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KINETICS OF THE FLUORESCENCE YIELD OF CHLOROPHYLL a_2 IN SPINACH CHLOROPLASTS AT LIQUID NITROGEN TEMPERATURE DU RING AND FOLLOWING A 16 μs FLASH

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SUMMARY

If, at liquid nitrogen temperature, the initial fluorescence yield of chlorophyll a_2 is high (*e.g.* after preillumination), a 16 μs flash produces in a few microseconds a decrease in fluorescence yield, followed by an increase, which occurs after roughly 10–20 μs, when the intensity of the flash has become negligible. It is concluded that during a flash, a quencher or quenching state T is formed, which disappears in a dark reaction in a time of the order of 10 μs. The kinetics are the same and can be interpreted in the same way as the kinetics at room temperature earlier reported by Duysens *et al.* (Abstr. VI. Int. Congr. on Photobiol. Bochum 1972, No. 277).

If the flash is given when the initial fluorescence yield is low, then the fluorescence yield increases only markedly at the end of the flash, when the intensity has become low. Even for a strong flash, the increase is only about 20% of the maximum increase attained after a large number of flashes. This indicates that at low temperature, in contradistinction to room temperature, the reduction of the primary oxidant Q is less efficient than the formation of the quencher T. For the interpretation of the experiments it was not necessary to introduce other light-induced quenchers than T, such as the oxidized primary reductant, P^+.

INTRODUCTION

Fluorescence yield changes have been extensively utilized as a monitor of the primary photochemistry in Photosystem II of green plant and algal photosynthesis[1]. According to the rationale of Duysens and Sweers[2], the increase in chlorophyll *a* fluorescence yield upon illumination arises from the reduction of a quencher entity, Q. Q can then be reoxidized by a secondary electron acceptor pool in 200–600 μs.

This communication presents some new results at liquid nitrogen temperature. These results can be explained by the hypothesis that just as at room temperature[3] in the light flash a chlorophyll *a* quencher T (possibly a carotenoid triplet) is formed, at low temperatures the quantum efficiency for this light reaction is about five times as high as the "normal" primary reaction of System II, which leads to an increase in the yield of the chlorophyll *a* fluorescence, due to the reduction of the primary acceptor, Q. At room temperature the normal reaction is more efficient, when the

reaction center is in the state Q. The results, taken together with experimental findings by Floyd *et al.*[4] and Butler *et al.*[5,6] further indicate that, if the re-reduction of $P680^+$ indeed takes 4–5 ms (ref. 4), it is unlikely that $P680^+$, as was stated previously[6], causes fluorescence quenching during a high-intensity light flash.

METHODS

Measurements of the fluorescence yield variation in spinach chloroplasts and *Chlorella pyrenoidosa* during transient excitation were made with the apparatus designed for rapid signal acquisition[7]. The excitation pulse from a GE FT230 xenon flash tube had a typical half width of about 16 μs. A series of filters (Calflex-C, Balzer K_1, and Schott BG18/2) permitted blue light with a maximum λ of approx. 420 nm to be directed onto the sample. The maximum intensity (100%) at the cuvette surface was determined to be approx. 250 $\mu J/cm^2$. At an energy higher than about 20–30 $\mu J/cm^2$, the maximum amount of oxygen was produced in a flash at room temperature. Attenuation of the excitation light by neutral density filters permitted fluorescence yield measurements to be made under different incident light intensities.

Both, the time course of the exciting light intensity, $I(t)$, and the variation of the fluorescence with time, $F(t)$, were monitored by an S-20 photomultiplier (EMI 9558 extended) and the resulting electrical currents were converted into digital form[7] and stored on a magnetic tape unit of a PDP-9 computer.

The fluorescence yield $\phi(t)=F(t)/I(t)$ was subsequently calculated from the stored data and plotted for analysis. With the xenon flash it was possible to routinely measure the fluorescence yield during the light flash in the time region of approximately 1–40 μs.

