

Charge accumulation and photochemistry in leaves studied by thermoluminescence and delayed light emission

(photosynthesis/electron transfer/oxygen-evolving enzyme/deactivation)

A. W. RUTHERFORD*, GOVINDJEE†, AND Y. INOUE

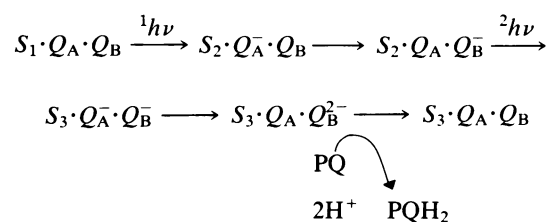
Solar Energy Research Group, The Institute of Physical and Chemical Research, Hirosawa, Wako-Shi, Saitama-Ken 351, Japan

Communicated by William Arnold, November 4, 1983

ABSTRACT A major breakthrough in our understanding of how plants oxidize water to molecular O₂ was the discovery by P. Joliot and co-workers that the O₂ yield per flash, in a series of light flashes, oscillates with a periodicity of 4. This led to the concept by B. Kok and co-workers that these reactions involve accumulation of four positive charges in independent “O₂-evolving centers,” which undergo a series of changes in their redox state (the so-called S states). In the present paper, we have applied optical techniques (such as thermoluminescence and delayed light emission, both discovered by W. Arnold and co-workers) to monitor charge storage on the O₂-evolving system in leaves from higher plants. We observed a period of four oscillations in both thermoluminescence and delayed light emission, with maxima on flashes 2 and 6, establishing a relationship with the charge accumulation process in photosynthesis. These measurements provided additional new information: the deactivation of the “O₂-evolving centers,” which cannot be measured by the O₂ method in the leaves, is in the 20- to 30-s range; and in the dark-adapted leaves, the secondary bound plastoquinone molecule (the so-called secondary electron acceptor Q_B) is in equal concentration in its reduced and oxidized forms. The origin of thermoluminescence and delayed light emission, in terms of the recombination of charges on the O₂-evolving and plastoquinone sides, is also discussed.

In spite of some new developments, the chemistry of oxidation of water to molecular O₂ remains a poorly understood process (1, 2). Joliot *et al.* (3), who measured the relative amount of O₂ evolved in a short flash, made an important discovery that the O₂ yield as a function of flash number oscillates with a periodicity of 4. Shortly thereafter, Kok *et al.* (4) suggested that each O₂-evolving center cycles through five different states (which they labeled as S₀, S₁, S₂, S₃, and S₄, with the subscripts reflecting the number of positive charges accumulated). To explain the first O₂ yield maximum on flash 3, they assumed that in dark-adapted samples, there is a preponderance of S₁ over S₀. On the acceptor side of photosystem II (PS II), Bouges-Bocquet (5) and Velthuys and Amesz (6) have shown that two negative charges accumulate on the secondary electron acceptor plastoquinone (designated Q_B) before electron flow can proceed further to the plastoquinone (PQ) pool.

This may be represented as follows:



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.

Here, Q_A stands for the first PQ electron acceptor, and the superscript on hν stands for the flash number.

The charge storage on both the O₂-evolving and the PQ sides also can be measured by optical techniques (such as luminescence). Luminescence in plants largely results from recombination of positive charges on photooxidized electron donors with electrons on photoreduced electron acceptors in PS II (7, 8). Thermoluminescence (TL) in plants was discovered by Arnold and Sherwood (9), and delayed light emission (DLE) in plants was discovered by Strehler and Arnold (10). TL and DLE have been reviewed by Inoue and Shibata (11) and by Lavorel (7), respectively. The flash-induced TL in control thylakoids has been identified as arising from S₂Q_B⁻ or S₃Q_B⁻ recombination (12). This TL corresponds to a slow phase of DLE (decaying in the seconds-to-minutes time scale) recorded after flash excitation of thylakoids at room temperature (13). When diuron, which inhibits electron flow from Q_A⁻ to Q_B, is added to thylakoids, the flash-induced TL band is shifted to a lower temperature (11, 12, 14) and the 30-s phase of DLE is replaced by one decaying within a few seconds (13, 15). The DLE in the presence of diuron is attributed to S₂Q_A⁻ and S₃Q_A⁻ recombination (12–16). These recent advances have allowed the measurement of slow DLE and TL to be used as probes of PS II photochemistry in thylakoids.

