

Insight into the relationship of chlorophyll *a* fluorescence yield to the concentration of its natural quenchers in oxygenic photosynthesis

(photosystem II/S states of oxygen-evolving complex/quinone acceptors/*Spinacia oleracea*)

VLADIMIR P. SHINKAREV*† AND GOVINDJEE*‡§

*Department of Physiology and Biophysics and †Department of Plant Biology, University of Illinois, Urbana, IL 61801

Communicated by Gregorio Weber, May 3, 1993

ABSTRACT Fluorescence of chlorophyll *a* (Chl*a*) is a noninvasive and very sensitive intrinsic probe of photosynthesis. It monitors the composition and organization of the photosystems, the exciton energy transfer, the photochemistry, and the effects of various types of stress on plants. It is the most used as well as the most abused tool in photosynthesis. Thus, an understanding of its relationship to photosynthesis has been of paramount importance. Both the oxidized primary plastoquinone, Q_A, and the oxidized primary reaction-center Chl*a*, P680⁺ (for short, P⁺), are known to be quenchers of Chl*a* fluorescence yield (ϕ_f) of photosystem II. Flash-number dependence of Chl*a* fluorescence yield shows either a period 4, due to the four-step charge-accumulation process of water oxidation (donor side), or period 2 behavior, due to the two-step reduction of the plastoquinone Q_B (acceptor side) of photosystem II reaction centers. We provide here a further insight into the relationship of variable Chl*a* fluorescence yield (ψ_f) to the concentration of the two quenchers. The observed time dependence of the ratio of ψ_f after flash 3 to that after flash 1 (or flash 5) in spinach thylakoids at pH 6 can be explained if we suggest that $1/\phi_f \approx a[\text{PQ}_A] + b[\text{P}^+] + c$, where *a*, *b*, and *c* are constants. From this it follows that the quenching of Chl*a* fluorescence by P680⁺ after a flash is dependent on Q_A: for low [Q_A] (when most reaction centers are closed, [PQ_A] is low) the quenching of Chl*a* fluorescence by P680⁺ predominates, while for high [Q_A] (when most reaction centers are open), the quenching of Chl*a* fluorescence is due predominantly to the increased concentration of the reduced form of P680 ([P⁺] is low).

The quantum yield of chlorophyll *a* (Chl*a*) fluorescence (ϕ_f) is determined by the expression (see, e.g., refs. 1 and 2)

$$\phi_f = k_f / (k_f + k_\Sigma + k_{\text{phot}}), \quad [1]$$

where k_f is the rate constant of fluorescence, k_Σ is the sum of the rate constants for radiationless deexcitation, excluding photosynthesis, and k_{phot} is the (pseudo)monomolecular rate constant of photosynthesis, which reflects photochemical utilization. In oxygenic photosynthesis, there are two photochemical reactions, labeled I and II, that operate in series (e.g., ref. 3). Photosystem II (PSII) oxidizes water and reduces plastoquinone, whereas photosystem I (PSI) oxidizes plastoquinol, via several intermediates, and reduces pyridine nucleotide, NADP⁺. It is the photochemistry of PSII, not that of PSI, that controls the ϕ_f of plants (e.g., ref. 2). The quenching of Chl*a* fluorescence in photosynthesis was suggested (4) to be mainly determined by the oxidized form of the primary quinone acceptor (Q_A) of the PSII

reaction center. Considering that $k_{\text{phot}} = k_p[\text{Q}_A]$, we may rewrite Eq. 1 as

$$\phi_f = k_f / (k_f + k_\Sigma + k_p[\text{Q}_A]), \quad [2]$$

where k_p is the bimolecular rate constant for PSII photochemistry.

