Proc. Nat. Acad. Sci. USA
Vol. 69, No. 11, pp. 3420–3422, November 1972

On the Primary Nature of Fluorescence Yield Changes Associated with Photosynthesis

(cytochrome *b*/chloroplasts/primary photoreaction/C-550)

WARREN L. BUTLER*

Biophysical Laboratory of the State University, Schelpenkade 14 A, Leiden, Netherlands

Communicated by Daniel I. Arnon, September 18, 1972

ABSTRACT **Absorbance changes of C-550 and cytochrome b_{559}, and fluorescence-yield changes were measured during irradiation of chloroplasts at −196°. The photoreduction of C-550 proceeded more rapidly than the photooxidation of cytochrome b_{559}, and the fluorescence-yield change had similar kinetics to the cytochrome b_{559} change. The fluorescence yield of chloroplasts exposed to a 16-μsec flash at −196° did not increase during the flash, but increased in the dark after the flash. Both of these experiments indicate that the fluorescence yield follows the dark reduction of the primary electron donor of Photosystem II, not the photoreduction of the acceptor. This explanation would also account for the recent results of Mauzerall [*Proc. Nat. Acad. Sci. USA* (1972) 69, 1358–1362] showing that the fluorescence yield of chloroplasts at room temperature requires about 20 μsec to reach a maximum after a very brief flash.**

Fluorescence-yield changes of chlorophyll in green plants were related to the photochemical and biochemical processes of photosynthesis by Duysens and Sweers (1). They proposed that the primary electron acceptor of photosystem II (PSII), denoted *Q* by them, quenched chlorophyll fluorescence in its oxidized state, but not in its reduced state. Thus, the primary photochemical reduction of *Q* by PSII resulted in an increase of fluorescence yield, while the dark oxidation of *Q*H by subsequent electron-transfer components reinstated the quenching. The association of fluorescence yield with the primary electron acceptor of PSII has made fluorescence a useful and important tool for the study of primary photochemical mechanisms of photosynthesis. Recently, however, this primary nature of fluorescence-yield changes has been brought into question (2).

Measurements of fluorescence from chloroplasts at liquid-nitrogen temperature (3) were taken to indicate that fluorescence yield might be determined by the redox state of the primary electron donor to PSII, the reaction-center chlorophyll P_{680}, as well as by the redox state of the primary electron acceptor, C-550 [absorbance changes in the 550-nm region have been related to the redox state of the primary electron acceptor of PSII (4)]. The reaction center complex of PSII was assumed to consist of redox components D·P·A, where D is cytochrome b_{559}, P is P_{680}, and A is C-550. The normal photochemical reaction at low temperature

$$\mathrm{D\cdot P\cdot A} \xrightarrow{h\nu} \mathrm{D\cdot P^{+}\cdot A^{-}} \rightarrow \mathrm{D^{+}\cdot P\cdot A^{-}}$$

resulted in a high fluorescence yield, about 5-fold greater than the initial F_0 level (the D·P·A state). However, when cytochrome b_{559} was oxidized before freezing, the photochemical reaction

$$\mathrm{D^{+}\cdot P\cdot A} \xrightarrow{h\nu} \mathrm{D^{+}\cdot P^{+}\cdot A^{-}}$$

resulted in a fluorescence yield that was only twice the F_0 level (F_0 being the same in both cases). This observation was taken to indicate that of the possible redox states of the primary electron-transfer couple, P·A, $P^{+}\cdot A$, $P^{+}\cdot A^{-}$, and $P\cdot A^{-}$, only $P\cdot A^{-}$ gave the high fluorescence yield. Subsequently, the time course of the photooxidation of cytochrome b_{559}, the photoreduction of C-550, and the light-induced fluorescence-yield increase has been followed in chloroplasts at low temperature (5). The results of this latter work, some of which will be presented here, support the view that fluorescence yield is determined by dark reactions of electron transport that follow the primary charge separation, as well as by the redox state of the primary electron acceptor.

Mauzerall (2) recently reported that fluorescence-yield changes in *Chlorella* did not reflect the primary photochemical electron-transfer reaction, as should be expected if fluorescence yield was determined solely by the redox state of the primary electron acceptor, since the fluorescence yield did not reach a maximum until 20 μsec after a brief (10 nsec) saturating flash. The contention of this article is that the time required for the fluorescence yield to increase after such a brief flash represents a dark reaction of electron transport from an electron donor on the water-splitting side of PSII to P^{+}_{680}.

METHODS

Changes of C-550, cytochrome b_{559}, and fluorescence yield were measured during continuous irradiation (630 nm) of spinach chloroplasts at liquid-nitrogen temperature. C-550 was monitored by the transmission at 543 nm and cytochrome b_{559} by the transmission at 556 nm. Fluorescence at 692 nm was measured with identical samples (1 mg of chlorophyll per ml in 1-mm cuvettes) with the same 630-nm actinic source and optical geometry. Measurements of the fluorescence yield at −196° during a 16-μsec Xenon flash were

Abbreviation: PSII, photosystem II.

