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DIFFERENT TYPES OF QUENCHING INVOLVED IN PHOTOSYSTEM II CENTERS

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SUMMARY

Low-temperature fluorescence rise curves are studied in an apparatus which allows fast variation and equilibration of the temperature.

1. Comparison of the effects of low temperature (−40 to −70 °C) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea shows that this inhibitor not only blocks the electron transfer between Q and A, but also removes a fraction of the quencher of Photosystem II centers.

2. Low-temperature fluorescence rises (−40 to −70 °C) depend on the number of oxidizing equivalents stored on the donor side of System II.

3. Three types of quenching can be experimentally distinguished: a quenching Q_F which is suppressed by a short saturating flash, a quenching Q_S destroyed under continuous illumination by a low efficiency process, and a quenching Q_R which cannot be destroyed at low temperature, but is removed by preillumination before cooling the sample.

4. Only the Q_F quenching seems to be related to the normal electron transfer which leads to O_2 formation.

5. It is suggested that destruction of Q_S is associated with photooxidation of cytochrome 559.

INTRODUCTION

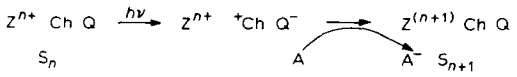
O_2 formation requires the extraction of 4 electrons from water. The measurement of O_2 evolved by a series of brief flashes[1,2] led Kok *et al.*[2] to propose a model for System II in which four series photoreactions occur in the same reaction center. This model is summarized in the following scheme:

$$S_0 \overset{h\nu}{\rightarrow} S'_0 \rightarrow S_1 \overset{h\nu}{\rightarrow} S'_1 \rightarrow S_2 \overset{h\nu}{\rightarrow} S'_2 \rightarrow S_3 \overset{h\nu}{\rightarrow} S'_3 \rightarrow O_2 \quad (1)$$

The four photoactive states S_n differ according to the number of oxidizing equivalents accumulated, which is equal to the state number n. States S_2 and S_3 deactivate slowly to S_1 with a half-time varying between 5 s and 2 min, depending upon the material. In dark-adapted material, the centers are a mixture of states S_0 and S_1, generally with a large excess of the state S_1.

Abbreviation: DCMU, 3,(3,4-dichlorophenyl)-1,1-dimethylurea.

From the experiments of Döring *et al.*[3] we know that the photoact is associated with a spectral change (bleaching) of one photoactive chlorophyll, Cha_{II}, with a turnover time $t = 200\ \mu s$. If we suppose that this bleaching reveals the photooxidation of Cha_{II}, a transition $S_n \rightarrow S_n{}^+{}_1$ can be written as follows:



Whatever the chemical interpretation of the Cha_{II} spectral change, it is likely that the positive charge formed by the photoreaction is not immediately stabilized at the level of the electron donor Z. Thus, the index n is equal to the number of positive charges stabilized on Z both in states S_n and S'_n.

Duysens and Sweers[4] demonstrated that the fluorescence yield of chlorophyll *in vivo* was a function of the oxidation state of a quencher Q which is the System II electron acceptor. The destruction (reduction) of Q occurs during the photochemical transition $S_n \rightarrow S_n'$. The reoxidation of Q^- is one of the dark reactions involved in the transition $S_n' \rightarrow S_{n+1}$.

More recently, it has been established[5–8] that the fluorescence yield is also dependent on the number of oxidizing equivalents stored, *i.e.* upon the state number (see Eqn 1).

First, the fluorescence yield which corresponds to the photoactive states S_2 and S_3 is slightly higher than that for states S_0 and S_1 (ref. 4). Second, Delosme[6,7] showed that the fluorescence yield is higher for photoinactive states S_0' and S_1' than for states S_2' and S_3'. These experiments were performed at room temperature by measuring the fluorescence yield reached during brief saturating flashes.

In order to study the fluorescence rise curves which correspond to the photochemical transition $S_n \rightarrow S_n'$, one can block the following dark steps by lowering the temperature[8]. We showed, under these conditions, that both the time course of the fluorescence rise curve and the fluorescence yield depend upon the state number. In this paper, we wish to analyse in more detail the basic features of these low-temperature fluorescence rise curves.

TECHNIQUES

Apparatus

The apparatus (Fig. 1) is designed to measure the fluorescence rise curves at any fixed temperature between -70 and $+30$ °C. The temperature of the sample is established by a Peltier effect device (Cambion thermoelectric module No. 801-3959-01). The photosynthetic material is placed on a circular cuvette (1 mm thick and 13 mm diameter). The circular wall is made of epoxy (Araldite) which supports 3 needles (cross-sectional diameter 8 mm). Two of these are used to renew the material in the cuvette while a thermocouple T_1 (not indicated in Fig. 1) is inserted in the third one to measure the temperature of the sample. The cuvette is closed on one side by the thermoelectric module. The other side is closed by a 0.2-mm-thick plastic sheet. The thermoelectric module is fixed with a heat-conductive glue on a large copper heat pipe, which is inserted in a Dewar flask containing the freezing mixture (solid CO_2 + ethanol).