Luminescence techniques have one great advantage over the majority of other probes of PS II: they can be adapted easily to the study of leaves. In this work, the recent developments in our understanding of DLE and TL have been applied to a study of PS II in leaves, and several of the phenomena associated with PS II that previously have been reported only in isolated thylakoids and algal cells have been measured in leaves. (i) Charge storage on the oxygen-evolving enzyme has been monitored as a period of four oscillations of a TL band at around 30°C and of a slow phase of DLE after a series of flashes. The luminescence is attributed to recombination of S₂Q_B⁻ and S₃Q_B⁻. (ii) From the oscillation pattern (maxima on flashes 2 and 6) and the effect of illumination given at 77 K upon the TL intensity, it is suggested that Q_B⁻ is stable in the dark in approximately 50% of the centers. This is higher than seen in thylakoid membranes but is similar to that seen in intact algae and probably reflects a more reduced PQ pool in dark-adapted living systems. (iii) An experimental procedure is described using TL in which deactivation of S₂ state is measured. This allows S₂ deactivation to be measured separately in centers containing Q_B⁻ or Q_B; the centers in the state S₂Q_B⁻ deactivate faster than those

Abbreviations: TL, thermoluminescence; DLE, delayed light emission; PS, photosystem; cyt b₅₅₉, cytochrome b₅₅₉; PQ, plastoquinone.

*Permanent address: Service Biophysique, Department de Biologie, Centre d'Etudes Nucleaires Saclay, BP 2, 91191 Gif-sur-Yvette, France.

†To whom reprint requests should be addressed at the permanent address: University of Illinois at Urbana-Champaign, 289 Morrill Hall, 505 South Goodwin Avenue, Urbana, IL 61801.

in the state S_2Q_B . This is taken as evidence that recombination is the major deactivation pathway in S_2Q_B centers. (iv) Infiltration of leaves with diuron results in a shift of the flash-induced thermoluminescence to a lower temperature (from 30°C to 10°C). The 10°C TL band is attributed to S_2Q_A recombination. A preliminary report was made at the Sixth International Congress on Photosynthesis (17).

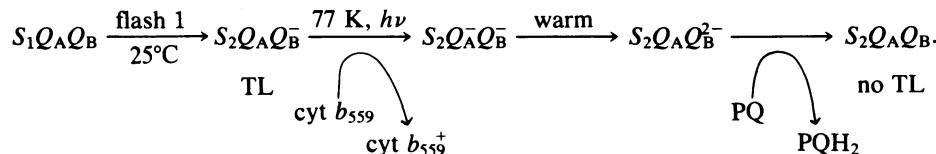
METHODS

Market spinach leaves were dark-adapted for at least 2 hr before being used. Leaf discs 1.2 cm in diameter were cut and used immediately for TL experiments. TL was measured in an apparatus as described (18). Reproducible TL measurements (within 10%; usually 5%) were obtained when discs from leaves of the same size and shade of green color,

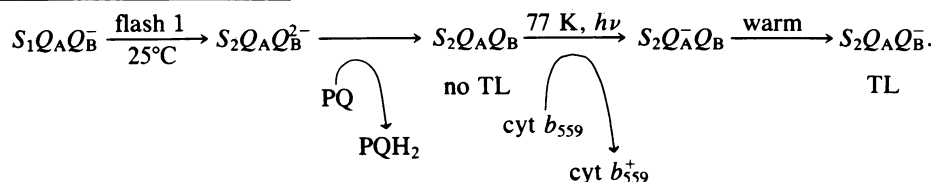
measured by other techniques (20, 21). Preillumination of thylakoids at 77 K or at room temperature resulted in an oscillation pattern with maxima on flashes 2 and 6 (12, 22). This was attributed to increased stable Q_B in the dark (12). Similarly, the flash pattern observed here in leaves may be explained by assuming that a higher proportion of stable Q_B is present in dark-adapted leaves than in dark-adapted thylakoids.

In thylakoids, low-temperature illumination before or after the flash resulted in an inversion of the Q_B/Q_B^- ratio. This is due to the introduction of a single electron into the acceptor complex at the expense of a donor other than the S states, probably cytochrome b_{559} (cyt b_{559}) (12). This effect is explained schematically as follows:

(i) In centers where S_1Q_B is present in the dark:



(ii) In centers where $S_1Q_B^-$ is present in the dark:



firmly clamped to the heater, were used. The heating rate was approximately 0.3°C/s. Flash and continuous illumination in TL experiments were as described (12). In some cases, leaf discs were infiltrated with 10 μM diuron under alternatively low and high pressure in a sealed syringe.