* On leave from the Department of Biology, University of California San Diego, La Jolla, Calif., 92037.

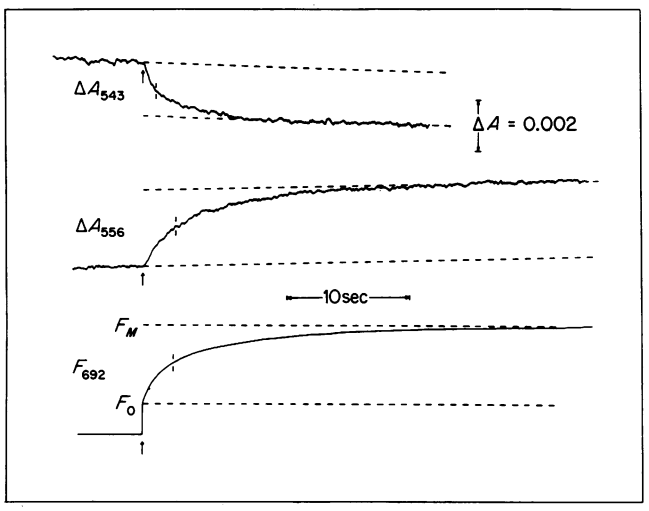

FIG. 1. Time course of the absorbance at 543 nm and 556 nm (1-nm pass bands) and of the fluorescence at 692 nm (20-nm pass band) during continuous irradiation (on at *upward arrows*) of spinach chloroplasts (1 mg of chlorophyll per ml in 1-mm cuvette) with 630-nm light (165 μW/cm^2) at $-196°$.

made in an apparatus developed by Duysens and coworkers†. The yield measurements are obtained by dividing the time course of fluorescence $F(t)$, measured during the flash, by the time course of the flash intensity, $I(t)$-$\phi(t) = F(t)/I(t)$. The energy in a flash was several times greater than that needed to saturate photosynthesis.

RESULTS AND DISCUSSION

The time course of the absorbance increase at 543 nm (due to the photoreduction of C-550), the bleaching at 556 nm (due to the photooxidation of cytochrome b_{559}), and the fluorescence at 692 nm during continuous irradiation with 630-nm actinic light are shown in Fig. 1. Semilogarithmic plots of these data are shown in Fig. 2. It is clear that the light-induced increase in fluorescence yield follows the photooxidation of cytochrome b_{559}, not the photoreduction of C-550. Direct measurements of P_{680} in the same type of experiment have proved more difficult, and have not as yet been made. The rate of oxidation of cytochrome b_{559}, however, is taken to indicate the rate of reduction of the photooxidized P_{680}.

The fluorescence yield of spinach chloroplasts frozen to $-196°$ was also measured during the time course of a Xenon flash. Flash excitation of such samples at room temperature causes the fluorescence yield to rise severalfold during the 16 μsec duration of the flash†. With the samples frozen to $-196°$, no increase of yield occurred during the first flash. However, at the onset of the second flash, given 1 sec later, the fluorescence yield was about twice the F_0 level, having increased in the dark after the first flash. The fluorescence yield at the onset of subsequent flashes (given 1-sec apart) was progressively higher, approaching the F_M level obtained with continuous irradiation of the frozen samples (F_M was about five times greater than F_0 in these experiments). Even though the flash energy was sufficient to saturate normal photosynthesis, a single flash at $-196°$ was not capable of increasing the fluorescence yield to the maximal, F_M, level. Similar results were also obtained with *Scenedesmus* cells frozen to $-196°$. (Flash excitation of the flashed or irradiated samples caused the high initial fluorescence yield to be quenched to the F_0 level during the 16-μsec duration of the flash, but the fluorescence yield returned to a high level during the 1-sec dark period between flashes. This photochemical quenching process, which was noted previously in measurements at room temperature†, is currently under investigation in Prof. Duysens' laboratory.)

It is clear from the time-course measurements of fluorescence yield and the electron-transport reactions during continuous irradiation at $-196°$ that fluorescence yield does not indicate the primary electron-transfer reaction, but rather a subsequent dark reaction, probably the reduction of P^+_{680}. The time course for the increase of fluorescence yield after a brief flash at room temperature (2) or at $-196°$ is also consistent with this view. The 20-μsec delay between the flash and the appearance of the maximum fluorescence yield found by Mauzerall at room temperature can be taken as the time required to reduce P^+_{680} by an electron donor on the water-splitting side of PSII.