Fluorescence yield changes in a time interval from about 150 μs to several minutes after ignition of a saturating actinic flash were measured by utilizing a small detecting xenon flash (EG and G FX76), fired at preset time intervals after the ignition of the photochemistry-inducing flash. The value of ϕ_0 was determined by igniting the detecting flash, immediately prior to application of the actinic light pulse.

Spinach chloroplasts, prepared as described by Amesz *et al.*[8] were suspended in a buffer consisting of 0.05 M *N*-tris(hydroxymethyl)methylglycine (Tricine), 0.01 M KCl, 0.002 M $MgCl_2$ and 0.4 M sucrose. All samples were diluted to an absorbance of 0.1 ($\lambda=680$) in a path-length of 1 mm. For experiments at liquid nitrogen temperature, the chloroplast sample was layered on an aluminium cuvette, designed by Dr W. Butler, which maintained thermal contact with the liquid nitrogen. Sample temperature was monitored by means of an iron-constantan thermocouple in the chloroplast suspension. We used the same computer programs as used by Duysens *et al.*[3].

RESULTS

The fluorescence yield in chloroplasts or algal samples during "a medium intensity flash" increases at room temperature to a maximum value of about 3 times ϕ_0 (ref. 3). The highest intensity used (about 250 $\mu J/cm^2$) is called 100%. "Medium" intensities then are around 10%. At "medium" intensities the half time of the fluorescence yield increase was 5–10 μs. In chloroplasts the increase above ϕ_0 depends to a large extent on the quality of the spinach. If the intensity of the exciting flash is

higher than 10%, a light intensity-dependent decrease in the fluorescence yield is observed[3,9]. This quenching process persists during the time that the flash intensity remains high, and following the flash the yield increases to the maximum value observed. The intervention of a metastable triplet carotenoid, which acts as an energy sink, has been postulated as an explanation of this quenching phenomenon[3].

Measurements during the flash at liquid nitrogen temperature, however, show a different behavior (Fig. 1)[6]. The dark frozen sample was subjected to a series of 100% flashes, and the fluorescence yield variation was monitored during each flash. The dark time between the flashes was 0.8 s. The first obvious conclusion that can be drawn from this experiment is that the quenching mechanism first noted at room temperature is also present at 77 °K, and also that the fluorescence yield increases when the flash intensity decreases in the "tail" of the flash (after 20 μs). However, one salient difference must be emphasized. At 77 °K the yield after the first flash increases only to about 1.5 times ϕ_0 (in chloroplasts)[6]. This increase in fluorescence yield occurs in the time interval from about 16–40 μs and is relatively independent