In PSII, light initiates the electron transfer from the primary Chl*a* donor (P680) to the primary electron acceptor pheophytin (Pheo), forming P680⁺ and Pheo⁻ (5). The latter transfers its electron to Q_A, which, when reduced, transfers it to the secondary quinone acceptor (Q_B). Unlike Q_A, which is a one-electron carrier, Q_B is a two-electron acceptor (see ref. 6). Plastoquinol (Q_BH₂), generated after a double turnover of PSII, then exchanges with an oxidized molecule of the plastoquinone pool (see review in ref. 7). This two-step process, also called the two-electron gate, leads to a period 2 oscillation in Chl*a* fluorescence yield because electron flow from Q_A⁻ to Q_B is faster than that from Q_A⁻ to Q_B⁻ (8). On the other hand, P680⁺, produced in the photochemical reaction of PSII, transfers its positive charge to a manganese complex (its redox state being labeled as S), via an intermediate, Y_Z (a tyrosine moiety). Here, however, four positive charges must accumulate before water is oxidized to molecular O₂. This leads to a periodicity of 4 in the O₂ evolution when measured as a function of flash number (e.g., ref. 9; reviews in refs. 10 and 11).

An increase in the Chl*a* fluorescence yield after short flashes in the nanosecond to submicrosecond time range (12) suggested that P680⁺ is also a quencher of Chl*a* fluorescence (13). To accommodate the quenching character of both P680⁺ and Q_A, Sonneveld *et al.* (14, 15) assumed that the most fluorescent state of PSII is PQ_A⁻ (where P represents P680). With such an assumption, it was easy to explain the dependence of the rate of Chl*a* fluorescence-yield changes upon the redox state of not only the acceptor side, but also the donor side, of PSII (16). Depending on the experimental conditions, one can observe, when plants are exposed to a series of flashes, a periodicity of 4 in the fast (microsecond) (17, 18) or in the slow (millisecond and second) components of Chl*a* fluorescence yield (18, 19) as affected by the four-step charge-accumulating process in the oxygen-evolving complex. The flash number-dependent period 4 oscillation in the Chl*a* fluorescence yield is explained by the different rate of

Abbreviations: Chl, chlorophyll; PSII, photosystem II; P680 (or P), electron donor of PSII; Q_A, primary one-electron quinone acceptor of PSII; Q_B, secondary two-electron quinone acceptor; S_k, charge-accumulating state on the donor side of PSII (where *k* is 0, 1, 2, 3, or 4); Y_Z, a tyrosine of PSII that is the electron donor to P680⁺.

†On leave from Biophysics Section, Department of Biology, Moscow State University, Moscow (Russia).

§To whom reprint requests should be addressed at: Department of Plant Biology, University of Illinois at Urbana-Champaign, 265 Morrill Hall, 505 South Goodwin Avenue, Urbana, IL 61801-3707.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

reduction of the quencher P680⁺ by Y_Z, which is dependent upon the redox state of the water oxidation complex, the S states.

In this paper, we analyzed the changes in Chl_a fluorescence yield that occur from 70 μs to 10 ms after actinic flashes in spinach thylakoids at pH 6. In view of the two-step reduction of plastoquinone discussed above, alternate flashes would place the electron acceptor side in the same status. Thus to diminish contributions of the acceptor side, we used the ratios of the Chl_a fluorescence yields after flashes 1, 3, and 5 (flash 3/flash 1 and flash 3/flash 5). Analysis of such data in terms of the proposed relationship $1/\phi_f \approx a[\text{PQA}] + b[\text{P}^+] + c$ (where [PQA] is the normalized concentration of the open traps, [P⁺] is the normalized concentration of P680⁺, and *a*, *b*, and *c* are constants) leads us to conclude that the quenching of ϕ_f by P680⁺ is dependent upon the concentration of Q_A: for low [Q_A], quenching increases with increased [P680⁺], but for high [Q_A], quenching increases with decreased [P680⁺].

MATERIALS AND METHODS

Spinach (*Spinacia oleracea*) thylakoids were isolated as described (20). The reaction medium contained 0.4 M sorbitol, 50 mM NaCl, 2 mM MgCl₂, and 20 mM Mes (pH 6). The concentration of Chl in the sample was 10 μM. When used, benzoquinone was at 40 μM. All the additions were made in the dark.