DLE was measured at room temperature in an apparatus described in ref. 13 except that a Teflon holder was fitted diagonally into the 1-cm cuvette. A 0.5-cm diameter leaf disc was mounted on to the Teflon holder so that it was at 45° to the flash and 45° to the cooled photomultiplier.

RESULTS AND DISCUSSION

Charge Accumulation in Photosystem II. Fig. 1A shows TL recorded on dark-adapted spinach leaf discs after a series of flashes. As in thylakoids, a single band (often referred to as the "B" band, ref. 11) was observed with a peak around 30°C. The amplitude of this band varied depending upon flash number. A period of four oscillations was present, with maxima on flashes 2 and 6 (Fig. 1A and B). A qualitatively similar flash pattern was always observed, even when leaves were dark-adapted for up to 18 hr.

Fig. 1C shows DLE recorded at room temperature from leaf discs after a series of flashes. Phases of DLE decaying in a few seconds and in the tens-of-seconds-to-minutes time scale were present. At all time ranges (30 s, 1 min, and 2 min), the oscillations of DLE amplitude with flash number were observed (Fig. 1D). The oscillation pattern was similar to that seen in TL (cf. Fig. 1B with 1D), showing maxima on flashes 2 and 6. By analogy with work done on thylakoids (12, 13), the origin of the TL and the DLE is ascribed to $S_2Q_B^-$ and $S_3Q_B^-$ recombination.

In thylakoids, it was demonstrated that the flash pattern of TL (in particular, the amplitude after flash 1 relative to that after flash 2) was dependent upon the ratio of Q_B to Q_B^- in the dark (ref. 12; see also refs. 14 and 19). In dark-adapted thylakoids, the oscillation pattern obtained (i.e., maxima on flashes 1 and 5) corresponded to ratio Q_B/Q_B^- of 70:30 as

When illumination at 77 K was given to leaves after one or two flashes, very little change in the amplitude of the TL was observed (Fig. 2). In this case a slight decrease in amplitude was observed, whereas, in other experiments with different batches of leaves, slight increases were sometimes observed. If the ratio Q_B/Q_B^- were 70:30 and that of S_1/S_0 were 75:25, and if TL were observed only when $S_2Q_B^-$ or $S_3Q_B^-$ were present, an examination of the above equations shows that 77 K illumination after flash 1 would lead to a large decrease in TL. Flash 2 would give a lower TL than flash 1, but the 77 K illumination after flash 2 would lead to a large increase in TL. On the other hand, if the Q_B^-/Q_B ratio were 50:50, the same analysis shows that flash 2 would give a much higher TL than flash 1, but the 77 K illumination would not significantly change the TL after either flash 1 or 2, as observed here (Fig. 2). Thus, in leaves, the population of centers having Q_B^- is approximately equal to that with Q_B . A slight shift to lower temperatures of the emission peaks after low-temperature irradiation is evident in Fig. 2; this might be explained by a destabilizing effect of oxidized cyt b_{559} , which is thought to be present under these conditions.

Deactivation of Charge-Accumulating States S_2 and S_3 . Because the TL band after flash 1 arises from $S_2Q_B^-$ recombination, the deactivation of S_2 in these centers can be measured by varying the time between the flash and the freezing of the sample and then measuring the extent of the TL band remaining. Fig. 3A shows the results of such a measurement. Although the nature of measurement results in some scatter in the points, it seems that the decay has a $t_{1/2}$ of around 20 s (flash 1 = 1f in Fig. 3). The $t_{1/2}$ varied from experiment to experiment, and a $t_{1/2}$ for this decay as slow as 35 s was observed. It is likely that this variation was due to slight differences in the room temperature rather than to inherent differences in the experimental material.

