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TEMPERATURE AND PREILLUMINATION DEPENDENCE OF DELAYED FLUORESCENCE OF SPINACH CHLOROPLASTS

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SUMMARY

Delayed fluorescence (luminescence) from spinach chloroplasts, induced by short saturating flashes, was studied in the temperature region between 0 and −40 °C. At these temperatures, in contrast to what is observed at room temperature, luminescence at 40 ms after a flash was strongly dependent, with period four, on the number of preilluminating flashes (given at room temperature, before cooling). At −35 °C luminescence of chloroplasts preilluminated with two flashes (the optimal preillumination) was about 15 times larger than that of dark-adapted chloroplasts. The intensity of luminescence obtained with preilluminated chloroplasts increased steeply below −10 °C, presumably partly due to accumulation of reduced acceptor (Q^-), and reached a maximum at −35 °C.

In the presence of 50 mM NH_4Cl the temperature optimum was at −15 °C; at this temperature luminescence was increased by NH_4Cl; at temperatures below −20 °C luminescence at 40 ms was decreased by NH_4Cl. At room temperature a strongly enhanced 40-ms luminescence was observed after the third and following flashes. The results indicate that both the S_2 to S_3 and the S_3 to S_4 conversion are affected by NlH_4Cl.

Inhibitors of Q^- reoxidation, like 3-(3, 4-dichlorophenyl)-1, 1- dimethylurea, did only slightly affect the preillumination dependence of luminescence at sub-zero temperatures if they were added after the preillumination. This indicates that these substances by themselves do not accelerate the deactivation of S_2 and S_3.

INTRODUCTION

Delayed fluorescence of green plants is presumably due to a back reaction between photoproducts of Photosystem 2 (see [1–3], and [4–6] for reviews of older literature). In the sub-ms region its intensity is strongly dependent upon the so-called S-state, i. e. the number of accumulated positive charges in the pathway to water [2]. This is also true at longer times after illumination for *Chlorella* [7, 8], but not for isolated spinach chloroplasts [8].

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

In the present paper we report the results of a study of delayed fluorescence of isolated chloroplasts in the temperature region between 0 and −40 °C. At these temperatures, in contrast to what is observed at room temperature, luminescence in the 40-ms region was clearly dependent upon the S-state, with a pronounced maximum in State S_4. In relation to these measurements the results are also reported of experiments concerning the effect of NH_4Cl and 3-(3, 4-dichlorophenyl)-1,1-dimethylurea (DCMU), which both are known to affect, amongst other things, the donor side of Photosystem 2.

When NH_4Cl was added to chloroplasts in State S_0, S_1 or S_2, both the S_2 to S_3 and the S_3 to S_4 conversion appeared to be affected. DCMU, if added between preillumination and cooling, did not abolish the preillumination dependence of luminescence at temperatures below 0 °C, indicating that it did not accelerate the decay of the positive charges stored in the pathway to water.

MATERIALS AND METHODS

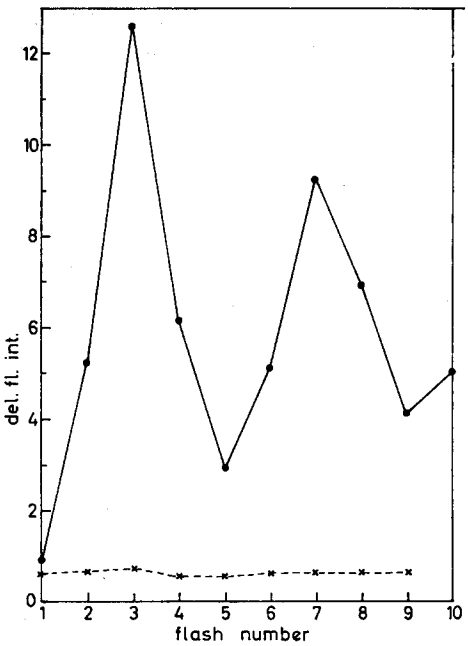
Chloroplasts from spinach were prepared as described in [9]. They were suspended in a solution of pH 7.8, containing 0.2 M sucrose, 0.06 M KCl, 0.04 M NaCl and 0.025 M sodium morpholinopropane sulfonate (MOPS) buffer, and kept in a darkened vessel at 0 °C until used. The chlorophyll concentration was 0.10 mM. The measurements were performed using the apparatus of Kraan et al. [10] adapted for low-temperature measurements. Actinic illumination was given in the form of short saturating flashes from a xenon flash tube, transmitted by a Balzers IR mirror and a Corning CS 4-76 glass filter. The measuring compartment was equipped with a double-walled Perspex chamber. A brass cuvette holder could be placed inside. The cuvette, made of Perspex, had a 1-mm path length and an area of 4 cm × 5 cm. Luminescence was detected from an area of approx. 3 cm × 2 cm in the center. A thermocouple at the center of this area, and in direct contact with the sample, was used to measure the temperature.