Chl_a fluorescence yields after single-turnover saturating flashes were measured as described (21). Measurements of relative fluorescence yield, at different times after a flash, were made on 10-min-dark-adapted spinach thylakoid suspensions. The initial fluorescence intensity, *F*₀, was measured with a weak (exciting only 1% of reaction centers), short (2.5 μs), blue (CS 4-96 Corning glass) xenon flash. Decays of relative fluorescence yield, after single-turnover saturating blue flashes, were then measured from 70 μs to 10 ms, also with weak flashes, used previously to monitor *F*₀. The data were plotted as $(F - F_0)/F_0$. This is nothing else but the variable Chl_a fluorescence yield, normalized to the yield of "constant" fluorescence. The latter is proportional to the yield prior to photochemistry (when [Q_A] = 1, the maximum) and is emitted in competition only with the excitation energy transfer to the reaction center (16).

RESULTS AND DISCUSSION

Kinetics of Chl_a Fluorescence Induced by the First, Third, and Fifth flashes. Fig. 1 shows the kinetics of Chl_a fluorescence of spinach thylakoids at pH 6 induced by the first, the third, and the fifth flash, in the absence (A) or presence (B) of 40 μM benzoquinone. Benzoquinone was added to ensure that the quinone acceptor complex of the reaction center of PSII was mostly in the Q_AQ_B state (22, 23). Thus, subsequent flashes of light were expected to induce the binary oscillation of the quinone acceptor complex, producing the semiquinone form of Q_B⁻ after the first, third, etc. flashes. Our results on Chl_a fluorescence-yield decay (Fig. 1, compare A and B) show that in our samples the addition of benzoquinone did not make any significant difference. Thus, in our samples PSII was mostly in the Q_AQ_B state.

Fig. 2 shows the ratio of the relative Chl_a fluorescence yields induced by the third and the first as well as by the third and the fifth flash for spinach thylakoids in the absence (A) or presence (B) of 40 μM benzoquinone. These ratios increased with a characteristic time of about 0.3 ms and decreased with a characteristic time of about 2 ms. To understand the observed time dependence of these ratios we will first discuss the well-known view of the relationship of

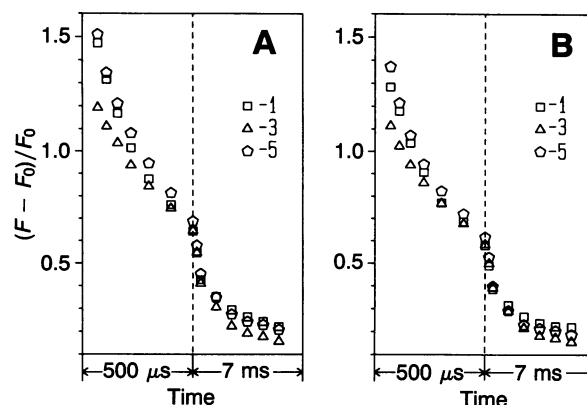


FIG. 1. Kinetics of the variable Chl_a fluorescence yield, $\psi_f = (F - F_0)/F_0$, induced by the first (□), the third (Δ), and the fifth (○) single-turnover actinic flash in spinach thylakoids, plotted in two time scales. Dark time between flashes, 1 s. The size of the data symbols includes the error bar and the uncertainty in the measurement. Suspension medium was 20 mM Mes, pH 6/0.4 M sorbitol/50 mM NaCl/2 mM MgCl₂ without (A) or with (B) 40 μM benzoquinone.

Chl_a fluorescence yield to the concentration of quenchers and then describe our understanding of the phenomenon.