A light pipe L resting directly against the plastic wall of the cuvette permits

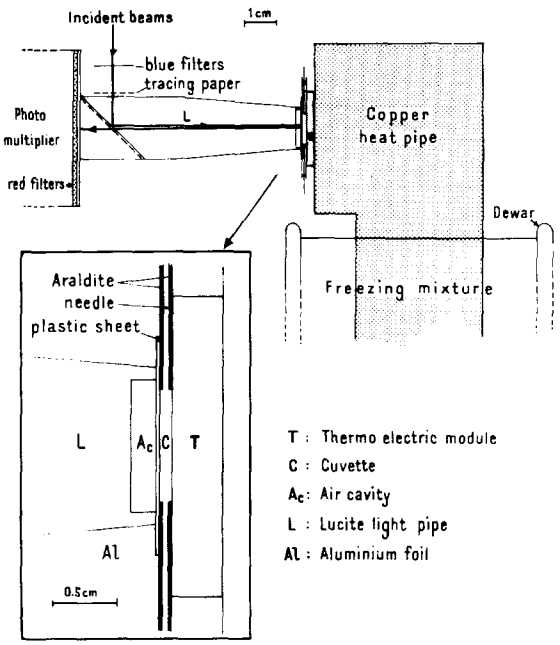


Fig. 1. Low-temperature experiments device. See text.

illumination of the material and collection of the fluorescent light. A 2-mm-thick air cavity A_c at the extreme end of the light pipe decreases considerably the heat loss on this side of the cuvette. The illuminating beams are partially reflected by a 45° cut in the light pipe (lucite to air reflection). To assure homogeneous illumination of the sample, a piece of tracing paper is placed in the incident beams. The sample can be illuminated continuously and/or by flashes (Verre and Quartz Co., Model XOD 22, 1.3 J, 5 μs). The light intensity of the continuous beam is about 5000 ergs $\cdot$ $cm^{-2} \cdot s^{-1}$. The two beams are focussed on the tracing paper sheet and are filtered by a 20-mm-thick blue Schott filter BG38. Fluorescence light is directed by the light pipe to the photocathode Model 9558 EMI photomultiplier and passes through two red filters (Rubylith Ulano and Wratten Kodak No. 70).

Temperature control

The electronic device to control the temperature has been realized by B. Frilley.

The thermocouple T_1 associated with a reference thermocouple T_0 (0 °C) is connected to a galvanometer. The temperature inside the cuvette is recorded on a Photodyne Sefram PH2D 118, which is also used to control the electric power supply of the thermoelectric module. The temperature can be regulated at ± 0.1 °C. Two temperatures can be preselected and fast transients are obtained by applying a direct current (5 A) of either polarity.

Experimental process

In the experiments reported in this paper, the higher of the two preselected temperatures is adjusted to a value between 0 and 20 °C where the sample can be

easily renewed. To reach this temperature, the thermoelectric module is used as a heater, the temperature of the upper part of the heat pipe being close to −60 °C. The lower preselected temperature is adjusted to a level below 0 °C, generally between −40 and −60 °C. Due to the low temperature on the heat pipe, heat losses through the thermoelectric module and the Peltier effect induced by a maximum direct current all contribute to the rapid decrease in temperature. In presence of glycerol, the rate of the decrease in temperature is about 3 °C/s in the +30 to −30 °C range, and slower in the lower temperature range.

Material

Spinach chloroplasts, prepared according to Avron[9], were either freshly prepared or stored at −60 °C, in the presence of 30% (v/v) glycerol or 5% (v/v) dimethylsulfoxide. These three types of chloroplast preparations gave similar results. During the experiments, the chloroplasts are suspended in 0.05 M Tris buffer (pH 7.5) with 0.4 M saccharose and 0.1 M NaCl *plus* 25% or 50% (v/v) glycerol. The chlorophyll concentration used is 30–40 μg/ml.

RESULTS

Effects of glycerol concentration

Fluorescence rise curves previously reported in ref. 8 were performed in the presence or absence of 25% (v/v) glycerol. In these conditions, the frozen sample is crystallized and, though the cuvette is only 1 mm thick, diffuses the actinic light, thus causing an inhomogeneity of illumination. This inhomogeneity increases all the characteristic levels of fluorescence emission and modifies significantly the time course of the rise curves.

Increasing the glycerol concentration to 50% allows the sample to be frozen as a perfect glass, with optical properties similar to those of the liquid phase sample. However, for periods longer than 2 min at low temperature (−50 to −60 °C), this glass begins to crystallize slowly, resulting in a situation similar to the one always observed in presence of 25% (v/v) glycerol. For these reasons, most of the experiments reported here are performed in 50% (v/v) glycerol, for periods not longer than 2 min at low temperature. At room temperature, the oxygen-evolving capacity of the chloroplasts is markedly decreased after 30 min in 50% (v/v) glycerol. In our experiments, a new sample is suspended in buffer with this glycerol concentration and used only during 20 min. Within this time period, we checked that the fluorescence rise curves measured at room temperature with or without 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) are not significantly dependent on glycerol concentration and particularly, present a well-marked inflection point. In the same time range, oxygen measurements in flashing light performed by Bouges-Bocquet in our laboratory showed a slight increase in the "misses" coefficient α (refs 2 and 10) which is responsible for a faster damping of the oscillatory pattern. Long incubation time in 50% (v/v) glycerol (>30 min) can modify also slightly the fluorescence induction curve in presence of DCMU.

Comparative effect of low temperature and DCMU

It is generally assumed that DCMU blocks the electron transfer between the

electron acceptor Q and the pool A (plastoquinone). Low temperatures act in a similar way by decreasing the rate of most of the dark reactions[11]. The comparison between effects of DCMU and low temperature has been recently discussed by Malkin and Michaeli[12].