According to the reactions outlined in schemes *i* and *ii*, a deactivation experiment carried out as described above (i.e., a flash at room temperature followed by a variable dark time

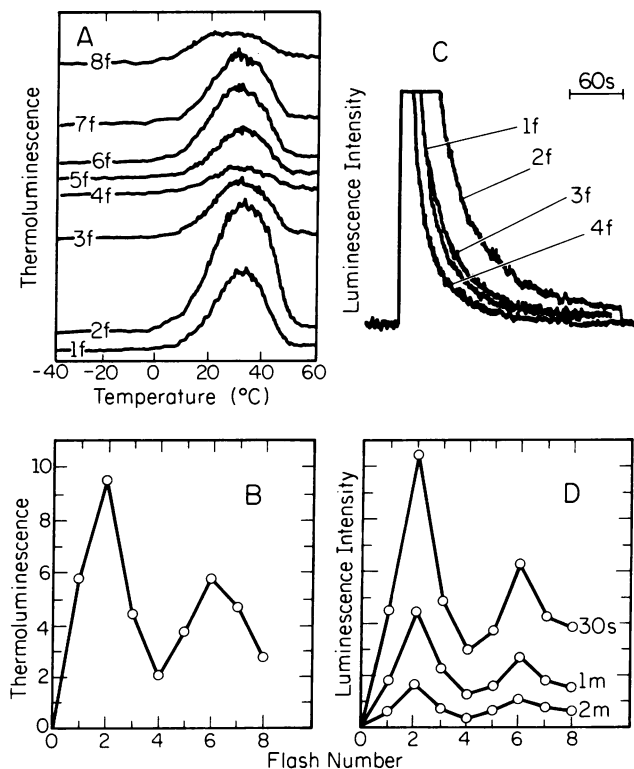


FIG. 1. (A) TL recorded from spinach leaf discs after a series of flashes (f). The traces show luminescence intensity (arbitrary units) as a function of temperature. (B) TL intensity at 35°C plotted as a function of flash number. (C) DLE recorded from spinach leaf discs after a series of flashes. (D) DLE intensity at 30 s, 1 min, and 2 min after a flash, plotted as a function of flash number. All flashes were given at room temperature.

before freezing) but with the inclusion of an additional period of continuous illumination at 77 K before TL is recorded should reflect deactivation of S_2 in centers where Q_B is present after the flash (Fig. 3A, $1f + h\nu$ 77 K). Although there was a significant fast phase, the deactivation of S_2Q_B centers ($t_{1/2} = 150$ s) was apparently much slower than S_2Q_B centers ($t_{1/2} = 20$ – 30 s). This suggests that Q_B is a major source of the electron responsible for the deactivation of S_2 in centers in the state S_2Q_B . Further support for this idea comes from the fact that the kinetics of DLE attributed to S_2Q_B recombination (Fig. 1C) are comparable to the fast phase of S_2 deactivation

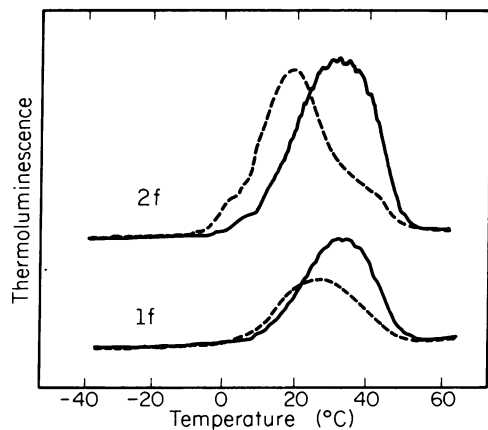


FIG. 2. TL intensity as a function of temperature from spinach leaf discs recorded after one (1f) or two (2f) flashes given at room temperature (—). The same after one or two flashes but followed by 1 min of continuous red light ($\lambda > 640$ nm; $12 \text{ mW} \cdot \text{cm}^{-2}$) illumination at 77 K (---).

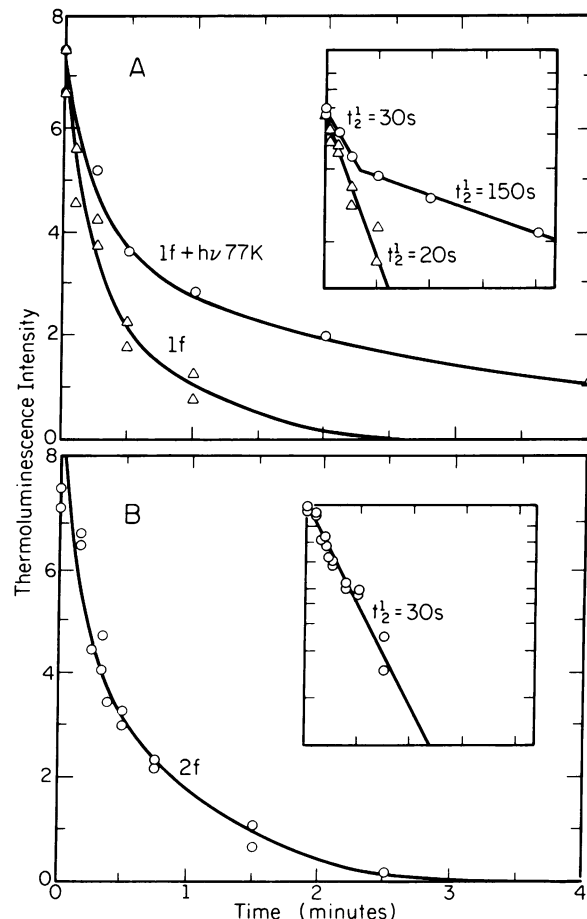
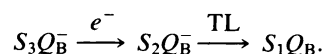