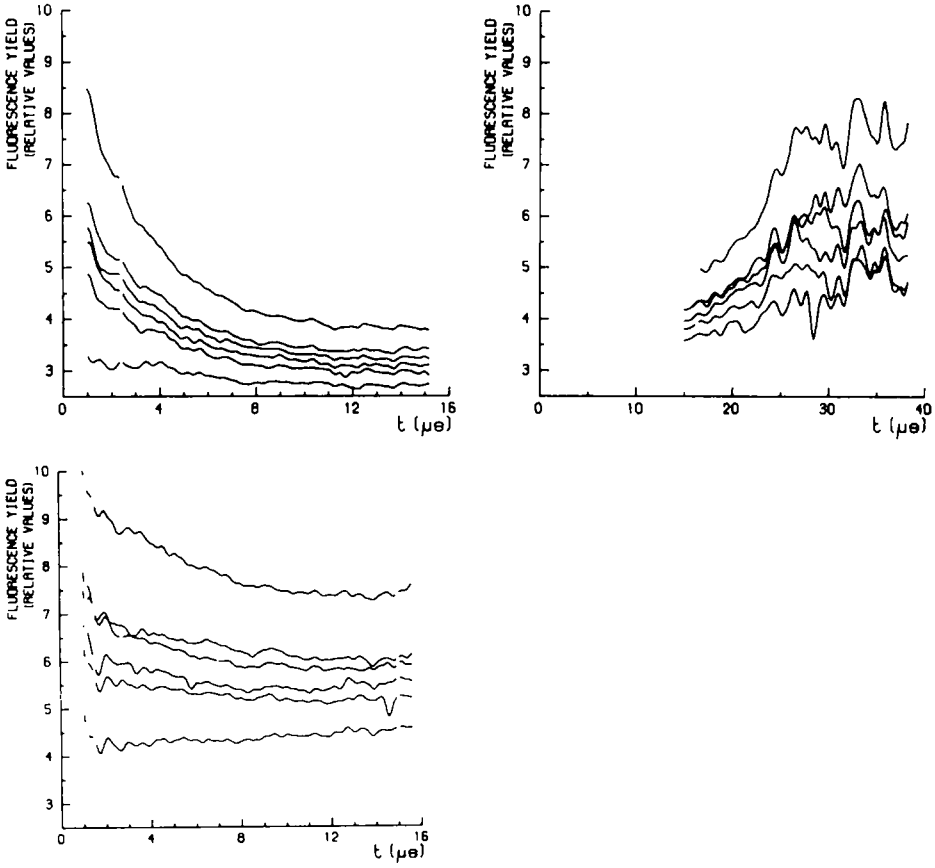

Fig. 1. Fluorescence yield kinetics of spinach chloroplasts at 77 °K. A and B, flash intensity 100%; C (bottom), flash intensity 14%. Number of flashes after dark: 1–5 and 20, shown in vertical sequence. Different samples were used for the experiments shown in Figs A, B and C.

of the intensity of the flash, provided that the actinic intensity is above 14% (Fig. 1C). In contrast to room temperature behavior, the fluorescence increase appears to be irreversible on a time scale of seconds (a decrease by about 30% taking a couple of minutes often occurs in *C. pyrenoidosa* after dark adaptation following a flash series). After a large number of flashes (*i.e.* 30), the fluorescence yield obtains a value of roughly 3 times ϕ_0, and the quenching process is quite prominent during the flash. Application of continuous background illumination results in a final yield of 4 times ϕ_0.

An increase in fluorescence yield after the second saturating flash was only observed if the first and second flashes were separated by a time interval of about 40 μs or longer. At shorter intervals the two overlapping flashes induced a fluorescence yield increase equal to the increase after a single flash. This suggests that charge separation is inhibited during the presence of the quenching state.

A typical experiment resulted in an increase of $1.44 \pm 0.05 \times \phi_0$ when two flashes were separated by 10 μs, and $1.60 \pm 0.07 \times \phi_0$ when these flashes were separated by 100 μs. To obtain sufficient accuracy an average of six identical samples was taken.

Similar experiments with *Chlorella* yielded qualitatively identical results, however, the relative variable fluorescence yield in the algal sample was lower than that observed with chloroplasts. Fig. 2 shows that the fluorescence yield in the region of 200 μs to 13 ms after a saturating flash remains constant within experimental error. The fluorescence is still the same after several seconds. At room temperature the fluorescence yield rapidly decreases after a flash.

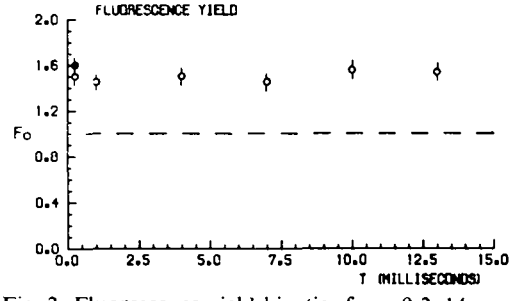

Fig. 2. Fluorescence yield kinetics from 0.2–14 ms after one saturating flash at 77 °K in spinach chloroplasts. $\phi = 1.5 \times \phi_0$. Different samples of the same preparation were used for each time. The ϕ_0 value was arbitrarily set to 1.0 for each time. The two points at time 0.2 ms were the first and last measured.

DISCUSSION

If at room temperature the fluorescence yield of chlorophyll is high, *e.g.* in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea and continuous background illumination, an intense flash causes a decrease in fluorescence yield, followed by a rise when the flash intensity decreases[3], in the same way as in the top curves of Figs 1A and 1B, which were measured at liquid nitrogen temperature.

