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STIMULATION OF MICROSECOND-DELAYED FLUORESCENCE FROM SPINACH CHLOROPLASTS BY UNCOUPLERS AND BY PHOSPHORYLATION

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Summary

Delayed fluorescence, as measured with a laser phosphoroscope, is stimulated not inhibited by uncouplers during the first 100 μs after the light is turned off. This is true only when uncouplers cause an increase in the rate of electron transport. When ADP and P_i cause an increase in the electron transport rate, microsecond-delayed fluorescence is also increased. Indeed, there is a complex quantitative relationship between the rate of electron transport and the initial intensity of delayed fluorescence under a wide range of conditions.

Uncouplers or ADP and P_i also increase the rate of decay of delayed fluorescence so that after about 150 μs they become inhibitory, as already reported by many authors.

Microsecond-delayed fluorescence continues to rise with rising light intensities long after the rate of reduction of exogenous acceptor is light-saturated.

These observations suggest a correlation of the rate of electron transport both with the intensity of the 5—100 μs-delayed fluorescence and with the rate of decay in the intensity of delayed fluorescence. The data imply that the decrease in intensity of millisecond-delayed fluorescence which has often been noted with uncouplers is probably not due to the elimination of a membrane potential. It seems more likely that the decrease in millisecond-delayed fluorescence is a reflection of the rate of disappearance of some other electron transport-generated condition, a condition which is uncoupler-insensitive. Certainly stimulations of microsecond-delayed fluorescence by electron transport which has been uncoupled by gramicidin suggest that ion gradients are not an essential component of the conditions responsible for delayed fluorescence.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine. DF*, values of delayed fluorescence corrected for changes in the efficiency of fluorescence of excited chlorophyll and expressed as the ratio of delayed fluorescence counts to prompt fluorescence counts multiplied by 1000.

Introduction

The delayed fluorescence of green cells and isolated chloroplasts appears to be a reversal of the initial photochemical and thermochemical events associated with Photosystem II [1] (for a review, see ref. 2). This fluorescence persists for milliseconds or longer and is envisioned as arising from a charge recombination within the Photosystem II reaction center involving the reduced primary electron acceptor (Q^-) and the oxidized form of some primary electron donor [3].

Uncouplers of photophosphorylation inhibit that part of the fluorescence which is delayed 3 ms or more after the light is extinguished [4]. Since uncouplers also inhibit the development of ion gradients across the lamellar membranes, it was suspected that delayed fluorescence in some way depends on a transmembrane hydrogen ion or salt concentration difference. This suspicion was greatly strengthened by Mayne's 1968 observation [5] that preilluminated chloroplasts emit light when shifted from an acidic to a basic medium and by Miles and Jagendorf's 1969 observation [6] that abrupt increases in salt concentration cause a similar burst of fluorescence (see also ref. 7). Fleischmann [8], Crofts et al. [9] and others suggested that photosynthetic electron transport establishes an electrochemical potential gradient across the membrane and that this membrane potential lowers the activation energy required for the recombination of the electron in Q^- with a hole in an electron donor, Z^+. The initial photochemical charge separations of photosynthesis probably occur across the membrane, reducing Q to Q^- toward the outside and oxidizing Z to Z^+ toward the inside [10]. If a membrane potential arises (positive to the inside), it would then facilitate the return of an electron from Q^- to Z^+. To explain the stimulation of delayed fluorescence by a pH gradient, Crofts et al. [9] suggested that the electron-carrying redox couples Q/Q^- and Z/Z^+ are in equilibrium with pools of secondary electron donors and acceptors which are hydrogen-carrying redox couples. These pools are supposed to be themselves in equilibrium with the aqueous phases on the opposite sides of the membrane and their redox potentials dependent on the pH of these phases. Thus, the pH difference across the membrane could increase the availability of either Q^- or Z^+ or both.

The first hint that the Fleischmann hypothesis might be wrong came from the observations of Neumann et al. [11] and Felker et al. [12]. They showed that, under certain conditions, uncoupling by ammonium salts enhanced millisecond-delayed fluorescence instead of inhibiting it. However, it was by no means certain that ammonia uncoupling actually abolished the membrane potential. Furthermore, no conditions were found where other uncouplers failed to inhibit millisecond-delayed fluorescence. Even methylamine, which seems to uncouple in the same manner as ammonia, was always inhibitory. Thus the Fleischmann model remained tenable.