Quantum Yield of Chl_a Fluorescence: The Well-Known View. The quantum yield of Chl_a fluorescence can be described by an extension of Eq. 1, the classical Stern-Volmer equation for PSII (24):

$$\phi_f = k_f / (k_f + k_\Sigma + k_p[\text{PQA}]). \quad [3]$$

When the reaction centers are open—i.e., when [Q_A] is maximal—the relatively high concentration of reaction centers in the state PQA is responsible for the decrease of fluorescence yield. However, Eq. 3 does not include the well-known quenching of Chl_a fluorescence by P680⁺ (13). To resolve this problem, Duysens and coworkers (14, 15, 24) have assumed that the relative Chl_a fluorescence yield, ψ_f , is proportional to the relative concentration of state PQA⁻ (also see ref. 25):

$$\psi_f \propto [\text{PQA}^-]. \quad [4]$$

This relationship qualitatively describes the Chl_a fluorescence quenching by both P680⁺ (since more P means less P⁺ and vice versa) and Q_A.

From Eq. 4, and the fact that after alternate flashes, the electron acceptor side is almost identical, it follows that the ratio of Chl_a fluorescence yield induced by the third [$\psi_f(3)$] and first [$\psi_f(1)$] flashes (as well as the third and fifth flashes) will mainly depend on the donor side of the reaction center:

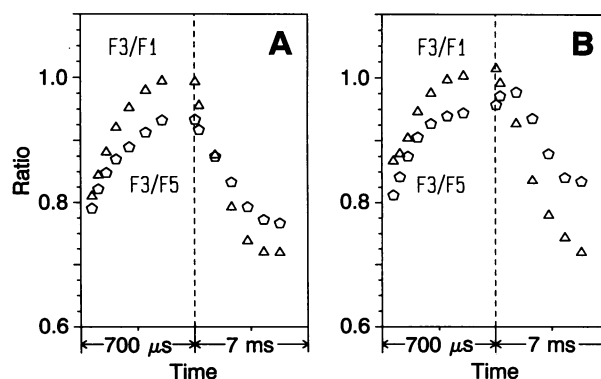


FIG. 2. Ratio of Chl_a fluorescence yields induced by the third (F3) and the first (F1) flash (Δ) and by the third (F3) and the fifth (F5) flash (○) in the absence (A) or presence (B) of 40 μM benzoquinone.

$$\begin{aligned}\psi_f(3)/\psi_f(1) &\approx P_3(t)/P_1(t) \\ \psi_f(3)/\psi_f(5) &\approx P_3(t)/P_5(t),\end{aligned}\quad [5]$$

where $P_k(t)$ is the concentration of the reduced form of P680 at time t after the k th flash, normalized to the reaction-center concentration.

Eq. 5 shows that the ratio $\psi_f(3)/\psi_f(1)$ in the microsecond to millisecond time scale must be determined only by the kinetics of the P680⁺ reduction as affected by the four-step charge-accumulating S states.

The kinetics of P680⁺ reduction after a flash in the microsecond to millisecond time domain is determined by a sequence of the monomolecular transitions: $S_k Y_Z P^+ \rightleftharpoons S_k Y_Z^+ P \rightleftharpoons S_{k+1} Y_Z P$. The transition $S_k Y_Z P^+ \rightleftharpoons S_k Y_Z^+ P$ occurs in the submicrosecond time domain, while the transition $S_k Y_Z^+ P \rightleftharpoons S_{k+1} Y_Z P$ occurs in the micro- to millisecond time domain. Hence, the fast component of the P680⁺ reduction (due to the reaction $S_k Y_Z P^+ \rightleftharpoons S_k Y_Z^+ P$) is out of the time resolution of our experiments, but we can consider the following kinetics by assuming that a quasi-equilibrium is reached in this reaction. Reduction of Y_Z in the reaction $S_k Y_Z^+ P \rightleftharpoons S_{k+1} Y_Z P$ will "force" the further reduction of P^+ . Only this slow component of P^+ reduction could be responsible for the period 4 modulation of Chla fluorescence yield considered below. The times of the S transitions are known to be approximately 30, 100, 300, and 1000 μ s for $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, and $S_3 \rightarrow S_0$ transitions, respectively (e.g., ref. 26).