FIG. 3. TL as a function of time after flash in spinach leaf discs. (Insets) Logarithm of TL as a function of time after flash for the respective curves in A and B. These experiments were designed to measure deactivation of S states. (A) S_2 deactivation. S_2Q_B recombination data (Δ) were recorded after the following procedure. Dark-adapted spinach leaf discs were given a single flash at room temperature, and a variable dark time (0–4 min) was allowed before they were plunged into liquid nitrogen and cooled to 77 K. TL was recorded during subsequent heating of the sample. The results are interpreted as a measurement of S_2 deactivation in centers in the state S_2Q_B . \circ , Data recorded following the same procedure but with an additional period of illumination at 77 K (see legend to Fig. 2) given just before TL was recorded. These results are interpreted as measuring the deactivation of S_2 in centers in the state S_2Q_B . (B) S_3 deactivation. An experiment using the same procedure as in A (no 77 K $h\nu$) but after two flashes. See text for discussion of S_3 deactivation measured by TL.

measured here by TL and in thylakoids with the O_2 electrode (e.g., refs. 23 and 24).

The corresponding experiment to measure S_3 deactivation in S_3Q_B centers (i.e., two flashes; then vary time, freeze, and measure TL) is shown in Fig. 3B. An approximately monophasic decay was observed with a $t_{1/2} = 30$ s. However, the results are more complicated to interpret because, if S_3 decays via S_2 to S_1 , S_3Q_B deactivation could result in the formation of either S_2Q_B (if recombination takes place) or S_2Q_B (if the electron comes from a different source). In the latter case, TL additionally arises from S_2Q_B , and the deactivation experiment would monitor S_2 decays as well as (or instead of) S_3 . In fact, the TL and DLE associated with S_3Q_B could be due to the S_2Q_B recombination if the following reactions were dominant:



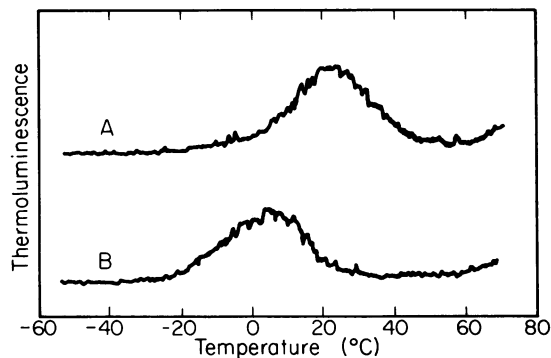


FIG. 4. TL as a function of temperature in spinach leaf discs with and without diuron. (A) Control showing TL after a single flash given at -10°C . (B) The same as A but after infiltration of the disc with $10\ \mu\text{M}$ diuron (in $\approx 10\%$ ethanol).

The analogous experiment to measure S_3Q_B deactivation (i.e., two flashes; then vary time, freeze, illuminate at 77 K, and record TL) runs into even more problems because any S_2Q_B that might be formed by S_3Q_B or S_3Q_B deactivation will be converted to S_2Q_B by the 77 K illumination. Attempts to carry out this measurement gave complicated but generally slower kinetics than those obtained in Fig. 3B (not shown). However, S_3 deactivation may be probed by this kind of TL measurement under conditions where the S_2Q_B and S_3Q_B bands can be distinguished from one another. Experiments on thylakoids have demonstrated that at pH 5.5 the TL band associated with S_3Q_B remains at around 25°C , whereas that associated with S_2Q_B shifts to about 40°C (e.g., see refs. 25 and 26). This probably reflects the involvement of a proton in the $S_2 \rightarrow S_3$ step but not the $S_1 \rightarrow S_2$ step (17). A similar shift was observed in leaves when they were infiltrated with pH 5.5 buffer (17). Deactivation experiments carried out with thylakoids at pH 5.5 indicate that S_3 does deactivate via S_2 (25). Similar results have been obtained in preliminary experiments with leaves (unpublished data). More detailed studies of S_3 deactivation at pH 5.5 in leaves and thylakoids using the experimental rationale described above would provide useful information not available from other techniques.