We wish now to report an entirely different pattern of uncoupler effects on delayed fluorescence which is observed when the measurements are made a few microseconds after the excitation light is extinguished. The development of a laser phosphoroscope which is capable of making reliable measurements of delayed fluorescence within microseconds [13] allowed us to investigate the

effects of uncouplers in this time range (see Methods).

It should be emphasized that our selection of a phosphoroscope was deliberate and not motivated by the convenience of rapidly summing the signals. We were particularly concerned about the relationship of delayed fluorescence to the cumulative effects of frequently repeated periods of illumination on that energized state of the membrane which is responsible for ATP formation and is abolished by uncouplers. The concern arose from the fact that this state was reputed to be involved in delayed fluorescence [8,9]. Single flash experiments on dark-adapted chloroplasts would have provided us with no information on these matters and, indeed, it is not at all clear why such single flash experiments should be affected by uncouplers at all. It seemed to us that single flash experiments on preilluminated chloroplasts would have suffered from the ambiguities of the phosphoroscope without the advantages. Both the phosphorescope and the single flash following preillumination have the disadvantage that the delayed fluorescence following the period of illumination may be superimposed on the residual delayed fluorescence associated with the previous period of illumination. On the other hand, the phosphoroscope has the added advantage that we are able to measure electron transport and delayed fluorescence in the same experiment and this is patently impossible with a single flash.

The series of rapidly repeated illuminations with the strong light employed in the laser phosphoroscope provided an average intensity capable of saturating the electron transport system and, indeed, the rate of electron transport with this intermittent light was no less than with continuous light of the same intensity. Therefore, it is reasonable to suppose that the electron carrier pools at the end of each millisecond illumination period were close to the conditions achieved during steady-state electron transport.

We found that, in stimulating electron transport, uncouplers also increase the intensity of the delayed fluorescence measured at any time between 5 and 100 μs after light extinction. However, the rate of decline in the intensity of the delayed fluorescence with time after light extinction is also increased by uncouplers, so that within milliseconds uncouplers appear to inhibit delayed fluorescence. These observations seem to preclude the possibility that any energized state of the membrane system which is sensitive to uncouplers can be required for the delayed fluorescence reported here, be it a membrane potential, an ion gradient, or a transmembrane pH difference.

Materials and Methods

Chloroplast lamellae were isolated from commercial spinach by the method of Ort and Izawa [14] and resuspended in a solution containing 0.2 M sucrose, 5 mM HEPES-NaOH (pH 7.4) and 2 mM $MgCl_2$.

Nigericin and gramicidin were added in ethanol. The final ethanol concentration in the reaction mixture was always less than 3%. This concentration of ethanol had no effect on the phenomena being studied.

The laser phosphoroscope employed was a modification of that described by Beall and Haug [13]. Briefly, the light produced by a continuous output argon laser (principle lines at 488 and 515 nm) was focussed by a microscope objective and chopped by a rotating sector at the focal point. This gave a cycle

of 1 ms light and 1 ms dark with a light-dark transition time of less than 250 ms. The light beam was then collimated and directed through the bottom of a $1 \times 1 \times 4.5$ cm quartz cuvette. Prompt and delayed fluorescence were measured at a right angle to the actinic beam by a single photomultiplier tube cooled with solid CO_2. A 690 nm interference filter was placed between the cuvette and the photomultiplier to shield it from stray actinic light. The 50 Ω output of the photomultiplier was in discrete pulses 1—3 ns in length, each pulse associated with individual photons striking the cathode surface. To measure the delayed fluorescence, only those pulses generated during a specified period after light extinction were counted. To do this its timing circuit was activated at the instant of light extinction. This timing circuit created a counting "window" by opening a gate from the photon detector to the counter for a prescribed time. Under most circumstances this window began 5 μs after light extinction and ended 10 μs after light extinction. The pulses arriving during 10 000 of these gated counting periods were summed (one counting period every 2 ms for 20 s). The duration and time of commencement of the counting window could be varied independently so that the rate of decay of delayed fluorescence could be determined. When longer delays occurred before the fluorescence was measured, the rate of photon counting was so much slower that the window was extended considerably, to 25 μs when the delay was 500 μs and to 100 μs when the delay was 1 ms. However, in Figs. 1 and 2, the data presented have taken into account this change in counting time. Prompt fluorescence was measured with the same photomultiplier by allowing the gate to open to the counter while the actinic light was still on.