Each $P_k(t)$ in Eq. 5 must be an increasing function of time due to the stabilization, through the S states, of the reduced form of P680 in the microsecond time domain. This means that the ratios in Eq. 5 must increase with a characteristic time of about 1000 μ s (numerator in Eq. 5), corresponding to the reduction of P680⁺ through the S_3 state, and decrease with a characteristic time of about 100 μ s (denominator in Eq. 5), corresponding to the reduction of P680⁺ through the S_1 state. In contrast to this prediction, the experimentally measured ratios (Fig. 2) decrease in the millisecond time range. Even if one considers that, in dark-adapted samples, the initial state of the donor side of PSII is a mixture of 75% S_1 and 25% S_0 states of the oxygen-evolving complex (9, 10), the same contradictory behavior persists. This is the observation that led to a further understanding of the factors which control Chla fluorescence yield.

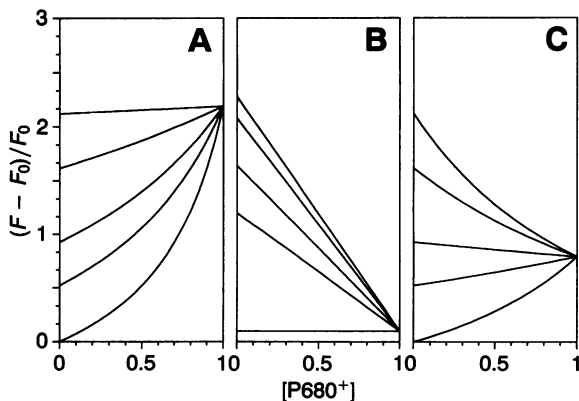


FIG. 3. Theoretical dependencies of the relative quantum yield of variable Chla fluorescence, $\psi_f = (F - F_0)/F_0$, on the oxidized form of P680, P680⁺, for concentrations of Q_A equal to 0.01, 0.1, 0.3, 0.5, and 1 per reaction center (from top to bottom). Concentration of the PQ_A state was calculated by assuming independence of P and Q_A ($[PQ_A] \approx [P][Q_A]$). In our calculations, $k_p/k_f = 35$; $k_\Sigma/k_f = 15$, and $k_q/k_f = 12.5$ were used. (A) Calculated on the basis of Eq. 3. (B) Calculated on the basis of Eq. 4 plus a constant. (C) Calculated from Eq. 6, which specifically includes quenching due to P680⁺.

We assume here that one of the reasons leading to the erroneous predicted behavior of the ratio in Eq. 5 is in the generally accepted view that Chla fluorescence yield is proportional to the concentration of state PQ_A^- (e.g., ref. 14). We now describe the expression for the quantum yield of Chla fluorescence which allows us to qualitatively describe the time dependence of the ratios in Eq. 5.

Quantum Yield of Fluorescence: Our View. To describe the quenching of Chla fluorescence by open reaction centers as well as by the oxidized form of P680, we suggest that Eq. 3 be expanded as follows:

$$\phi_f = k_f / (k_f + k_\Sigma + k_p[PQ_A] + k_q[P^+]). \quad [6]$$

Here, the concentration of reaction centers in the state $[PQ_A]$ is responsible for the decrease of fluorescence when the reaction centers are open, and k_q is the rate constant of quenching of Chla fluorescence by P680⁺.

Interestingly, an increase in $[P^+]$ in Eq. 6 produces two opposite effects: it decreases the number of open reaction centers (the term $k_p[PQ_A]$), and thus increases Chla fluorescence yield, while it decreases Chla fluorescence yield as P^+ is a quencher. The sum of these two effects will be determined by the values of the rate constants k_p and k_q as well as by Q_A concentration.

If $[Q_A] \approx 0$ (most reaction centers are closed), ϕ_f is mainly determined by $[P^+]$:

$$\phi_f \approx k_f / (k_f + k_\Sigma + k_q[P^+]). \quad [7]$$

That is, P^+ quenches fluorescence, in agreement with experiments of Mauzerall (12) and Sonneveld *et al.* (14).

