Biochimica et Biophysica Acta, 638 (1981) 268–274
Elsevier/North-Holland Biomedical Press

BBA 48183

DELAYED FLUORESCENCE INDUCTION IN CHLOROPLASTS

IRRADIANCE DEPENDENCE

STEVEN W. McCAULEY and R.H. RUBY

Natural Sciences Division, University of California, Santa Cruz, CA 95060 (U.S.A.)

(Received March 9th, 1981)
(Revised manuscript received July 22nd, 1981)

Key words: Luminescence induction; Photosystem II; Irradiance dependence; (Spinach chloroplast)

We have studied the delayed fluorescence in spinach chloroplasts produced 0.5 ms after each of a pair of (sub)-microsecond flashes. We observe an increase in the delayed fluorescence from the second flash relative to that produced by the first. This increase is proportional to the product of the first and second flash irradiances, appearing as an I^2 dependence if both flashes are increased together. The enhancement is observable at very weak flash levels (roughly 1 photon absorbed/100 PS II centers). If the irradiance of the first flash is increased, but the irradiance of the second held constant, the delayed fluorescence from the second flash is observed to increase, but then to saturate well below the first flash irradiance at which the delayed fluorescence from the first flash itself saturates. For most experiments, the dark time between flashes was 30 ms. If the dark time is varied, the enhancement changes, reaching a half-maximal value for a dark time of approx. 300 μs. The enhancement is stopped by hydroxylamine, but not by gramicidin, valinomycin, DCMU, or mild heating. These experiments are consistent with the notion that there are two different types of Photosystem II centers if we assume that only one type is responsible for the induction we see and has an optical cross-section about 4-times the size of the other type of center.

Introduction

Different results have been reported for the dependence of delayed fluorescence on irradiance at low light levels. Jones [1], Stacy et al. [2] and Lavorel [3], all using phosphoroscopes with light pulses lasting about a millisecond, observed an I^2 dependence at low irradiance levels and a linear dependence at higher light levels. Ruby [4], Zankel [5] and Jursinic and Govindjee [6], using microsecond and submicrosecond width light pulses, observed a linear dependence at all irradiance levels. A millisecond duration light pulse has been necessary to produce the I^2 dependence. This may indicate the need for sequential turnovers at a single PS II reaction center (double hitting) for this dependence to be observed. It is puzzling that the I^2 dependence should occur only at low irradiance levels, where the probability of two photons being absorbed at a single reaction center is very low.

In an effort to reconcile these observations, we examine here the delayed fluorescence in spinach chloroplasts produced by sequences of microsecond and submicrosecond duration light pulses. We observe an enhancement of the delayed fluorescence from the later flashes relative to that produced by the first flash. We show that this enhancement increases as the square of the irradiance while the delayed fluorescence from a single flash increases linearly. This behavior is consistent with both phosphoroscope and pre-

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS, photosystem; Chl, chlorophyll.

vious microsecond flash studies.

The enhancement is observed to saturate at an irradiance well below the irradiance at which delayed fluorescence from a single flash saturates. This may be taken to support the suggestion of Melis and Duysens [7] that there are two different antenna sizes associated with PS II centers if we assume that the large antenna centers have a greatly increased delayed fluorescence signal after two flashes.

Delayed fluorescence transients have been shown to be caused by many mechanisms, including charge and pH gradients [8], acceptor availability [9], donor availability [10,11], and charge states of the oxygen-evolving system [5,12,13]. These and other delayed fluorescence phenomena have been reviewed by Govindjee and Jursinic [14]. The effects of ionophores, electron-transport inhibitors and heating on the enhancement are reported.

Materials and Methods

Chloroplasts were prepared from market spinach according to the method of Avron [15]. After being isolated, chloroplasts were made into a dense suspension with 30% ethylene glycol, 0.1 M sucrose, 0.1 M KCl, 20 mM Tricine (pH 7.8), and kept at 77 K until needed. Before use, the frozen chloroplasts were thawed and then resuspended in a solution of 0.1 M KCl and 20 mM Tricine (pH 7.8). The concentration used for measurements was approx. 2.1 μg CHl/ml, as determined by using the extinction coefficients of MacKinney [16].