Delayed fluorescence is reported as the ratio of counts during 5 μs divided by the prompt fluorescence in 5 μs for the following reasons; we are interested not so much in the actual delayed fluorescence as in the thermochemical reactions responsible for the excitation of the chlorophyll. Since delayed fluorescence and prompt fluorescence seem to come from the same chlorophyll, it is reasonable to assume that the chemically excited chlorophyll responsible for delayed fluorescence fluoresces with the same efficiency as the light-excited chlorophyll responsible for prompt fluorescence. Thus, dividing by the prompt fluorescence should help to correct for variations in the fluorescence yield of excited chlorophyll although, to be sure, the prompt fluorescence yield was not measured and could not be measured at the same instant as the delayed fluorescence. Nevertheless, these "corrected" values for delayed fluorescence should reflect the recombination reactions we wish to measure more accurately than the delayed fluorescence itself. The "corrected" values have the added advantage of normalizing the results of the various experiments since each data point represents a separate experiment and there are inevitable small variations in the actinic light intensity, chloroplast density and chloroplast condition.

In the same experiments electron transport was measured as the reduction of ferricyanide. This was done by observing the changes in the ferricyanide absorbance at 420 nm. A weak light was passed through a 420 nm interference filter before it passed through the cuvette. This beam was then detected with a photomultiplier which was protected by another 420 nm interference filter to screen out the actinic light. The signal from the photomultiplier was processed by a logarithmic amplifier before it was fed into a strip chart recorder so that

rates of change in the concentration of ferricyanide could be directly determined.

In all cases the values given for delayed fluorescence intensity are the mean of 5 determinations. Prompt fluorescence was measured after 20 s of preillumination and immediately before and after the delayed fluorescence determinations. All experiments were conducted at room temperature, approx. 23°C. The intensity of the light from the laser impinging on the reaction cuvette was approximately 80 $mW \cdot cm^{-2}$. The reaction mixture occupied a cubic volume 1 cm on each side.

The contrast ratio of the measurements of the apparent delayed fluorescence and actual prompt fluorescence observed when there was really no delayed fluorescence at all, was determined by replacing the chloroplast-containing reaction mixture with a solution of chlorophyll in acetone. With this chlorophyll solution, the counts falsely attributed to the 5—10 μs delayed fluorescence were always less than one per 10 000 counts of prompt fluorescence measured over the same length of time. The intensity of the delayed fluorescence from chloroplasts was routinely ten times or more higher than this noise level.

Results

Uncouplers of photophosphorylation and phosphorylating conditions (ADP + P_i) increase the intensity of delayed fluorescence for the first 100 μs after the light is turned off (Figs. 1 and 2). Ammonium ions routinely increase the number of photons emitted 5—10 μs after illumination about 3-fold. Increases due to other uncouplers and phosphorylating conditions are somewhat smaller, viz., 50—100%. These stimulations of delayed fluorescence are real and not artifacts of our method of correcting for the fluorescence efficiency of excited chlorophyll (see Methods), since they are present and of a similar magnitude whether or not such corrections are made. On the other hand, the rate at which delayed fluorescence dies away after the light is off is markedly increased by uncoupling and by ADP and P_i. Consequently uncoupler-influenced delayed fluorescence is usually less than in the control after about 150 μs. As has been noted previously, delayed fluorescence disappears almost entirely in 1—2 ms with many uncouplers.

It is clear from these observations that the intensity of delayed fluorescence cannot be dependent on any uncoupler-inhibited state of the membrane system. On the contrary, some process which is enhanced by uncouplers or ADP and P_i must be determining delayed fluorescence. Since all uncouplers share with ADP + P_i the ability to increase the electron transport [15] and the ability to increase the intensity of 5-μs delayed fluorescence, we sought and found a correlation between electron transport rates and the initial intensity of delayed fluorescence.

Table I illustrates the correlation between electron transport and 5-μs delayed fluorescence. In these experiments, electron transport was varied in three ways: It was greatly diminished by omitting an exogenous electron acceptor or almost abolished with the inhibitor DCMU. In both cases the intensity of μs-delayed fluorescence was much lowered, in the case of DCMU almost