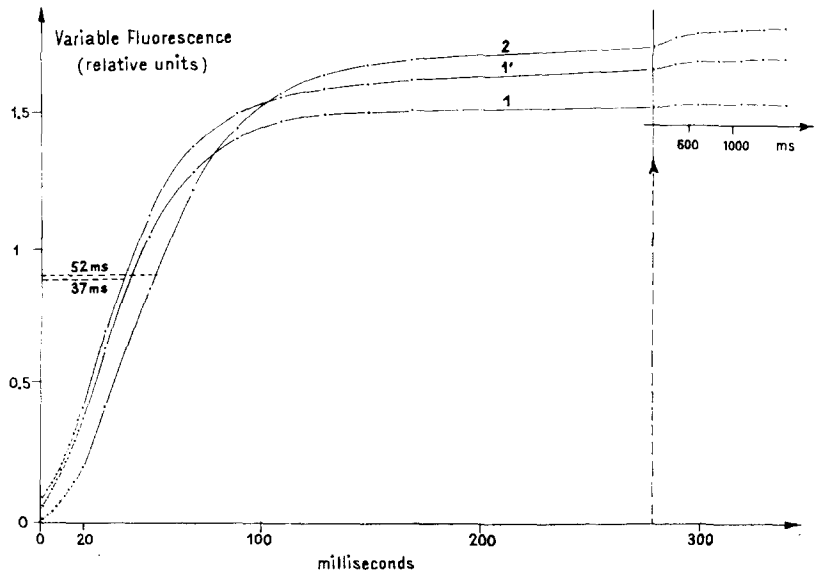


Fig. 2. Comparative effects of low temperature and DCMU on variable fluorescence curves. Chloroplasts *plus* 50% (v/v) glycerol. Curve 1, T = +5 °C, 20 μM DCMU; Curve 1', $T = -25$ °C, 20 μM DCMU. The same curves are observed for $T = -52$ °C or $T = -61$ °C. Curve 2, $T = -52$ °C, no DCMU.

We checked first the effect of temperature on fluorescence rise curves in presence of 20 μM DCMU (Fig. 2). When one decreases the temperature from +5 (Curve 1) to −25 °C (Curve 1'), a slight increase of the maximum fluorescence yield is observed, which is probably due to the block of the back reaction $Z^+ Q^- \rightarrow Z Q$ (ref. 13). A further decrease in temperature to −61 °C does not induce any further change in the Curve 1'. Thus the freezing process itself which occurs at about −32 °C in 50% (v/v) glycerol does not modify the light distribution in the sample and the efficiency of the photochemical reaction.

A comparison between the low-temperature rise curves (−52 °C) with or without DCMU (Curves 1' and 2, respectively) shows significant differences: in the presence of DCMU, the rise curve is faster ($t_{\frac{1}{2}} = 37$ ms) than when it is absent ($t_{\frac{1}{2}} = 52$ ms). In addition, the lag phase at the onset of illumination is more pronounced in the absence of DCMU. Depending upon the batch, the decrease in the half-time induced by DCMU varies between 0 and 40%. With *Chlorella* cells, this effect is more pronounced and more reproducible, the decrease in half-time being always close to 50%. This result can be interpreted in several ways:

(1) It can be due to a change in the quantum yield of the reaction, or to an increase of the light collected by the harvesting pigments.

(2) DCMU can decrease the number of active quenchers. This last interpretation agrees with recent experiments of Bennoun and Li[14].

Fluorescence oscillations

We previously showed[8] that the low-temperature fluorescence rise curves depend upon the states of Photosystem II centers, as defined in the model of Kok *et al.*[2]. In order to vary the distribution of the S states, dark-adapted chloroplasts are preilluminated at 0 °C by a series of flashes (Fig. 3). Immediately after the last preilluminating flash, the temperature of the sample was rapidly lowered to −50 °C.

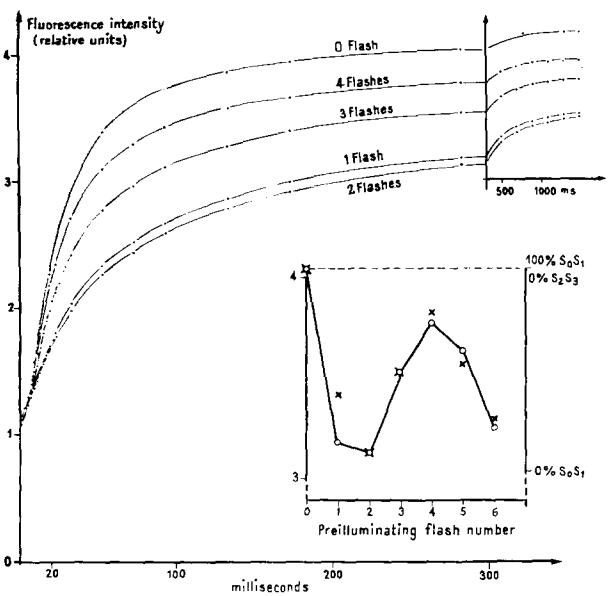


Fig. 3. Effect of preillumination by saturating flashes at 0 °C on fluorescence rise curves observed at −50 °C. Chloroplasts in 25% (v/v) glycerol. The number of preilluminating flashes is indicated for each rise curve. The start of the cooling process is synchronized with the last flash. Insert: ○, fluorescence yield reached at the end of the fast rise at −50 °C as a function of the preilluminating flash number; ×, S_0+S_1 concentration, computed from independent O_2 measurements during a series of flashes.