The apparatus was similar to that used in an experiment previously described [4]. Either flashlamps (full width at half maximum about 1 μs) or a nitrogen-pumped dye laser (full width at half maximum 7 ns at a wavelength of 490 nm) were used for a light source. The sample volume used was 4 ml, except when the laser was used. The laser usable beam diameter of 0.5 cm led to a sample volume of 0.8 ml. The irradiance was varied by interposing neutral density filters and, for the flashlamps, mesh screens between the light source and the sample. The output from the photomultiplier was displayed on an oscilloscope and videotaped at 60 frames/s. By rerunning the videotape frame by frame, we could measure the signals of interest.

Usually the sample was dark adapted for 10 min before use. The samples that were heat treated were raised quickly to 50°C, held there for 3 min and then quickly brought back to room temperature. They were then dark adapted for 10 min before use. In experiments with exogenous agents (DCMU, hydroxylamine, gramicidin, and valinomycin), the sample was dark adapted for 9 min, the agent was injected in the dark, and the sample was dark adapted for 1 min more before signals were measured.

Results

We first observed induction effects with a series of weak flashes (we estimate about 1 photon absorbed/100 PS II centers) spaced 30 ms apart. Fig. 1a shows the magnitude of delayed fluorescence at 0.5 ms after each flash in such a series. There was sample-to-sample variability, but we usually saw a rise of a few per cent from the first to the second flash, and a near doubling by the tenth. We were surprised to observe such large induction effects with such weak flashes. We examined the dependence of both delayed fluorescence emission by single flashes and the induction effect on irradiance. We used two flashes if equal strength with 30 ms dark time between the flashes. We varied the strength of both flashes and observed the delayed fluorescence at 0.5 ms after the first flash, $D^f_{0.5}$, and the delayed fluorescence at 0.5 ms after the second flash, ${}^f_{30}D^f_{0.5}$. These flashes are over an order of magnitude below the value needed to saturate delayed fluorescence. At this irradiance level, most of the centers hit by the second flash will not have been hit by the first. We assume that the signal from the second flash is made of a base level nearly equal to the signal from the first flash, due to centers hit only by the second flash, plus an additional signal due to centers hit by both the first and the second flashes. We also assume that induction occurs because the delayed fluorescence emitted after a flash is larger if a center has been hit by a previous flash. If these assumptions are true, then the difference between the two signals, ${}^f_{30}D^f_{0.5} - D^f_{0.5} = \Delta D$, should increase as the square of the irradiance. To test these assumptions, we plotted $D^f_{0.5}$ and ΔD vs. irradiance in Fig. 2a and b on log-log plots. For comparison, we show slopes of 1 and 2. It is clear that the single flash delayed fluorescence increases linearly with irradiance, while the enhancement increases as I^2.