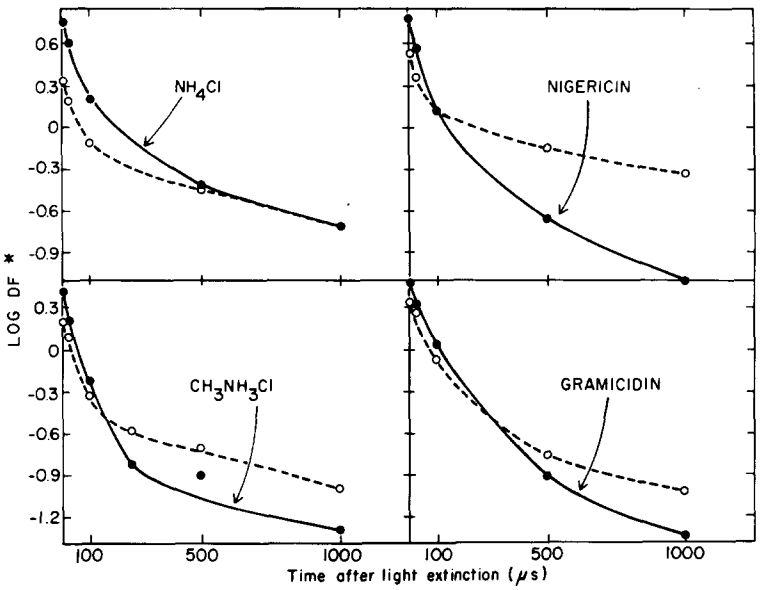

Fig. 1. Delayed fluorescence in spinach chloroplasts and its rate of decay in the presence and absence of uncouplers. Reaction mixtures contained in 1.0 ml: chloroplasts with 5—10 μg chlorophyll; $MgCl_2$, 2 μmol; Tricine-NaOH (pH 7.8—8.1), 50 μmol; potassium ferricyanide, 0.5 μmol; sucrose, 100 μmol. When added: NH_4Cl, 5 μmol; methylamine-HCl, 5 μmol; gramicidin, 5 μg; nigericin, 5 μg. Ordinates are log *DF**, where *DF** is 1000 times the ratio of the number of delayed fluorescence photons counted in 5 μs in the dark to the number of prompt fluorescence photons counted in 5 μs in the light. At 500 and 1000 μs after the light was turned off, the periods of counting of delayed fluorescence photons were increased to 25 and 100 μs, respectively, with appropriate corrections. Solid lines represent decays of delayed fluorescence in the presence of the uncoupler and dotted lines represent decays of delayed fluorescence in the absence of the uncoupler. Note that the decay is polyphasic whether or not uncouplers are present. This suggests that different reactions probably limit the back-reaction responsible for the chemical excitation of chlorophyll at different times.

absent. Or electron transport was greatly increased by the use of uncouplers in which case, as already noted, the increase in microsecond-delayed fluorescence was marked. It is particularly important to note that the increase in delayed fluorescence caused by uncouplers, which is noted in the presence of an exo-

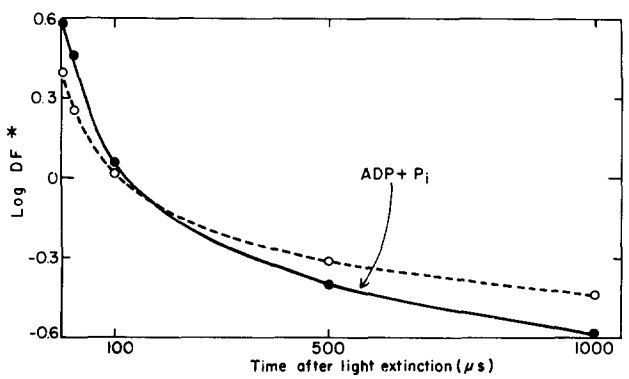

Fig. 2. Delayed fluorescence in spinach chloroplasts and its rate of decay in the presence and absence of ADP and orthophosphate. Reaction conditions and data presentations as in Fig. 1. When added, ADP was 1.5 μmol, and K_2HPO_4, 5 μmol.

TABLE I

THE EFFECTS OF FERRICYANIDE, UNCOUPLERS, AND DCMU ON THE RELATIVE INTENSITIES OF 5—10 MICROSECOND-DELAYED FLUORESCENCE AND ELECTRON TRANSPORT

Reaction conditions as in Fig. 1. DCMU when used was 100 nmol. Rates of electron transport are in μmol electrons $\cdot h^{-1} \cdot mg^{-1}$ Chl Rate of electron transport was measured with an oxygen electrode in a parallel experiment as oxygen production in the presence of ferricyanide and as oxygen consumption in its absence.