The freezing process is long compared to the dark relaxation times $S_n' \rightarrow S_{n+1}$ (see Eqn 1), but much shorter than the life time of states S_2 and S_3 formed by the preillumination at 0 °C. Fig. 3 represents the fluorescence rise curves in continuous light at −50 °C, after 0 to 4 preilluminating flashes at 0 °C. These rise curves depend strongly on the number of preilluminating flashes; the maximum difference is observed between 0 and 2 flashes preillumination. In the insert, a good correlation is obtained between the fluorescence level at the end of the fast rise, and the concentration of S_0+S_1 before continuous illumination as determined by independent O_2 measurements.

We studied the dependence of the fluorescence rise on the light intensity. After 0 or 2 preilluminating flashes given at room temperature, fluorescence rise

curve is measured at -72 °C for two intensities of the continuous beam, 1 and 0.016, respectively. Even in such a light intensity range, fluorescence yield depends upon the $I \cdot t$ product. Thus, low temperature fluorescence rise is essentially driven by photochemical reactions.

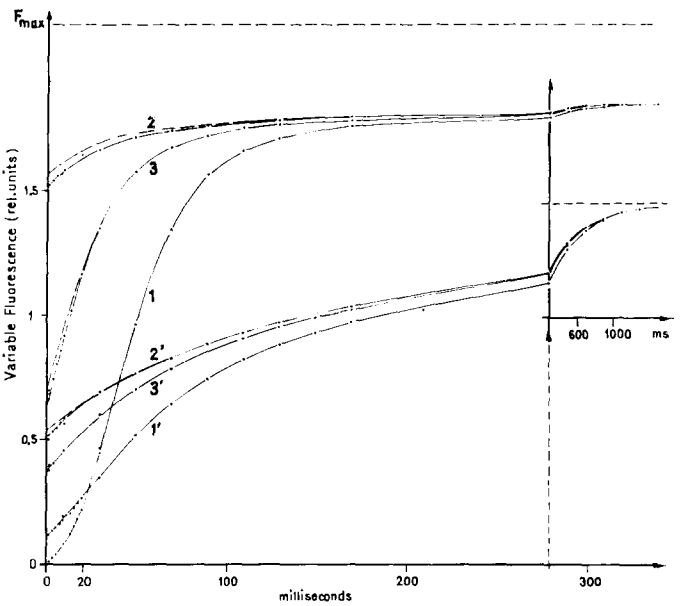


Fig. 4. Fluorescence rise curves at -52.5 °C. Chloroplasts in 50% (v/v) glycerol. Curves 1–3, dark-adapted chloroplasts. At time $t=0$, the centers are in S_0+S_1 states. Curve 1, control; Curve 2, a saturating flash (relative energy = 1) is added at -52.5 °C, a time $t_d=20$ ms before the continuous illumination; Curve 3, same as Curve 2, but with 0.041 relative flash energy. Curves 1′–3′, identical to Curves 1–3 except that chloroplasts are preilluminated at $+6$ °C by 2 saturating flashes (at an interval of 300 ms), the start of the cooling process being synchronised with the last flash; at time $t=0$ the centers are in S_2+S_3 states. The half-times for Curves 1 and 1′ are 48 and 98 ms, respectively. F_{max}, maximum fluorescence yield reached under continuous preillumination starting 30 s before the cooling process. The dashed lines above Curves 2, 2′ and 3 are the extrapolated curves for a time $t_d=0$ between the flash and the continuous illumination (see insert Fig. 9).

The experiments of Fig. 3 were performed in a medium which crystallizes at low temperature (buffer *plus* 25% (v/v) glycerol). We were able to reproduce these experiments in 50% (v/v) glycerol. In Fig. 4 the data obtained after 0 or 2 preilluminating flashes (Curves 1 and 1′) given before freezing are presented. With dark-adapted material, only states S_0 and S_1 are present, while after two preilluminating flashes, about 95% of the centers are in the S_2 and S_3 states. We determined that the relationship between states S and the fluorescence yield is independent of the glycerol concentration, however, significant differences exist in the time courses of comparable curves in Figs 3 and 4, due to inhomogeneity of illumination.

In Fig. 4, Curves 1 and 1′ differ by the fluorescence level attained in continuous light and by the half rise times, which are 2 times longer for Curve 1′ (S_2+S_3) than for Curve 1 (S_0+S_1). Curves 2 and 2′ represent the rise curves obtained when a

flash is added at the onset of the continuous illumination. In both cases, this additional flash does not destroy the totality of the quencher. Doschek and Kok[16] reported that with dark-adapted chloroplasts at room temperature in the presence of DCMU, a flash does not remove the totality of the quencher. In our experiments, the remaining slow rise curve observed after the flash is much more pronounced in Curve 3′ than in Curve 2. We checked that the light intensity of the flash was saturating at both low temperature and at room temperature. Decreasing the relative flash energy from 1 to 0.4 does not change the rise Curves 2 and 2′. Curves 3 and 3′ are obtained with a relative flash energy of 0.041, which is therefore not saturating in both cases. The dashed line F_{max} is obtained when the chloroplasts are preilluminated in strong white light to reduce completely pool A before freezing. This level is always significantly higher than the level reached at low temperature with dark-adapted chloroplasts (states $S_0 + S_1$).