Dark-adapted chloroplasts, contained in the measuring cuvette, were preilluminated with various numbers of flashes, given at 1-s intervals. Within 5 s after the last flash the cuvette, plus holder, was put into liquid N_2, cooled to a temperature about 10 °C lower than the desired temperature, and transferred to the measuring compartment. When the temperature of the sample had increased to the desired temperature, one or more flashes were given and luminescence recorded.

For some experiments (Figs 4 and 6) the chloroplasts were preilluminated in a preillumination chamber [10] and mixed with an equal volume of medium, containing inhibitor (like NH_4Cl or DCMU), before they were transferred to the measuring cuvette.

RESULTS AND INTERPRETATION

Luminescence of spinach chloroplasts at temperatures below 0 °C was strongly dependent on preillumination. Fig. 1 shows luminescence at −35 °C, measured 40 ms after a flash, as function of the number of preilluminating flashes given before cooling. The flash number in the figure includes the final flash given after cooling. A large oscillation in amplitude, with a period of four, is apparent.



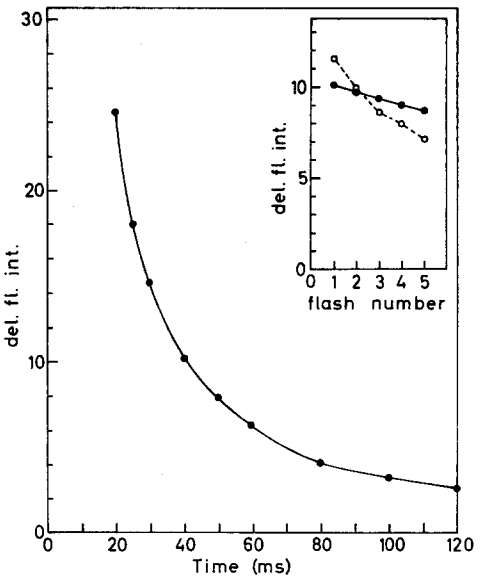


Fig. 1. Delayed fluorescence of spinach chloroplasts at 40 ms as function of flash number. × - - - ×, temperature 15 °C, ● — ●, the last flash of a series was given at −35 °C, the preceding flashes at 15 °C. For this and the other figures the intensity of delayed fluorescence is given in arbitrary units, which are roughly comparable for different figures.

Fig. 2. Kinetics of delayed fluorescence induced by a flash at −40 °C. Chloroplasts were preilluminated by two flashes at 15 °C. Insert: Delayed fluorescence at 40 ms as function of the number of flashes given at −30 °C (broken line) or −40 °C (solid line). Chloroplasts were preilluminated by two flashes at 15 °C.

The oscillation is in phase with that of O_2 evolution (at room temperature) [11, 12], indicating that the luminescence was strongest in State S_4. Above 0 °C the luminescence in this time region was much weaker, and only very little dependent on preillumination (Fig. 1, broken line; see also [8]). The kinetics of luminescence in the region around 40 ms, at −40 °C, are given in Fig. 2. With repeated flashes, at −40 °C, the luminescence decreased only little (Fig. 2, insert). This suggests that the decay of luminescence at −40 °C is mainly governed by recombination, and not by a reaction of the luminescence precursors with secondary electron donors and acceptors.