Additions	Relative *DF**	Electron transport
None	46	64
Methlyamine-HCl	46	65
Gramicidin	51	59
Ferricyanide	100	280
Ferricyanide, methylamine-HCl	187	1170
Ferricyanide, gramicidin	147	1240
Ferricyanide, DCMU	14	46

genous electron acceptor, does not occur when the electron transport rate is limited by the absence of an exogenous electron acceptor. That is to say, uncouplers do not increase the intensity of the μs-delayed fluorescence when the uncouplers fail to increase the electron transport rate.

Under some conditions, μs-delayed fluorescence is a nearly linear function of the rate of electron transport (Fig. 3). However, this linear relationship may only apply to a rather narrow range of special situations. For instance, the partial inhibition of electron transport by DCMU, which blocks transport before

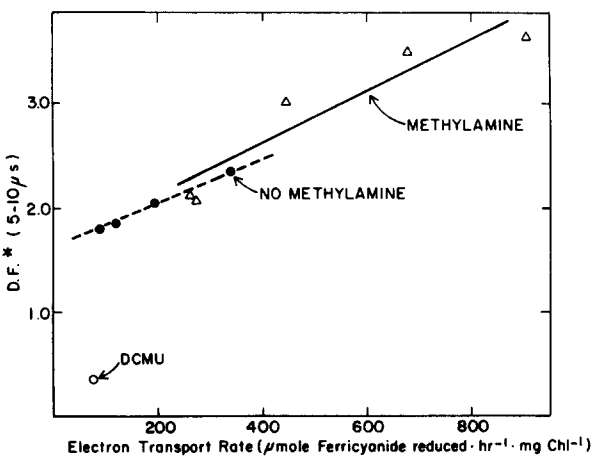

Fig. 3. The relationship of electron transport rate to the intensity of delayed fluorescence. In order to lower the electron transport rate without lowering the light intensity, the chloroplasts were pretreated with KCN, thus inactivating a portion of the plastocyanin [16]. A variety of electron transport rates were achieved by varying the length of the KCN pretreatment (up to 48 min) and by uncoupling with methylamine. △, experiments with methylamine addition. Cyanide pretreatment was in an ice bath in the dark with the following mixture: Tricine-NaOH (pH 8.0), 100 mM; sucrose, 100 mM; $MgCl_2$, 1 mM; KCN, 50 mM; and potassium ferricyanide, 50 μM. Reaction mixture as in Fig. 1. Methylamine-HCl when used, 5 mM. Numbers in parentheses represent minutes of incubation of the chloroplasts in the KCN. The values of the point labelled "DCMU" were calculated from the data of Table I.

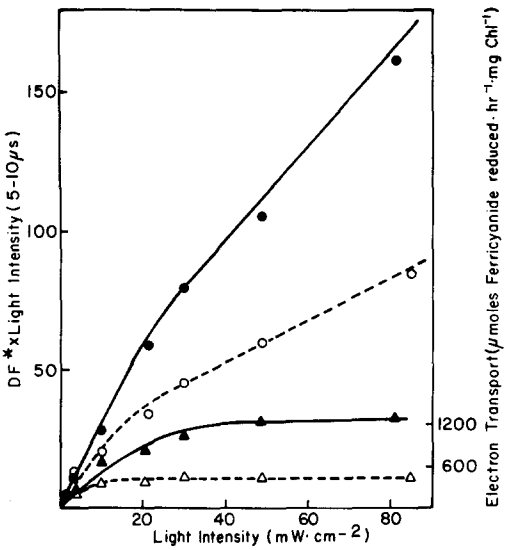

Fig. 4. Delayed fluorescence and electron transport as functions of the exciting light intensity in the presence and absence of the uncoupler gramicidin. The value for "corrected" delayed fluorescence (*DF**) is obtained by dividing the observed delayed fluorescence by the prompt fluorescence. Since prompt fluorescence under any one set of conditions is usually proportional to the incident light intensity, for the purposes of the comparison made here it was necessary to multiply *DF** by the light intensity. The maximum rate of electron transport in the absence of gramicidin was over 400 μmol ferricyanide reduce · $h^{-1} \cdot mg^{-1}$ Chl, which suggests that these chloroplasts had probably been inadvertantly partially uncoupled.

plastoquinone, gives much more inhibition of μs-delayed fluorescence than would be predicted from an extrapolation of the straight line in Fig. 3 where the electron transport rate was varied by inhibiting plastocyanin. For that matter, this straight line does not extrapolate through the origin of the graph and, therefore, it would be wrong to conclude that delayed fluorescence even requires the net transfer of electrons to an exogenous acceptor. Furthermore, different uncouplers sometimes give different intensities of delayed fluorescence at the same rate of reduction of the exogenous acceptor (data not shown).