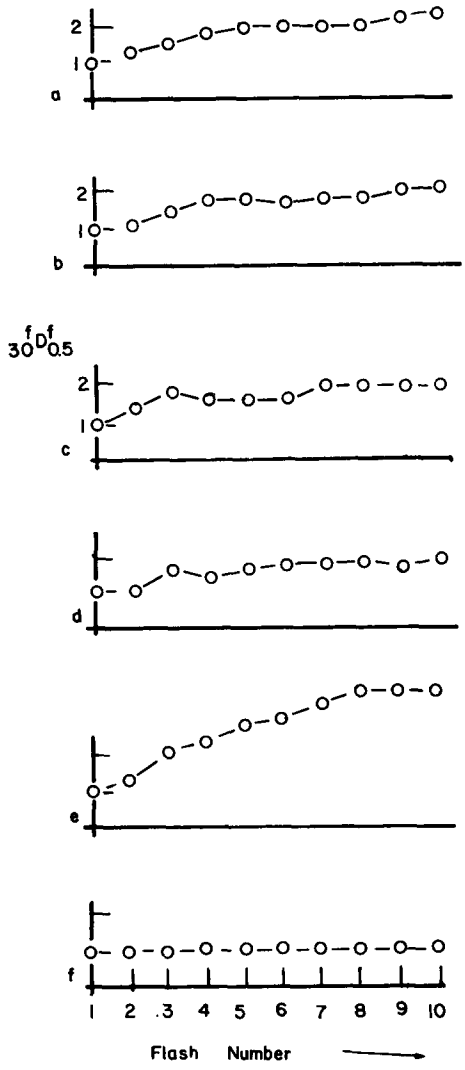

Fig. 1. ${}_{30}^{f}D_{0.5}^{f}$, the delayed fluorescence from a series of weak flashes 30 ms apart measured 0.5 ms after each flash in the series, plotted vs. the flash number. The signals are normalized to their respective initial values. The flash has about 10^{11} photons/pulse per cm^2. About 1 photon absorbed/100 PS II centers. The flash full width at half maximum is 1 μs. (a) Control, (b) 0.1 μM valinomycin, (c) 0.2 μM gramicidin, (d) 10 μM DCMU, (e) heating for 3 min at 50°C, (f) 1.0 mM hydroxylamine. Incubation times are described in Materials and Methods.

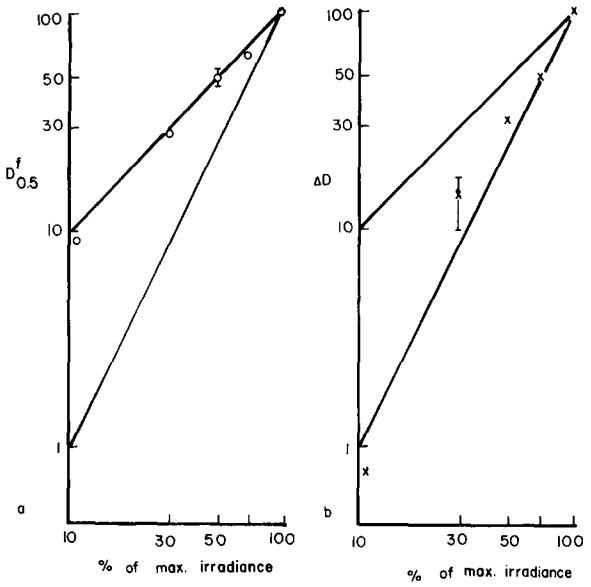

Fig. 2. Log-log plot of delayed fluorescence produced by a pair of equal strength flashes 30 ms apart as a function of irradiance. Each point is an average of nine runs. (a) $D_{0.5}^{f}$, the delayed fluorescence 0.5 ms after the first flash; (b) ΔD, the delayed fluorescence 0.5 ms after the second flash minus the delayed fluorescence 0.5 ms after the first flash. Both signals are normalized so that their maximum values are equal to 100. Slopes of one and two are shown for comparison. Maximum flash strength is about 10^{13} photons/pulse per cm^2. The flash full width at half maximum is 1.8 μm.

The time for the production of the enhancement was measured by using two flashlamps of nearly equal strenght and varying the time between firing them. In control experiments we measured the signals produced by each flashlamp alone. We call the signal at 0.5 ms after the second flash alone $D_{0.5}^{f}$, the signal at the variable time after the first flash alone D_{t}^{f}, and the signal from the pair of flashes, 0.5 ms after the second flash ${}_{t}^{f}D_{0.5}^{f}$. With weak flashes, in the absence of enhancement effects, the signals should be independent of one another, so that the ratio:

$$\frac{{}_{t}^{f}D_{0.5}^{f}}{(D_{t}^{f}+D_{0.5}^{f})}$$

should be unity. An increase in this ratio means that the first flash is having an effect on the delayed fluorescence produced by the second. In Fig. 3 we plot this ratio as a function of the time between the flashes. For short times the ratio is close to unity. At longer times, the ratio increases. The half-time for the rise is approx. 300 μs, consistent with an ob-