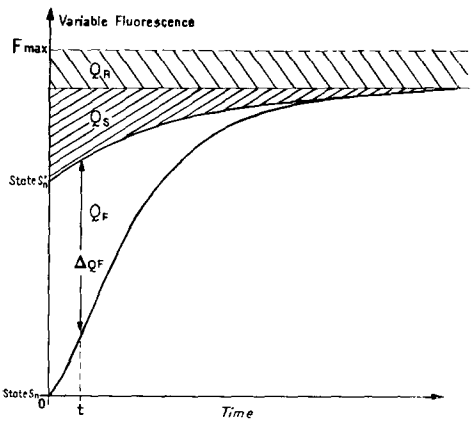


Fig. 5. Schematic representation of the three types of quenching Q_F, Q_S and Q_R. See text.

We can now define three different types of quenching, schematically represented in Fig. 5:

– a quenching Q_F, which can be destroyed by one saturating flash;

– a quenching Q_S, which remains after a saturating flash and is slowly destroyed upon continuous illumination. The total destruction of quenching Q_S requires a series of saturating flashes (10–20);

– a quenching Q_R, which at low temperature cannot be destroyed. Continuous preillumination at room temperature is required to abolish this quenching.

It must be borne in mind that these definitions are purely experimental and qualitative. For this reason, we prefer to use the unprecise term of "quenching" to the term "quencher", which presupposes a possible identification with a definite biochemical entity.

The amplitude of these three quenching types oscillates as a function of the number of preilluminating flashes (Fig. 6). States S_0 and S_1 show a large Q_F and small Q_S and Q_R, while the reverse is true for states S_2 and S_3. The faster damping of these oscillations compared to those depicted Fig. 3 is due to the presence of higher glycerol concentration. A similar effect is observed for the oxygen sequence.

The phase of the fluorescence oscillations is reproducible but their amplitude can vary among the chloroplasts preparations. We observed variations from 1.8 to 3 for the ratio $\Delta Q_F(S_0+S_1)/\Delta Q_F(S_2+S_3)$.

Fig. 7 shows the effect of 0.2 mM ferricyanide on the low temperature fluorescence rise curve (same chloroplasts as in Fig. 4). The addition of ferricyanide specifically decreases Q_S, and increases Q_R; for example in states S_2+S_3, Q_S is 4.5 times smaller in presence of ferricyanide though Q_F is almost unchanged. Some chloroplasts

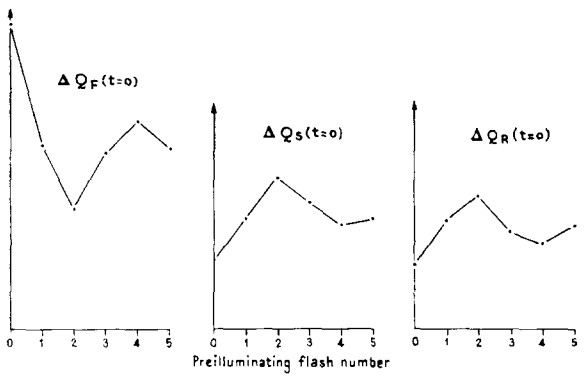


Fig. 6. Fluorescence oscillations of Q_F, Q_S and Q_R at -52.5 °C as a function of the preilluminating flash number at $+5$ °C. Chloroplasts in 50% (v/v) glycerol, *plus* 0.1 mM methyl viologen as electron acceptor. The amplitude of each Q_F, Q_S and Q_R quenching is measured at time $t=0$ (see Fig. 5).

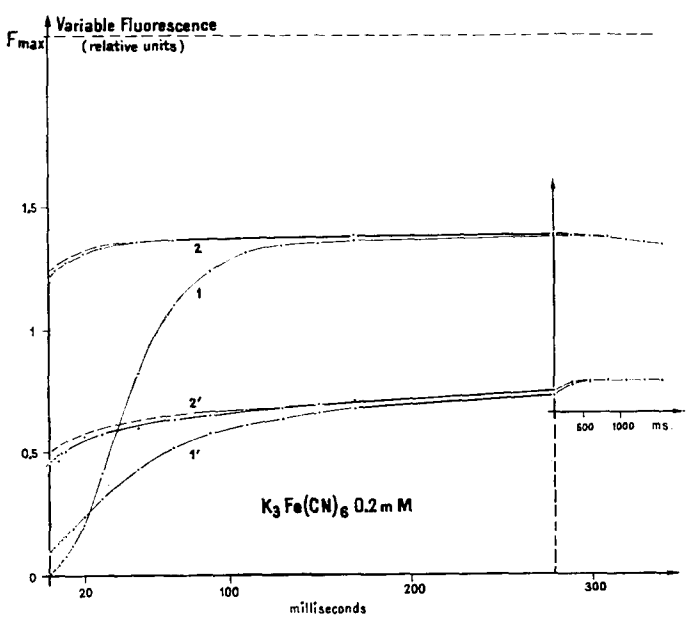


Fig. 7. Fluorescence rise curve at -52.5 °C in presence of 0.2 mM ferricyanide. Same chloroplasts and experimental conditions as in Fig. 4, Curves 1–2 and 1′–2′. F_{max}, maximum fluorescence yield measured as in Fig. 4, in absence of ferricyanide. Dashed curves, same process as in Fig. 4.

show a complete suppression of the quenching Q_S in the presence of ferricyanide.