Döring *et al.* (6, 7) measured the bleaching and recovery of P_{680} (Chl a_{II} in their terminology) in repetitive flash experiments at room temperature, and reported a 200-μsec time constant for the recovery of P_{680} after a flash. It has been pointed out, however, that the extent of P_{680} bleaching measured by Döring *et al.* was much less than should be expected (8); about 1 P_{680} per 10^4 chlorophyll molecules. Floyd *et al.* (9) measured P_{680} bleaching by single flashes at $-196°$ and obtained, with samples of comparable chlorophyll concentration, absorbance changes about 100-fold greater than those of Döring *et al.* It seems possible, therefore, that a major part of the P_{680} recovers with a time constant of the order of 10 μsec at room temperature, and that this part of the P_{680} was not observed in the measurements of Döring *et al.* Their measurements showed only a minor part, which recovered with a 200-μsec time constant.

Under most conditions, the influence of P_{680} on fluorescence yield will not be observed because of its fast recovery, and

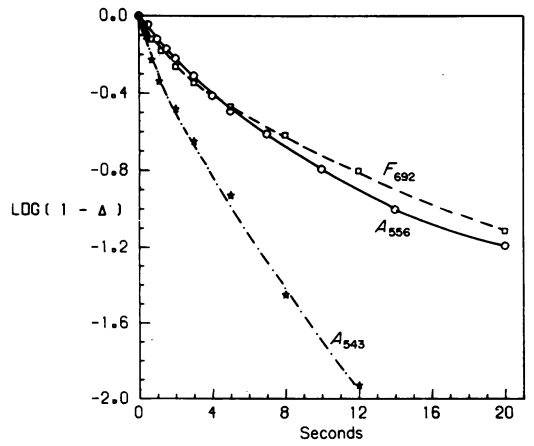

FIG. 2. Semilogarithmic plots of the data in Fig. 1. Log (1 $-$ Δ) against time, where Δ is the fraction of the maximal light-induced change.

† Duysens, L. N. M., Schatte Oliver, T. E. van der & Haan, G. A. den (1972) "Light-induced quenching of the yield of chlorophyll a_2 fluorescence with microsecond backreaction stimulated by oxygen," *VI International Congress on Photobiology*, Abstract, in press.

fluorescence yield will appear to be determined solely by the primary electron acceptor. The influence of P_{680}, however, can be observed at low temperatures, or in measurements at room temperature at very high time resolution. The absorbance change of C-550 appears to be a more direct indicator of the primary electron acceptor of PSII than is fluorescence yield.

I am indebted to J. W. M. Visser and H. L. Simons for assistance in measuring the absorbance and fluorescence changes in continuous light and to G. A. den Haan and F. B. de Mink for fluorescence-yield measurements during a flash. This work was performed under a National Science Foundation Senior Postdoctoral Fellowship. The work was supported in part by the Netherlands Foundation for Biophysics, financed by the Netherlands Organization for the Advancement of Pure Research (ZWO).

1. Duysens, L. N. M. & Sweers, H. E. (1963) "Mechanisms of two photochemical reactions in algae as studied by means of fluorescence," in *Studies on Microalgae and Photosynthetic Bacteria*, ed. Miyachi, S. (University of Tokyo Press, Tokyo), pp. 353–372.
2. Mauzerall, D. (1972) "Light-induced fluorescence changes in Chlorella and the primary photoreactions for the production of oxygen," *Proc. Nat. Acad. Sci. USA* **69**, 1358–1362.
3. Okayama, S. & Butler, W. L. (1972) "The influence of cytochrome b_{559} on the fluorescence yield of chloroplasts at low temperature," *Biochim. Biophys. Acta* **267**, 523–529.
4. Erixon, K. & Butler, W. L. (1971) "The relationship between Q, C-550 and cytochrome b_{559} in photoreactions at −196°C in chloroplasts," *Biochim. Biophys. Acta* **234**, 381–389.
5. Butler, W. L., Visser, J. W. M. & Simons, H. L. (1972) "The Kinetics of light-induced changes of C-550, cytochrome b_{559} and fluorescence yield in chloroplasts at low temperature," *Biochim. Biophys. Acta*, in press.
6. Döring, G., Stiehl, H. H. & Witt, H. T. (1967) "A second chlorophyll reaction in the electron transport chain of photosynthesis-registration by the repetitive excitation technique," *Z. Naturforsch. B* **22**, 639–644.
7. Döring, G., Renger, G., Vater, S. & Witt, H. T. (1969) "Properties of the photoactive chlorophyll a_{II} in photosynthesis," *Z. Naturforsch. B* **24**, 1139–1143.
8. Butler, W. L. (1972) "The relationship between P_{680} and C-550," *Biophys. J.* **12**, 851–857.
9. Floyd, R. A., Chance, B. & DeVault, D. (1971) "Low temperature photoinduced reactions in green leaves and chloroplasts," *Biochim. Biophys. Acta* **226**, 103–112.