The temperature dependence of luminescence at 40 ms after a flash is given in Fig. 3 (solid line). The chloroplasts were preilluminated by one or two flashes before cooling. The intensity of luminescence increased steeply below −10 °C and reached a maximum at about −35 °C. The temperature dependence appeared to be somewhat dependent on the preillumination: with two flashes preillumination the luminescence increase was relatively smaller and took place in a somewhat more narrow temperature range than after one flash. The temperature range in which the main increase of luminescence took place, −10 to −30 °C, is the same as the one in which, according to Malkin and Michaeli [13] (see also [14]), reoxidation of photoreduced Q by secondary acceptors is progressively inhibited. This makes it

probable that the increase in luminescence is at least partly due to the enhanced concentration of reduced Q after a flash at the lower temperatures. Below about −35 °C the luminescence intensity decreased again (Fig. 3). The total amount of light emitted between 40 ms and 1 s decreased similarly (data not shown) indicating that the decrease was not due to a change in decay time, but to a decrease in yield.

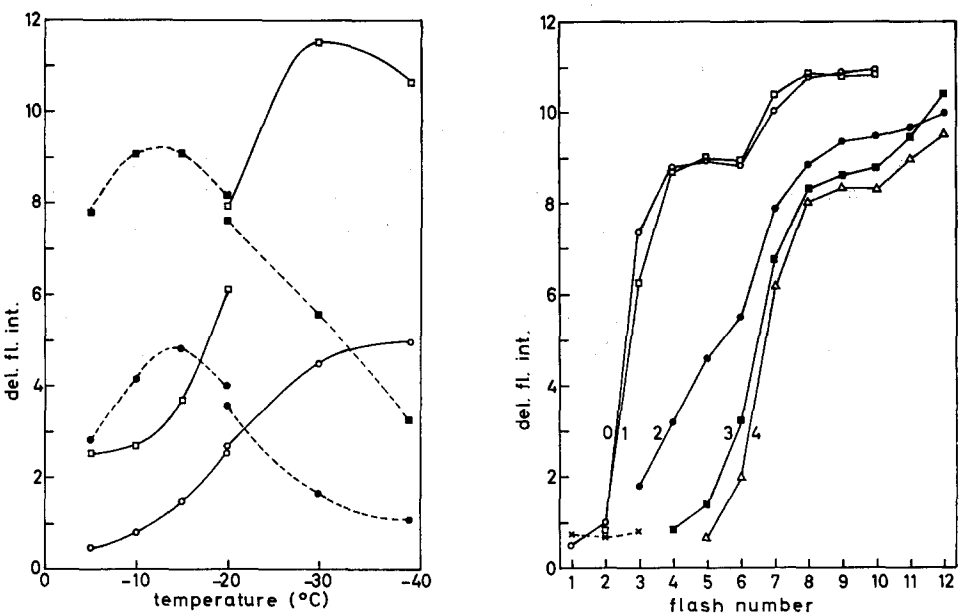


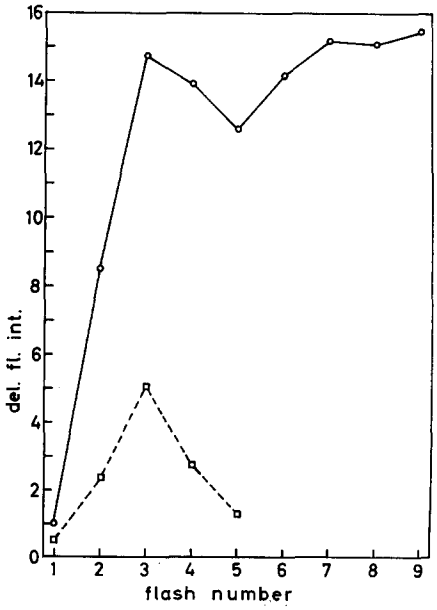
Fig. 3. Delayed fluorescence of preilluminated chloroplasts as function of temperature in the absence (solid lines) and presence (broken lines) of 50 mM NH_4Cl. Delayed fluorescence was measured at 40 ms after a flash. ● and ○, 1 flash preillumination, given at 15 °C; ■ and □, 2 flashes preillumination given at 15 °C. The temperature ranges −5 to −20 °C and −20 to −40 °C were measured with different preparations of chloroplasts.