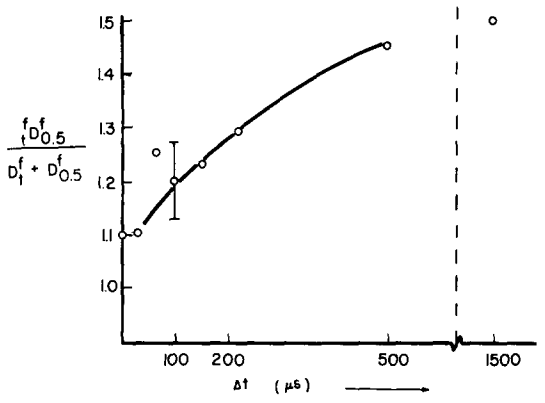

Fig. 3. Delayed fluorescence induction as a function of the time between two flashes. The circles represent the delayed fluorescence produced by the pair of flashes at a time 0.5 ms after the second flash, ${}_{t}^{f}D_{0.5}^{f}$, divided by the sum of delayed fluorescence from each flash separately, $D_t^f + D_{0.5}^f$. Both flashes have about $2 \cdot 10^{12}$ photons/pulse per cm^2. The flash full width at half maximum is 1 μs. Each point is an average of three runs.

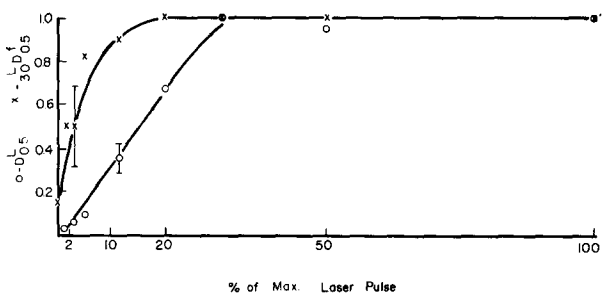

Fig. 4. Delayed fluorescence from the laser pulse and the following measuring flash as a function of laser pulse strength. Both signals are normalized to their respective maximum values. The time between the flashes was 30 ms. (o——o) Delayed fluorescence 0.5 ms after the laser pulse, $D_{0.5}^L$. The maximum laser pulse was $1.5 \cdot 10^{15}$ photons/pulse per cm^2. The laser pulse full width at half maximum was 7 ns at a wavelength of 490 nm. (x——x) Delayed fluorescence 0.5 ms after the measuring flash, ${}_{30}^{L}D_{0.5}^{f}$. The measuring flash always had about $3 \cdot 10^{11}$ photons/pulse per cm^2. The measuring flash full width at half maximum was 1 μs. With no preceding laser pulse, the measuring flash produces a signal of 0.15. This means that the half-saturating value for it is 0.57 rather than 0.5. This occurs at about 3.5% of our maximum laser pulse. The delayed fluorescence produced by the laser pulse does start from zero, so its half-saturating value is 0.5. This occurs at about 15% of our maximum laser pulse. The ratio of half-saturating values is about 4.

servation of Lavorel [17].

In the next series of experiments, we saturated the induction effect. The protocol was to use the laser as a variable first light pulse followed 30 ms later by the weak measuring flash from the flashlamp. The irradiance of the flashlamp was held constant, so that any changes in the delayed fluorescence produced by it were due to changes in the irradiance of the preceding laser pulse. In control runs without the measuring flash, the delayed fluorescence 30 ms after the laser pulse was too small to measure. In Fig. 4 we plotted the delayed fluorescence 0.5 ms after the laser fires, $D_{0.5}^L$, and the delayed fluorescence from the measuring flash, ${}_{30}^{L}D_{0.5}^{f}$, as a function of laser flash strength. Because of the smaller sample volume, the signal from the weak measuring flash was more difficult to measure than in the previous experiments. But it is clear that the effect of the laser flash on the delayed fluorescence produced by the second flash saturates well before the delayed fluorescence produced by the laser flash itself saturates. The half-saturation values differ by about a factor of 4. After a saturating laser flash, the flashlamp produces more than 5-times as much delayed fluorescence than it does with no preceding flash.