Changing the rate of electron transport by changing the light intensity has very different effects on the delayed fluorescence (Fig. 4). Long after the light intensity has been raised to a level where the electron transport system is saturated, that is to say long after the rate of reduction of exogenous electron acceptor has stopped increasing, the delayed fluorescence continuous to increase. This is true whether or not the chloroplasts have been uncoupled by gramicidin. However, regardless of the light intensity used, the uncoupler does increase the 5—10-μs delayed fluorescence.

Discussion

The effects of uncouplers on microsecond-delayed fluorescence in chloroplasts have been observed by Jursinic et al. [17] using a single flash with and

without preillumination. These authors observed no inhibition with concentrations of gramicidin sufficient to abolish any membrane potential-induced absorbance change at 518 nm. They also observed that there was no enhancement of 6—100 microsecond-delayed fluorescence in the presence of valinomycin in response to an abrupt increase in KCl concentration. Consequently they concluded that membrane potentials probably did not play any part in providing activation energy for microsecond-delayed fluorescence in their systems.

Our conclusions based on entirely different data are very similar. Uncouplers which should diminish or abolish ion gradients and membrane potentials actually increase microsecond-delayed fluorescence if electron transport is also increased. It should be emphasized that the latter observations are not at variance with the observations of Jurisinic et al. since their gramicidin effects were observed in the absence of an electron acceptor and therefore in the absence of a high rate of electron transport during preillumination. We also found that gramicidin neither increased nor decreased microsecond-delayed fluorescence in the absence of an exogenous electron acceptor (see Table I).

We also report that uncouplers and phosphorylating conditions increase the rate of decay of the delayed fluorescence so that after milliseconds the delayed fluorescence is diminished. Since the decay in the ability of chloroplasts to produce delayed fluorescence is complex, it is difficult to determine the nature of the processes involved in the decay. It seems likely that the delayed fluorescence measured microseconds after light extinction is the result of conditions which are unrelated to the membrane potential but are intimately associated with the rate of electron transport. It seems probable to us that the stimulation of microsecond-delayed fluorescence and the increased rate of decay are both due to some as yet unspecified effects of uncouplers of electron transport, effects that determine the levels of the reactant species (Z, Q, *P*-680) arrived at the light and also determine the rate at which they disappear in the dark by non-radiative mechanisms.

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References

1 Strehler, B.L. and Arnold, W. (1951) J. Gen. Physiol. 304, 809—820
2 Lavorel, J. (1975) in Bioenergetics of photosynthesis (Govindjee, ed.), pp. 223—317, Academic Press, New York
3 Arthur, W.E. and Strehler, B.L. (1957) Arch. Biochem. Biophys. 70, 507—526
4 Mayne, B. (1967) Photochem. Photobiol. 6, 189—197
5 Mayne, B. (1968) Photochem. Photobiol. 8, 107—113
6 Miles, C.D. and Jagendorf, A. (1969) Arch. Biochem. Biophys. 129, 711
7 Barber, J. and Kraan, G.P.B. (1970) Biochim. Biophys. Acta 197, 49—95
8 Fleischmann, D.E. (1971) Photochem. Photobiol. 14, 277—286
9 Crofts, A.R., Wraight, C.A. and Fleischmann, D.E. (1971) FEBS Lett. 15, 89—100

10 Kraan, G.P.B., Amesz, J., Velthuys, B.R. and Steemers, R.G. (1970) Biochim. Biophys. Acta 223, 129—145
11 Neuman, J., Barber, J. and Gregory, P. (1973) Plant Physiol. 51, 1069—1073
12 Felker, P., Izawa, S., Good, N.E. and Haug, A. (1974) Arch. Biochem. Biophys. 162, 345—356
13 Beall, H.C. and Haug, A. (1973) Anal. Biochem. 53, 98—107
14 Ort, D.R. and Izawa, S. (1973) Plant Physiol. 52, 595—600
15 Good, N.E. (1977) in Encyclopedia of Plant Physiology (Trebst, A. and Avron, M., eds.), Vol. 5, pp. 429—447, Springer-Verlag, Berlin
16 Ort, D.R., Izawa, S., Good, N.E. and Krogmann, D.W. (1973) FEBS Lett. 31, 119—122
17 Jursinic, P., Govindjee and Wraight, C.A. (1978) Photochem. Photobiol. 27, 61—71