These observations can be explained[3] by the reactions:

$$S \xrightarrow{h\nu} T \quad (1)$$

$$T \rightarrow S \quad (2)$$

T is a quencher which is formed in the light reaction (1), and disappears in the dark reaction (2) within a few microseconds. Our observation that the kinetics of the reactions following a flash are not appreciably altered at low temperature, is in accordance with the interpretation[3] that $S \xrightarrow{h\nu} T$ is a light reaction, the formation of a triplet, followed by triplet–triplet transfer in a chlorophyll *a*–carotenoid complex, and the reaction $T \longrightarrow S$, a triplet singlet transition: the rates of these reactions may be expected to be independent of temperature.

At room temperature the lowest curve of Fig. 1C would be quite different: the fluorescence yield would rise in 12 μs from a value $\phi_0 = 4.2$ to about $\phi = 8.4$. This rise has been attributed to the reduction of a quencher Q (ref. 3).

$$Q \xrightarrow{h\nu} Q^- \tag{3}$$

At room temperature, Reaction 3 is much more efficient than Reaction 1; Reaction 1 occurs with good yield when Q is reduced.

The simplest explanation for the curves in Fig. 1A and 1C is, that the light-induced reduction of Q, which causes the increase in fluorescence, occurs at liquid nitrogen temperature, with a quantum efficiency of only about 20% of that of the light-driven S→T reaction, and that, when T is present, the trapping in T is much more efficient than in Q so that during the remaining part of the flash, as long as T is present, no more Q is reduced. The 20% fraction of reduced Q formed at the beginning of the flash is responsible for the 20% increase in fluorescence yield when the quencher T disappears in a dark reaction (see lower curve of Fig. 1B). We have assumed that at liquid nitrogen temperature the units are separated. If that is not the case more than 20% of the Q is reduced in a flash. It is conceivable that the reaction center in state Q is a less efficient energy trap at liquid nitrogen than at room temperature.

Thorne and Boardman[10] also concluded from measurements at low intensity that the quantum efficiency for reduction of Q was about 4.4 times less at 77 °K than at room temperature. The above hypothesis is consistent with this.

We now consider an alternative explanation for the low fluorescence increase in a saturating flash. It might be assumed that Reaction 3 proceeds *via* a non- or weakly fluorescing intermediate I, as follows:

$$Q \xrightarrow{h\nu} I \rightarrow Q^- \tag{4}$$

The bottom curve of Fig. 1B then indicates that the reaction $I \rightarrow Q^-$ proceeds in a time shorter than 30 μs. In order to explain the low quantum yield for Q^- formation, we then have to assume that the reaction I→Q occurs in a shorter time than 30 μs. If it is assumed that the reaction I→Q occurs in a time appreciably shorter than about 30 μs, in a 100% flash of 16 μs appreciably more Q^- would be formed than in a 14% flash, which is not observed. Experiments with shorter flashes or with higher precision will establish whether this hypothesis is correct.

Butler[6] proposed a scheme as given by Eqn 4 with specified compounds. He specified Q, I and Q^- as DPQ, DP^+Q^- and D^+PQ^-, in which P and Q were the primary electron donors and acceptors and D a secondary donor; not only Q but also P^+ was assumed to quench the fluorescence.

A part of a light-induced decrease in absorption at 680 nm occurring at low temperature, was attributed to oxidation of P (Floyd *et al.*[4]). This interpretation was also adopted in Butler's discussion. The dark restoration of this part of the absorption change occurred with a half time of 4 ms. We only observed an increase in fluorescence in the dark with a half time of roughly 23 μs or less. Thus, if P^+_{680} is considered to be a fluorescence quencher, it is unlikely that the re-reduction of P^+_{680} takes 4–5 ms at liquid nitrogen temperature[4]. If re-reduction of P^+_{680} occurs in the μs region, however, P^+_{680} quenching is, within the precision of our experiments, masked by the quenching by T.

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