Delayed fluorescence transients are known to be influenced by many factors, including charge and pH gradients [8], availability of electron acceptors and donors [9,10], and the amount of charge on the oxygen-evolving system [5,12,13]. We tried several treatments to see which of these factors was important for the weak flash induction. To test for the effects of the electric field associated with the H^+ gradient on the weak flash delayed fluorescence enhancement, we used the ionophore valinomycin (Fig. 1b). Valinomycin is supposed to allow K^+ but not H^+ across the membrane [8]. We used gramicidin D to test for the effect of the pH component of the gradient (Fig. 1c). At the concentrations used here, gramicidin is supposed to allow both K^+ and H^+ through the membrane [18]. We examined the effects of acceptor availability by using DCMU (Fig. 1d), which is believed to block on the acceptor side after Q [9]. We tested the donor side by mild heating (Fig. 1e), which cuts off the oxygen-evolving enzyme as a source of electrons, and prevents the cycle of four oscillations in delayed fluorescence due to charge accumulation

on the enzyme [5,12,19]. We also tested the donor side by using hydroxylamine (Fig. 1f). At the concentration we used, the primary mode of action of hydroxylamine is supposed to be blocking electron transfer from Y, the primary donor, to P [20,21]. For all of the treatments except hydroxylamine, the signal nearly doubled by the tenth flash. With hydroxylamine, there was very little enhancement of the delayed fluorescence from the later flashes relative to that produced by the first.

Discussion

Our results, displayed in Fig. 2, confirm that the delayed fluorescence at 0.5 ms after a microsecond width flash has a linear dependence on irradiance. This is what would be expected from a single-hit process. The enhancement, however, increases as the product of the two flash irradiances, as shown in Fig. 2b. This is the result that would be expected from a process that requires a hit from both the first and the second flashes. For equal strength flashes, this leads to an I^2 dependence.

The data in Fig. 4 shows that the effect of the first laser flash on the delayed fluorescence produced by the second flash saturates at a lower irradiance than does the delayed fluorescence produced by the laser flash itself. Our first laser pulse lasted only 7 ns. This is faster than the P^+ reduction time of 20–35 ns [22]. Thus, the differences in the saturation curves are not due to double hitting within the laser flash. It is possible that we are observing processes associated with two different antenna sizes. Jursinic [23] has found evidence for antenna heterogeneity based on oxygen-evolution studies. Melis and Duysens [7] have found evidence for two different types of PS II centers. They found that the optical cross-sections of the antennas for the two types of centers differed by about a factor of three. Our half-saturation values, shown in Fig. 4, differed by about a factor of 4. We believe that our results can be explained by a model with two antenna sizes. We assume that there are two types of PS II centers with antenna sizes that differ by a factor of 3 or 4. We also assume that the 0.5 ms delayed fluorescence from the small antenna centers is relatively constant for the first two flashes, but that the 0.5 ms delayed fluorescence emitted by the larger centers is negligible after the first flash and greatly enhanced after the second flash. If these assumptions are true, then the enhancement effect should saturate at lower light levels than does delayed fluorescence. This is what we observed, as shown in Fig. 4.

The investigators who have used phosphoroscopes have had effective pulse widths ranging from hundreds of microseconds to milliseconds [1–3]. The data displayed in Fig. 3 show that significant induction occurs only with separations between the first and second flashes of at least a few tens of microseconds. The relatively long duration of each phosphoroscope flash would lead to double hitting within the pulse. This could lead to an apparent square law dependence of delayed fluorescence on irradiance. At very low light levels, the large antenna centers would be receiving single hits and the small antenna centers would receive very few hits so little delayed fluorescence would result. At higher light levels the large antenna centers would be receiving two hits, and the small antenna centers single hits. If the yield of delayed fluorescence from the doubly hit large antenna centers were much larger than the delayed fluorescence yield from the singly hit small antenna centers, most of the delayed fluorescence would follow a square law. At these light levels phosphoroscopes would show an I^2 dependence. As the light level is increased even more, the large antenna centers would saturate and produce a constant amount of delayed fluorescence, while more and more small centres would be hit. This would lead to a linear dependence at high light levels. Whether this actually occurs would depend on the relative number of small and large antenna centres, their relative antenna sizes, and their delayed fluorescence yields after one and two flashes. The only one of these parameters which we can determine unambiguously from our data is the ratio of the antenna sizes, which, as mentioned previously, is about 4. If the centres and antennas were homogeneous, precisely the opposite dependence would be expected. A linear dependence would be observed at low light levels, when double hitting is very improbable, and a square dependence at higher levels, when the chances of double hitting become significant. Some of the earlier models used to explain the I^2 dependence, such as triplet-triplet annihilation [2], the influence of neighboring traps due to the $L = J\phi$ relationship [24], or the semiconductor model, with