The kinetics of destruction of Q_F can be computed from the difference between the rise curves with and without a flash at low temperature (see $\Delta Q_F = f(t)$ Fig. 5). This computation is only approximate because it assumes that destruction of both Q_F and Q_S are independent phenomena (no photochemical competition) which is probably not the case. Fig. 8 shows this computation of ΔQ_F for S_0S, and S_2S_3 in the absence (solid lines) or in the presence (dashed lines) of ferricyanide. It can be clearly seen that all the curves are far from exponential functions and present a characteristic lag. Furthermore, the half-times for Q_F destruction are close for states S_0S_1 and states S_2S_3. Moreover, no essential modification is induced by ferricyanide on Q_F quenching.

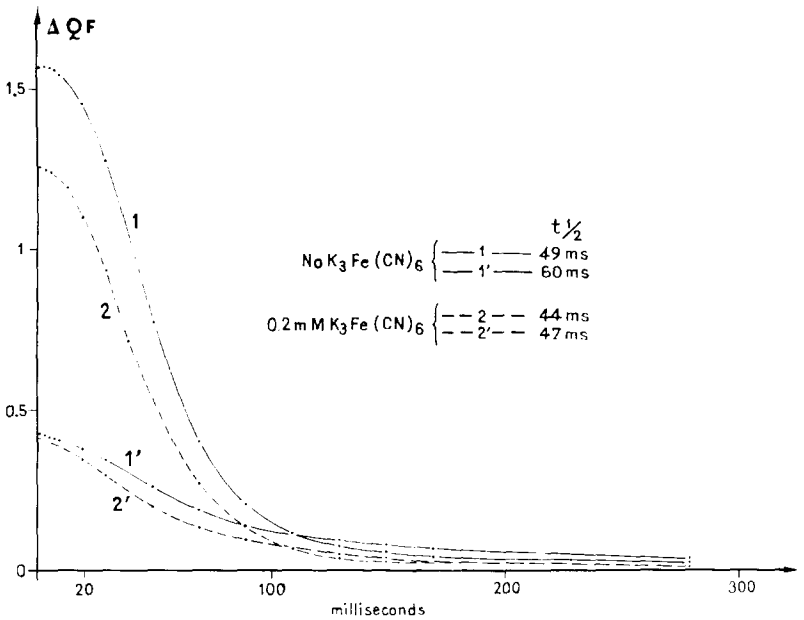


Fig. 8. $\Delta Q_F = f(t)$ as defined in Fig. 5. Curves 1 and 2, dark-adapted chloroplasts. Curves 1′ and 2′, chloroplasts are preilluminated at +5 °C by 2 saturating flashes (at an interval of 300 ms). Curves 1 and 1′, No ferricyanide, computed from Fig. 4 (Curve 2 *minus* Curve 1, and Curve 2′ *minus* Curve 1′). Curves 2 and 2′, 0.2 mM ferricyanide, computed from Fig. 7.

Regeneration of the quenchers at low temperature

Experiments performed between −60 and −40 °C show that some of the reactions which restored the quenchers are not completely blocked. Fig. 9 gives an example of this type of process with the chloroplasts placed in states S_2+S_3 before freezing: the dashed curves are obtained when a saturating flash is added a dark time t_d before the onset of the continuous illumination. It must be pointed out that even for dark time t_d longer than 30 s, the quencher can never be completely regenerated. Similar results are obtained when the same type of experiment is performed from S_0S_1 states, but a smaller fraction of the quencher is restored. To inter-

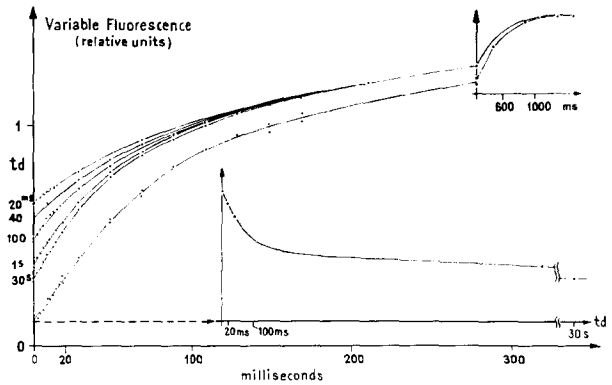


Fig. 9. Regeneration of Q_F at -52.5 °C. Chloroplasts in 50% (v/v) glycerol. The chloroplasts are preilluminated at $+6$ °C by 2 saturating flashes which convert the centers in States S_2 and S_3. Solid line, no flash given at low temperature. Dashes lines, one saturating flash is added at -52.5 °C a time t_d before the onset of continuous illumination. Insert: fluorescence yield at time $t=0$ as a function of t_d.