If, however, $[Q_A] \approx 1$, and $k_p > k_q$, then ϕ_f is mainly determined by the concentration of open reaction centers ($[P] + [P^+] = 1$):

$$\begin{aligned}\phi_f &\approx k_f / (k_f + k_\Sigma + k_p[P] + k_q[P^+]) \\ &\approx k_f / \{ (k_f + k_\Sigma + k_q + [P](k_p - k_q)) \}.\end{aligned}\quad [8]$$

That is, the quenching of Chla fluorescence is mainly determined by the concentration of the reduced form of P680.

Fig. 3 shows the dependence of quantum yield of variable Chla fluorescence (ψ_f) on P680⁺ concentration calculated on the basis of Eq. 3 (A), Eq. 4 (B), and Eq. 6 (C) with the assumption that $[PQ_A] \approx [P][Q_A]$. In the case of the Stern-

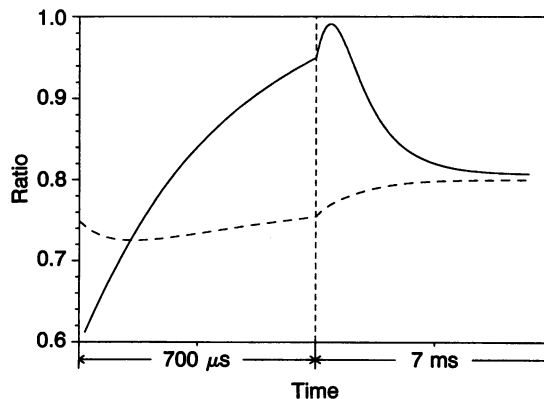


FIG. 4. Theoretical time dependence of the ratios of Chla fluorescence yield, calculated on the basis of Eq. 4 (dashed line) and Eq. 6 (solid line). The kinetics of $[PQ_A]$ was calculated by assuming independence of P and Q_A ($[PQ_A] \approx [P][Q_A]$) during dark relaxation. The increase in $[Q_A]$ after a flash was assumed to occur exponentially with $\tau = 0.4$ ms. P^+ reduction was assumed to occur exponentially with times 30, 100, 300 and 1000 μ s for $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, and $S_3 \rightarrow S_0$ transitions, respectively. In calculation of the solid line, values of $k_p/k_f = 35$; of $k_\Sigma/k_f = 15$, and $k_q/k_f = 12.5$ were used.

Volmer equation, Eq. 3, ψ_f increases with increasing $[P680^+]$ for all Q_A concentrations (Fig. 3A); i.e., the reduced form of P680 is the quencher of Chla fluorescence. This disagrees with the quenching nature of $P680^+$. In the case of Eq. 4, ψ_f is a linearly decreasing function of $[P680^+]$ for almost all Q_A concentrations (Fig. 3B); i.e., the oxidized form of P680 is the quencher of Chla fluorescence. Eq. 6 predicts that when $[Q_A]$ is very low, P^+ approximately linearly quenches fluorescence, whereas when most traps are open ($[Q_A] \approx 1$), the reduced form of P680 begins to act as a quencher of Chla fluorescence; i.e., ψ_f increases with increasing $[P680^+]$ (Fig. 3C). When $k_q = k_p$, Eq. 6 reduces to $\phi_f = k_f / \{k_f + k_\Sigma + k_p(1 - [PQ_A^-])\}$, which predicts the increase of ϕ_f with $[PQ_A^-]$, similar to Eq. 4.

Fig. 4 shows the values of the ratios $\psi_f(3)/\psi_f(1)$ and $\psi_f(3)/\psi_f(5)$ calculated from two different approaches (Eqs. 4 and 6). The dashed line in Fig. 4 shows the prediction, from Eq. 4, that has usually been considered to be satisfactory. However, the prediction shows a rise in Chla fluorescence yield in the millisecond time scale, whereas the actual data show a decline in Chla fluorescence yield (Fig. 2). The absence of fit with the experimental results clearly points out that this relationship is insufficient to explain the experimental data. The calculation based on the generalized Stern-Volmer equation (solid line, Eq. 6) is, however, in good qualitative agreement with that obtained experimentally (Fig. 2), indicating reasonable approximation of the quantum yield of Chla fluorescence by the relationship suggested in this paper.