recombination of electrons and holes from two different types of traps [25], predict results inconsistent with those we observed (Fig. 3). If these models were correct, there should be maximal induction when the time between the flashes is a minimum. The half-time for the enhancement of a few hundred microseconds dark time suggests that some process such as turnover at a reaction center, or establishment of a membrane potential, must occur before enhancement can occur.

The irradiance dependence is consistent with the notion that the induction is due to an enhanced delayed fluorescence yield from the second hit at the large antenna centers. In an effort to find what the mechanism producing this enhanced delayed fluorescence yield might be, we subjected the samples to the treatments shown in Fig. 1b–f. Of all the treatments, only hydroxylamine stopped the delayed fluorescence induction studied here (Fig. 1f). At high concentrations, hydroxylamine reduces P^+. At the concentrations used here, hydroxylamine is supposed to block electron transfer from Y to P [20,21]. This suggests that the enhancement is involved with the charge states on the donor side. Van Best [10] has previously suggested heterogeneity of PS II centers based on studies of hydroxylamine effects on fluorescence and delayed fluorescence.

In contrast to hydroxylamine, mild heating led to an increase in the weak flash induction effect (Fig. 1e). The amount of heating was critical. A few seconds too much heating would destroy the increase. The same heat treatment that increased the induction effects of weak flashes reduced the delayed fluorescence from a series of saturating flashes (data not shown). Several authors have observed a cycle of four oscillations in delayed fluorescence enhancement when saturating flashes are used [5,12,13]. This enhancement with saturating flashes is apparently due to the influence of the changes in the charge states of the oxygen-evolving S-state mechanism. We interpret our heating results to mean that we were heating enough to destroy the S-state mechanism responsible for the period of four enhancement seen with saturating flashes, but that another donor side carrier was responsible for the weak flash enhancement. The carriers close to the reaction center are Y, the primary donor, and D, the auxiliary donor to P [20]. The relatively long time of 30 ms between our flashes suggests that some fairly stable state is involved. D is known to transport electrons much more slowly than Y [21]. It is possible that the delayed fluorescence induction is due to a back reaction involving D and is associated only with the centers with large antennas. This could indicate a difference in the electron-transport rates on the donor side for the two types of centers. In the absence of concomitant absorption and fluorescence measurements, however, the delayed fluorescence data alone do not permit construction of a more detailed model.

A problem with this model is that DCMU would also be expected to block the enhancement effect by preventing Q^- from being oxidized by the secondary acceptor. This means that double hitting should not occur so that we should not see enhancement. Our data in Fig. 1d, however, show that there is still an increase after DCMU is added. This is a transient increase. The steady-state value is near the initial value. For the control, the steady-state value is several times the initial value (data not shown). It is possible that this indicates some enhancement due to membrane gradients. Our experiments with valinomycin (Fig. 1b) and gramicidin (Fig. 1c) showed that these ionophores, which should reduce the charge and pH gradients, did not stop the weak flash enhancement. Jursinic et al., [26], working with saturating flashes, showed that these gradients were much less important for submillisecond delayed fluorescence than for that emitted at longer times. The same authors, however, also showed that there were some effects that were not eliminated by valinomycin and gramicidin. So while we have no evidence for the involvement of membrane gradients in the enhancement that we observe, we cannot rule out this possibility.

It is also possible that our model suggesting that the enhancement is due to sequential electron transport is correct, and that the enhancement in the presence of DCMU indicates that there are two acceptors before the DCMU block. Multiple Q's have been suggested in parallel [27,28] and also in series configurations [29,30]. If two acceptors are available in the presence of DCMU, the transient rise followed by a decrease in delayed fluorescence would be expected as the pool is filled. A model with homogeneous antennas and multiple acceptors before the DCMU block would be sufficient to account for the data shown in Fig. 1d. But it would not be sufficient to account for the data in Figs. 2 and 4. The most straightfoward

explanation for the irradiance dependence is that the enhancement effects are due to an increased second flash delayed fluorescence yield from a population of PS II centers having optical cross-sections approximately 4-times the size of the others.