Fig. 4 shows the effect of infiltrating leaf discs with the inhibitor diuron. The presence of diuron results in a block of electron transport from Q_A^- to Q_B ; this is manifested as a shift in the emission temperature of the TL band. The band at 30°C attributed to S_2Q_B recombination is replaced by a band at around 10°C . The band observed in the presence of diuron is assigned to $S_2Q_A^-$ recombination in accordance with work done on thylakoids (ref. 12; see also refs. 14 and 27). This kind of peak shift is exactly as predicted in the theoretical framework of DeVault *et al.* (16).

CONCLUDING REMARKS

The results reported here, data on charge accumulation and on PS II photochemistry obtained in leaves, are of particular interest where they differ from those obtained in isolated thylakoids. This is the case in the measurement of the redox state of Q_B in the dark. In dark-adapted thylakoids, the ratio Q_B/Q_B^- is usually 70:30 (20, 21). However, the data reported here are interpreted as indicating that a ratio closer to 50:50 is present in leaves, even after long dark adaptation. It is of interest that, in whole algal cells, a ratio of 50:50 for Q_B/Q_B^- was found to be present in the dark (28), and this is also the case in intact chloroplasts (ref. 14; unpublished data). It seems possible that this ratio is the normal condition for *in vivo* systems. One question that remains is: what determines the redox state of Q_B in the dark. Previously, a relationship

between the amount of stable Q_B^- and the stable S states has been assumed (ref. 12; see also ref. 28). To simulate the TL data, it was assumed that Q_B^- was stable in the dark in centers where S_0 and S_1 were present, whereas it was unstable if S_2 and S_3 were present (due to recombination). In the simplest case, this results in 25% stable Q_B^- in the dark (12). However, deactivation in the presence of reduced PQ pool could result in a much higher proportion of stable Q_B^- (e.g., $S_2Q_B + PQH_2 \rightarrow S_1Q_B^- + 2H^+$ might occur). By extending this idea, the larger amount of stable Q_B^- in leaves could reflect a more reduced PQ pool. Unlike thylakoids, the PQ pool in whole algal cells is partly reduced in the dark (29). This is thought to be due to a feedback of electrons into the PQ pool (e.g., refs. 30–32). Such a system may be functional also in leaves.

The deactivation measurements made here are not only of interest because they appear to be the first to be carried out on leaves but also because they provide a mechanistic insight into the deactivation process. By necessity, the deactivation kinetics are divided into two separate measurements, the centers in the $S_2Q_B^-$ state and the centers in the S_2Q_B state. The results indicate that the recombination is the major deactivation process in center in the $S_2Q_B^-$ state. The source of electrons responsible for deactivation of S_2 in the S_2Q_B centers is not known but under some circumstances could be PQH_2 (cf. ref. 27), via the mechanism discussed above, resulting in $S_1Q_B^-$ formation.

The difficulties in measuring and interpreting S_3 deactivation have been discussed already, but experimental rationale has been developed that may prove useful for studies of this reaction in the future.

Luminescence, in particular TL, is an extremely useful probe of PS II photochemistry in leaves. Some of the potential applications of these techniques in leaves are demonstrated in this work in which they have been used to monitor charge storage on the O_2 -evolving enzyme and on the acceptor side of PS II, the decay of these charge storage states, and the effect of a herbicide on the quinone acceptor complex.

This work was supported by a Science and Technology Agency (Japan) Grant on Solar Energy Conversion by Means of Photosynthesis given to The Institute of Physical and Chemical Research, RIKEN (Japan) and, in part, by the Commission of the European Communities (Solar Energy Programme, Contract ESD-015-F) and by the National Science Foundation (PCM 78-24532). A.W.R. and G. were also recipients of Japanese Government Research Awards for Foreign Specialists.