We are indebted to Dr. Chunhe Xu for assistance in measuring Chla fluorescence decays after a series of flashes in dark-adapted spinach thylakoids. We thank the National Science Foundation for support (Grant 91:16838 to G.).

- Weber, G. (1960) in *Comparative Biochemistry of Photoreactive Systems*, ed. Allen, M. B. (Academic, New York), pp. 395-411.
- Govindjee, Ames, J. & Fork, D. C., eds. (1986) *The Light Emission by Plants and Bacteria* (Academic, New York).
- Govindjee, ed. (1982) *Photosynthesis, Vol. I*. (Academic, New York).
- Duysens, L. N. M. & Sweers, H. E. (1963) in *Studies on Microalgae and Photosynthetic Bacteria* (Jpn. Soc. Plant Physiol., Univ. of Tokyo Press, Tokyo), pp. 353-372.
- Wasielewski, M. R., Johnson, D. G., Seibert, M. & Govindjee (1989) *Proc. Natl. Acad. Sci. USA* **86**, 524-528.
- Velthuys, B. & Ames, J. (1974) *Biochim. Biophys. Acta* **333**, 85-94.
- Crofts, A. R. & Wraight, C. A. (1983) *Biochim. Biophys. Acta* **726**, 149-185.
- Bowes, J. M. & Crofts, A. R. (1980) *Biochim. Biophys. Acta* **590**, 373-384.
- Kok, B., Forbush, B. & McGloin, M. (1970) *Photochem. Photobiol.* **11**, 457-475.
- Joliot, P. & Kok, B. (1975) in *Bioenergetics of Photosynthesis*, ed. Govindjee (Academic, New York), pp. 388-413.
- Rutherford, A. W., Zimmermann, J.-L. & Boussac, A. (1992) *Top. Photosynth.* **11**, 179-229.
- Mauzerall, D. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1358-1362.
- Butler, W. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3420-3422.
- Sonneveld, A., Rademaker, H. & Duysens, L. N. M. (1979) *Biochim. Biophys. Acta* **548**, 536-551.
- Sonneveld, A., Rademaker, H. & Duysens, L. N. M. (1980) *Biochim. Biophys. Acta* **593**, 272-289.
- Lavorel, J. & Etienne, A. L. (1977) *Top. Photosynth.* **2**, 203-268.
- Delosme, R. (1971) in *Proceedings of the 2nd International Congress on Photosynthesis Research*, eds. Forti, G., Avron, M. & Melandri, A. (Junk, Dordrecht, The Netherlands), Vol. 1, pp. 187-195.
- Zankel, K. L. (1973) *Biochim. Biophys. Acta* **325**, 138-148.
- Joliot, P. & Joliot, A. (1971) in *Proceedings of the 2nd International Congress on Photosynthesis Research*, eds. Forti, G., Avron, M. & Melandri, A. (Junk, Dordrecht, The Netherlands), Vol. 1, pp. 26-38.
- Eaton-Rye, J. J. & Govindjee (1988) *Biochim. Biophys. Acta* **935**, 237-247.
- Eaton-Rye, J. J. & Govindjee (1988) *Biochim. Biophys. Acta* **935**, 248-257.
- Lavergne, J. (1982) *Biochim. Biophys. Acta* **679**, 12-18.
- Robinson, H. H. & Crofts, A. R. (1987) in *Progress in Photosynthesis Research*, ed. Biggins, J. (Nijhoff, Dordrecht, The Netherlands), Vol. 2, pp. 429-432.
- Duysens, L. N. M. (1979) in *Chlorophyll Organization and Energy Transfer in Photosynthesis*, Ciba Foundation Symposium 61 (Excerpta Med., Amsterdam), pp. 323-340.
- Kramer, D. M., Robinson, H. H. & Crofts, A. R. (1990) *Photosynth. Res.* **26**, 181-193.
- Dekker, J. P., Plijter, J. J., Ouwehand, L. & Van Gorkom, H. J. (1984) *Biochim. Biophys. Acta* **767**, 176-179.