cryptomonads. In Govindjee, Amesz J and Fork D C (eds) Light Emission by Plants and Bacteria, pp 451–496. Orlando: Academic Press

Govindjee and Papageorgiou G C (1971) Chlorophyll fluorescence and photosynthesis: fluorescence transient. In Giese A C (ed) Photophysiology 6: 1–46. New York: Academic Press

Givindjee and Satoh K (1986) Fluorescence properties of chlorophyll *b* and chlorophyll *c*-containing algae. In Govindjee, Amesz J and Fork D C (eds) Light Emission by Plants and Bacteria, pp 497–538. Orlando: Academic Press

Green B (1988) The chlorophyll-protein complexes of higher plant photosynthetic membranes or just what green band is that? Photosynthesis Research 15: 3–32

Hodges M and Barber J (1986) Analysis of chlorophyll fluorescence induction kinetics exhibited by DCMU-inhibited thylakoids and the origin of alpha and beta centers. Biochim Biophys Acta 828: 239–246

Horn M E, Sherrard J H and Widholm J M (1983) Photoautotrophic growth of soybean cells in suspension culture. Plant Physiol 72: 426–429

Joliot A and Joliot P (1964) Étude cinetique de la reaction photochimique liberant l'oxygene au cour de la photosynthèse. CR Acad Sc Paris t258: 4622–4625

Jursinic P and Govindjee (1977) The rise in chlorophyll *a* fluorescence yield and decay in delayed light emission in tris-washed chloroplasts in the 6–100 μs time range after an excitation flash. Biochim Biophys Acta 461: 253–267

Malkin S and Kok B (1966) Fluorescence induction studies in isolated chloroplasts. I. Number of components involved in the reaction and quantum yield. Biochim Biophys Acta 126: 413–432

Malkin S, Armond P A, Mooney H A and Fork D C (1981) Photosystem II photosynthetic unit sizes from fluorescence induction in leaves. Plant Physiol 67: 570–579

Mathis P and Paillotin G (1981) Primary processes of photosynthesis. In Hatch M D and Boardman N K, eds. The Biochemistry of Plants: Photosynthesis, Vol 8: pp 97–161. Sidney: Academic Press

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497

Murata N and Satoh K (1986) Absorption and fluorescence emission by intact cells, chloroplasts, and chlorophyll-protein complexes. In Govindjee, Amesz J and Fork D C (eds) Light Emission by Plants and Bacteria, pp 137–159. Orlando: Academic Press

Papageorgiou G C (1975) Chlorophyll fluorescence: an intrinsic probe of photosynthesis. In Govindjee (ed) Bioenergetics of Photosynthesis, pp 319–371. New York: Academic Press

Renger G and Schreiber U (1986) Practical applications of fluorometric methods to algae and higher plant research. In Govindjee, Amesz J and Fork D C (eds) Light Emission by Plants and Bacteria, pp 587–619. Orlando: Academic Press

Robinson H H and Crofts A R (1983) Kinetics of the changes in oxidation-reduction reactions of the Photosystem II quinone acceptor complex, and the pathway for deactivation. FEBS Lett 153: 221–226

Robinson H H and Crofts A R (1987) Kinetics of the changes in oxidation-reduction states of the acceptors and donors of Photosystem II in pea thylakoids measured by flash fluorescence. In Biggins J (ed) Proceedings of the 7th International Congress of Photosynthesis, Vol 2: pp 429–432. Dordrecht: Martinus Nijhoff Publishers

Rogers S M D and Widholm J M (1986) Photosynthetic characteristics of photoautotrophic cell suspensions of soybean. Plant Physiol 80: S-46

Rogers S M D and Widholm J M (1988) Comparison of photosynthetic characteristics of two photoautotrophic cell suspension cultures of soybean. Plant Science 56: 69–74

Rogers S M D, Ogren W L and Widholm J M (1987) Photosynthetic characteristics of a photoautotropic cell suspension culture of soybean. Plant Physiol 84: 1451–1456

Rutherford A W, Govindjee and Inoue Y (1984) Charge accumulation and photochemistry in leaves studied by thermoluminescence and delayed light emission. Proc Natl Acad Sci USA 81: 1107–1111

Sivak M N and Walker D A (1983) Some effects of CO_2 concentration and decreased O_2 concentration on induction of fluorescence in leaves. Proc Roy Soc London B 217: 377–392

Sivak M N, Dirtz K, Heber U and Walker D A (1985) The relationship between light scattering and chlorophyll *a* fluorescence during oscillations in photosynthetic carbon assimilation. Arch Biochem Biophys 237: 513–519

Sonneveld A, Rademaker H and Duysens L N M (1979) 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

Taoka S, Robinson H H and Crofts A R (1983) Kinetics of the reaction of the two-electron gate of Photosystem II: studies of the competition between plastoquinone and inhibitors. In Inoue Y, Crofts A R, Govindjee, Murata N, Renger G and Satoh K (eds) The Oxygen Evolving System of Plant Photosynthesis, pp 369–381. Tokyo: Academic Press

Velthuys B R (1981) Electron-dependent competition between plastoquinone and inhibitors for binding to Photosystem II. FEBS Lett 126: 277–281

Velthuys B R and Amesz J (1974) Charge accumulation at the reducing side of system II of photosynthesis. Biochim Biophys Acta 333: 85–94

Walker D A (1981) Secondary kinetics of spinach leaves in relation to the onset of photosynthetic carbon assimilation. Planta 153: 273–278

Walker D A, Sivak M N, Prinsley R T and Cheesbrough J K (1983) Simultaneous measurement of oscillations in oxygen evolution and chlorophyll *a* fluorescence in leaf pieces. Plant Physiol 73: 532–549

Witt H T, Schlodder E, Brettel K and Saygin O (1986) Reaction sequences from light absorption to the cleavage of water in photosynthesis — routes, rates and intermediates. Photosynth Res 10: 453–471

Wollman F A (1978) Determination and modification of the redox state of the secondary acceptor of Photosystem II in the dark. Biochim Biophys Acta 503: 263–273

Xu C, Blair L C, Rogers S M D, Govindjee and Widholm J M (1988) Characteristics of five new photoautotrophic suspension cultures including two Amaranthus species and a Cotton strain growing on ambient CO_2 levels, Plant Physiol, in press

Yamagishi A, Satoh K and Katoh S (1978) Fluorescence induction in chloroplasts isolated from the green alga *Bryopsis maxima III*. A fluorescence transient indicating proton gradient across the thylakoid membrane. Plant and Cell Physiol 19: 17–25