- Govindjee, ed. (1982) *Photosynthesis: Energy Conversion by Plants and Bacteria* (Academic, New York), Vol. 1.
- Govindjee (1983) in *Proceedings of the Sixth International Congress on Photosynthesis*, ed. Sybesma, C. (Martinus Nijhoff/Dr. W. Junk Publishers, Den Haag, Netherlands), in press.
- Joliot, P., Barbieri, G. & Chabaud, R. (1969) *Photochem. Photobiol.* **10**, 304–321.
- Kok, B., Forbush, B. & McGloin, M. (1970) *Photochem. Photobiol.* **11**, 457–475.
- Bouges-Bocquet, B. (1973) *Biochim. Biophys. Acta* **314**, 250–256.
- Velthuys, B. & Ames, J. (1974) *Biochim. Biophys. Acta* **333**, 85–94.
- Lavorel, J. (1975) in *Bioenergetics of Photosynthesis*, ed. Govindjee (Academic, New York), pp. 233–317.
- Govindjee & Jursinic, P. (1979) *Photochem. Photobiol. Revs.* **4**, 125–205.
- Arnold, W. & Sherwood, H. (1957) *Proc. Natl. Acad. Sci. USA* **43**, 105–114.
- Strehler, B. & Arnold, W. (1951) *J. Gen. Physiol.* **34**, 809–820.
- Inoue, Y. & Shibata, K. (1982) in *Photosynthesis: Energy Conversion by Plants and Bacteria*, ed. Govindjee (Academic, New York), Vol. 1, pp. 507–536.

12. Rutherford, A. W., Crofts, A. R. & Inoue, Y. (1982) *Biochim. Biophys. Acta* **682**, 457–465.
13. Rutherford, A. W. & Inoue, Y., *FEBS Lett.*, in press.
14. Demeter, S. (1982) *FEBS Lett.* **144**, 97–100.
15. Lavergne, J. & Etienne, A.-L. (1980) *Biochim. Biophys. Acta* **593**, 136–148.
16. DeVault, D., Govindjee & Arnold, W. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 983–987.
17. Rutherford, A. W., Govindjee & Inoue, Y. (1983) in *Proceedings of the Sixth International Congress on Photosynthesis*, ed. Sybesma, C. (Martinus Nijhoff/Dr. W. Junk Publishers, Den Haag, Netherlands), in press.
18. Ichikawa, T., Inoue, Y. & Shibata, K. (1975) *Biochim. Biophys. Acta* **408**, 228–236.
19. Inoue, Y. (1983) in *The Oxygen Evolving System of Photosynthesis*, eds. Inoue, Y., Crofts, A. R., Govindjee, Murata, N., Renger, G. & Satoh, K. (Academic, Tokyo), pp. 439–450.
20. Wollman, F. A. (1978) *Biochim. Biophys. Acta* **503**, 263–273.
21. Fowler, C. F. (1977) *Biochim. Biophys. Acta* **459**, 351–363.
22. Inoue, Y. & Shibata, K. (1978) *FEBS Lett.* **85**, 193–197.
23. Joliot, P., Joliot, A., Bouges, B. & Barbieri, G. (1971) *Photochem. Photobiol.* **14**, 287–305.
24. Forbush, B., Kok, B. & McGloin, M. (1971) *Photochem. Photobiol.* **14**, 307–321.
25. Rutherford, A. W. & Inoue, Y. (1983) in *Proceedings of the Sixth International Congress on Photosynthesis*, ed. Sybesma, C. (Martinus Nijhoff/Dr. W. Junk Publishers, Den Haag, Netherlands), in press.
26. Inoue, Y. (1981) *Biochim. Biophys. Acta* **634**, 309–320.
27. Sane, P. V., Desai, T. S., Rane, S. S. & Tatake, V. G. (1983) *Ind. J. Exp. Biol.* **21**, 401–404.
28. Velthuys, B. R. (1980) *Annu. Rev. Plant Physiol.* **31**, 545–567.
29. de Kouchkovsky, Y. (1966) in *Currents in Photosynthesis*, eds. Thomas, J. B. & Goedheer, J. C. (Ad Donker, Rotterdam, Netherlands), pp. 367–374.
30. Maison-Peteri, B. (1983) Dissertation (Univ. Paris Sud, Orsay, France).
31. Mills, J. D., Crowther, D., Slovacek, R. E., Hind, G. & McCarty, R. E. (1979) *Biochim. Biophys. Acta* **547**, 127–137.
32. Arnon, D. I. & Chain, R. K. (1979) *FEBS Lett.* **102**, 133–138.