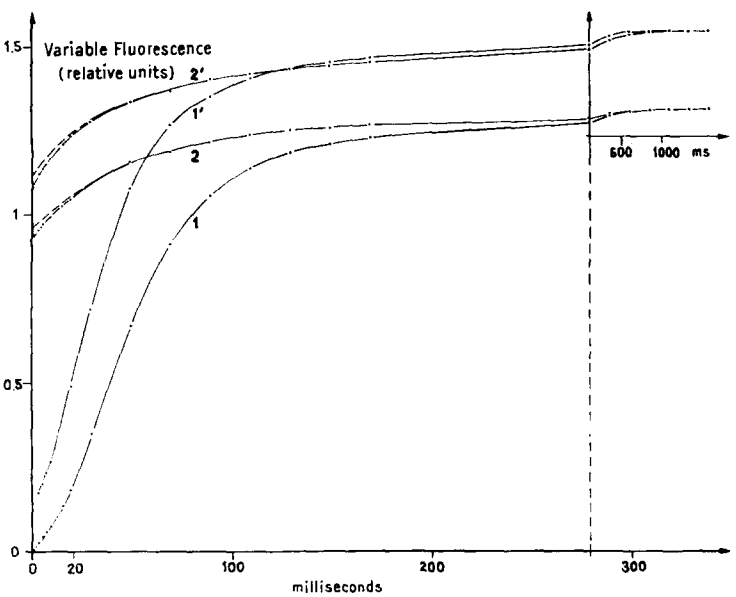


Fig. 10. Effect of continuous preillumination before cooling to $T=-52.5$ °C. Chloroplasts in 50% (v/v) glycerol. No electron acceptor. Curve 1, dark-adapted chloroplasts; Curve 2, same as Curve 1, but one saturating flash is added at -52.5 °C a time $t_d=20$ ms before the continuous illumination. Curve 1', chloroplasts are preilluminated for 30 s, white light at $+5$ °C, *plus* 1 min dark before cooling; Curve 2', same as Curve 1' but one saturating flash is added at -52.5 °C a time $t_d=20$ ms before the continuous illumination. Curve 1', chloroplasts are preilluminated for 30 s, white light at $+5$ °C, *plus* 1 min dark before cooling; Curve 2', same as curve 1' but one saturating flash is added at -52.5 °C a time $t_d=20$ ms before continuous illumination.

pret these phenomena, a more systematic study is required to determine whether this recovery is a back reaction or an electron transfer from reduced Q to A.

Effect of strong continuous preillumination

Fig. 10 shows an experiment in which chloroplasts have been preilluminated at +5 °C by 30 s of strong white light to completely reduce pool A. After a 1-min dark period at +5 °C, the sample is frozen. The fluorescence rise curve obtained after this pretreatment (Curve 1′) is compared to the rise curve observed for dark-adapted chloroplasts (Curve 1). One observes that a large fraction of the quencher is restored during the 1-min dark period, despite the fact that pool A remains practically reduced during this time. This result confirms that in the dark the equilibrium constant between pool A and Q is not equal to 1 (refs 8, 12 and 17). The main difference between Curves 1 and 1′ is the elevation of the fluorescence yield observed after preillumination. This increase probably reveals the same phenomenon that Delosme[18] described at room temperature. His experiments at very high light intensity were interpreted by means of a quencher R destroyed by a dark process. According to the terminology we defined above (Fig. 5), the preillumination at +5 °C suppresses the quenching Q_R.

The recovery of the three types of quenching Q_F, Q_S and Q_R was studied as a function of the dark time between the continuous preillumination at +5 °C and the start of the cooling process: Q_S is restored completely in less than 15 s, and Q_R in a much longer time (5–10 min). The recovery of Q_F is more complex: a main fraction is restored in about 15 s, but total recovery requires about 10 min.

DISCUSSION

Q_F quenching

We defined Q_F as the part of the quenching which is destroyed by a saturating flash. From O_2 measurements at room temperature, we know that such a flash induces the photoreaction $S_n \rightarrow S_n'$ for most of the centers (approx. 90% in chloroplasts). It seems reasonable to associate the Q_F destruction with this transition. In this hypothesis, the fluorescence yield measured few milliseconds after the low temperature flash is characteristic of the quenching of states S_n', since the following transitions $S_n' \rightarrow S_{n+1}$ are practically blocked at low temperature[19].

The quenching of states S_n' has been also studied at room temperature by Delosme[6,7], who measured directly the fluorescence yield 1 μs after the start of each flash in a series; this time is much shorter than the transitions $S_n' \rightarrow S_{n+1}$ at room temperature. A similar dependence of the fluorescence yield of state S_n' upon the state number is observed both at room and at low temperatures, with less variation of amplitude at room temperature. This difference is due to a secondary quenching process[20] which occurs during the flash and decreases the amplitude of the variable fluorescence (Delosme, R., unpublished).

Analogy between room-temperature and low-temperature experiments can also be seen in unpublished data of R. Delosme (Fig. 11): the fluorescence rise is measured at 0 °C under continuous high intensity illumination. With dark-adapted algae (Curve 1), the rise curve presents the characteristic sigmoidal shape. When the algae are preilluminated by a continuous weak light, which leads to equal concentration of the four photoactive states (*i.e.* 50% S_0+S_1, and 50% S_2+S_3), the rise curve is

slowed down (Curve 1'). The difference between the initial concentrations of states S_2+S_3 explains the less marked contrast between Curves 1 and 1' than in our experiments (see Fig. 4, Curves 1 and 1').

All the experiments we discuss here show that a much smaller variation of the fluorescence yield is associated with the transitions $S_2 \rightarrow S_2'$ and $S_3 \rightarrow S_3'$ than with the transitions $S_0 \rightarrow S_0'$ and $S_1 \rightarrow S_1'$. However, the time course of Q_F destruction is essentially independent of the state number (see Fig. 8). Thus, the quantum yield of the transition $S_n \rightarrow S_n'$ is independent of the state number; a conclusion that can also be drawn from O_2 measurements.