Acknowledgments

The authors would like to thank Shmuel Malkin and Anastasios Melis for stimulating ideas. Thanks are also due to Jane Rice and Jim Lewis for the loan of their laser and invaluable assistance. This research was supported by Faculty Research Funds granted by the University of California, Santa Cruz. S.W.McC. was partially supported by a grant from the National Institute of Health (Grant No. 2507RR0713510) during this work.

References

1 Jones, L. (1967) Proc. Natl. Acad. Sci. U.S.A. 58, 75–80
2 Stacy, W.T., Mar, T., Swenberg, C.E. and Govindjee (1971) Photochem. Photobiol. 14, 197–219
3 Lavorel J. (1971) Photochem. Photobiol. 14, 261–275
4 Ruby, R.H. (1971) Photochem. Photobiol. 13, 97–111
5 Zankel, K. (1971) Biochim. Biophys. Acta 245, 373–385
6 Jursinic, P. and Govindjee (1977) Photochem. Photobiol. 26, 617–628
7 Melis, A. and Duysens, L.N.M. (1979) Photochem. Photobiol. 29, 373–382
8 Wraight, C.A. and Crofts, A.R. (1971) Eur. J. Biochem. 19, 386–397
9 Ruby, R.H. (1976) Photochem. Photobiol. 26, 293–298
10 Van Best, J.A. (1977) Studies on Primary Reactions of System 2 of Photosynthesis by Means of Luminescence and Fluorescence, Ph.D. Thesis, University Leiden, The Netherlands
11 Mar, T., Brebner, J. and Roy, G. (1975) Biochim. Biophys. Acta 376, 345–353
12 Van Gorkom, H.J. and Donze, M. (1973) Photochem. Photobiol. 17, 333–342
13 Barbieri, G., Delosme, R., and Joliot, P. (1970) Photochem. Photobiol. 12, 197–206
14 Govindjee and Jursinic, P. (1979) in Photochemical and Photobiological Reviews (Smith, K., ed.), vol. 4, pp. 125–205, Plenum Press, New York
15 Avron, M. (1974) in Experimental Plant Physiology (San Pietro, A., ed.), p. 22, C.V. Mosby Co., Saint Louis, MO
16 MacKinney, G. (1941) J. Biol. Chem. 140, 315–322
17 Lavorel, J. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 223–317, Academic Press, New York
18 Witt, H.T. (1971) Q. Rev. Biophys. 4, 365–477
19 Izawa, S. and Good, N.E. (1972) Methods Enzymol. 24, 358–377
20 Den Haan, G.A., Duysens, L.N.M. and Egberts, D.J.N. (1974) Biochim. Biophys. Acta 368, 409–421
21 Joliot, A. (1977) Biochim. Biophys. Acta 460, 142–151
22 Sonneveld, A., Rademaker, H. and Duysens, L.N.M. (1979) Biochim. Biophys. Acta 548, 536–551
23 Jursinic, P. (1979) Arch. Biochem. Biophys. 196, 484–492
24 Lavorel, J. (1969) Progr. Photosynth. Res. 2, 883
25 Arnold, W. and Azzi, J. (1971) Photochem. Photobiol. 14, 233–240
26 Jursinic, P., Govindjee and Wraight, C.A. (1978) Photochem. Photobiol. 27, 61–71
27 Joliot, P. and Joliot, A. (1977) Biochim. Biophys. Acta 462, 559–574
28 Lavergne, J. and Etienne, A. (1980) Biochim. Biophys. Acta 593, 136–148
29 Bowes, J., Crofts, A. and Itoh, S. (1979) Biochim. Biophys. Acta 547, 320–335
30 Maison-Peteri, B. (1980) Biochim. Biophys. Acta 592, 338–348