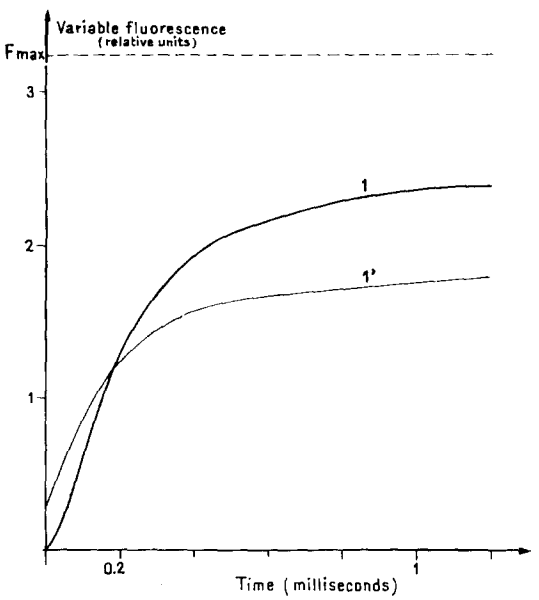


Fig. 11. Fluorescence rise curves at 0 ° and in high intensity illumination. *Chlorella* (from Delosme, R., unpublished). Curve 1, control with dark-adapted cells; Curve 1', 1 min preillumination in weak light (1 photon/300 ms per System II center). F_{max}, maximum fluorescence yield reached 5 s after the onset of illumination. Experimental method described in ref. 18.

We conclude that the destruction of Q_F is associated with the normal photoreaction which results in the storage of positive charges and O_2 evolution. Additional evidence is that neither Q_F quenching nor O_2 produced by a series of flashes is modified by ferricyanide, while the Q_S quenching is suppressed.

Q_S quenching

The photodestruction of Q_S appears as a slow process, *i.e.* a low efficiency process: the half-time of the destruction of Q_S from states S_2' and S_3' computed from Fig. 4 (Curve 2') is about 3 times longer than the half-time of destruction of Q_F. This low efficiency process indicates that Q_S destruction is not involved in the high efficiency process which leads to O_2 formation. Moreover, a single flash can destroy only a small fraction of Q_S, despite the fact that the flash energy is saturating.

Several arguments favor the hypothesis that destruction of Q_S is associated with the oxidation of cytochrome 559:

(a) At −196 °C, one observes the photooxidation of cytochrome 559, and the reduction of cytochrome C-550 (refs 21, 22) associated with the destruction of the fluorescence quencher[23].

At −196 °C, dark-adapted *Chlorella* show a slow fluorescence rise curve without a lag, in contrast to that obtained at −50 °C (refs 6 and 24). The transition from the fast to the slow rise curve occurs at about −100 °C (ref. 11). Actually, the fluorescence rise curves measured at −196 °C with dark-adapted material are similar or even slower than the rise curves observed at −50 °C with preilluminated material (S_2+S_3). Very low temperatures seem to partially suppress the Q_F quenching and enhance the Q_S quenching. Okayama and Butler[25] observed that the addition of ferricyanide markedly decreases the maximum fluorescence yield reached during the rise curve at −196 °C. This decrease of the maximum fluorescence yield is correlated with the oxidation of cytochrome 559 by ferricyanide, as shown by redox titration.

At −50 °C in States S_2+S_3 (Fig. 7, Curves 1′ and 2′) we observed a similar effect of ferricyanide which prevents the Q_S destruction. Thus, it seems reasonable to suggest that cytochrome 559 is the electron donor associated with the electron acceptor Q_S: a previous oxidation of cytochrome 559 by ferricyanide prevents a subsequent photoreduction of Q_S.

(b) Recently, Vermeglio and Mathis[26] showed for dark-adapted chloroplasts that only a small fraction of cytochrome 559 is photooxidized at −50 °C under continuous illumination. In the same experimental conditions, we observed a small Q_S quenching. However, when the chloroplasts have been preilluminated by one or two flashes at room temperature, a continuous illumination at −50 °C provokes a large photooxidation of cytochrome 559. For these conditions, we observed a large destruction of Q_S.

Photooxidation of cytochrome 559 and photoreduction of Q_S appear as a secondary process, which occurs when the normal donor Z has been previously oxidized. At −196 °C, the donor Z is partially inactived and photooxidation of cytochrome 559 becomes the main process. Recently, Cox and Bendall[27] concluded also that cytochrome 559 is not directly involved in the mechanism of O_2 evolution.

Q_R quenching

This quenching, which cannot be removed photochemically at low temperature, is only suppressed if pool A (plastoquinone) has been first reduced by a preillumination before freezing. This quenching may be due to the quencher R introduced by Delosme[18] to interpret the fluorescence rise curve observed at room temperature under high intensity illumination. A possible interpretation, already proposed by Delosme, is to assume that the oxidized form of plastoquinone exhibits some intrinsic quenching properties.

CONCLUSION

The three types of quenching Q_F, Q_S, Q_R have been introduced to take into account the experimental data reported here and do not imply any identification with definite biochemical entities.

We previously proposed[7] a model in which each photocenter includes two donor–acceptor couples Z_1Q_1 and Z_2Q_2 associated with one photoactive chlorophyll. Each Z can store a maximum of 2 positive charges and the photoactive quencher is

Q_1 in states S_0S_1, and Q_2 in states S_2S_3. In this model, Q_F would correspond alternatively to Q_1 or to Q_2 depending upon the initial S state. As only one quencher is reduced by a flash, the remaining quencher Q_2 or Q_1 would correspond alternatively to the Q_S quenching.

Since the validity of this model is not definitively established, it seems to us premature to extend this analysis further. We want essentially to point out the unexpected complexity of the fluorescence properties of chlorophyll *in vivo*. Whatever their exact functions, it is necessary to assume the intervention of two quenchers per photocenter. The destruction of these two quenchers involves two different types of photoprocesses, with high and low efficiencies.

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