Fig. 4. Effect of NH_4Cl on delayed fluorescence at 40 ms as function of flash number at 17 °C. Flashes were given at 5-s intervals. NH_4Cl (50 mM) was added before the first flash (Curve 0) or 2 s after the first, second, third or fourth flash (Curves 1, 2, 3, 4, respectively). ×- - -×, no NH_4Cl added.

Luminescence at sub-zero temperatures was strongly affected by a high concentration of NH_4Cl (Fig. 3). It enhanced the luminescence above about −20 °C and gave a decrease at lower temperatures. The optimum was now at −15 °C. At room temperature, the 40-ms luminescence was little affected for the first two flashes; it was strongly enhanced from the third and following flashes on (Fig. 4, Curve 0). Fig. 4 (Curves 1–4) also gives the results obtained when NH_4Cl was added after the first, second, third and fourth flash. The results indicate that, for large stimulation of 40-ms luminescence to occur, NH_4Cl must be present before State S_3 is reached, i. e. before three positive charges have been accumulated in the pathway to water. That already the S_2 to S_3 conversion is affected by NH_4Cl is also evident from Zankel's measurements [2]: luminescence at 0.5 ms, at room temperature, was already strongly enhanced after the second flash. Since at 40 ms only little stimulation occurs after the second flash (Fig. 4, Curve 0), one may conclude that the lumines-

cence precursor can decay in a time much shorter than 40 ms after the second flash. A similar decay is apparently not possible after the third flash. At −15 °C, the 40-ms luminescence is also strongly enhanced after the second flash, indicating that the decay of the luminescence precursor by secondary reactions is inhibited then also (Figs 3 and 5).

The fact that 40-ms luminescence at temperatures below 0 °C is strongly dependent upon the S-state can be used as a method to determine the S-state in conditions where the more usual method, i. e. by measurement of O_2 evolution, is not possible. We have applied this by studying the effect of DCMU, which, with chloroplasts, inhibits O_2 evolution ([15], see also [16–18]). After preillumination with various numbers of flashes, we added 10 μM DCMU. Approx. 20 s later the sample was cooled to −35 °C, where flash-induced luminescence was measured. Fig. 6 shows that the addition of the inhibitor only slightly affected the luminescence observed at this temperature; evidently, the inhibitor did not abolish the effect of preillumination. Similar results were obtained with atrazine and *o*-phenanthroline. These results indicate that these substances by themselves do not accelerate the deactivation of States S_2 and S_3 and that the inhibition of O_2 evolution occurring with these substances [15–18] cannot be explained by such a mechanism.



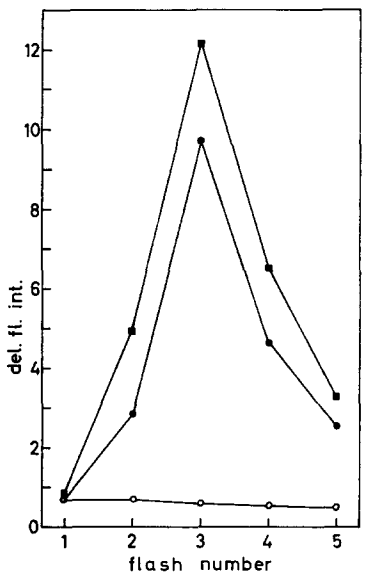


Fig. 5. Delayed fluorescence at −15 °C as function of flash number, in the absence (□ . . . □) and presence (○ − ○) of 50 mM NH_4Cl. The last flash of a series was given at −15 °C, the preceding ones at 15 °C.

Fig. 6. Effect of DCMU on delayed fluorescence of chloroplasts at −35 °C. ● − ●, chloroplasts were preilluminated (at 17 °C) by 0–4 flashes. 3 s after the last flash 10 μM DCMU was added. Approx. 20 s later the chloroplasts were cooled to −35 °C, where a flash was given and 40-ms luminescence recorded. ○ − ○, the DCMU was added before the preillumination; ■ − ■, no DCMU added. As solvent for DCMU, dimethylsulfoxide (final concn: 0.1 %) was used. It was also added to the control (■ − ■).

DISCUSSION

At room temperature sub-ms luminescence of isolated chloroplasts, in particular the 200-μs component, is strongly dependent on the S-state [2]. With dark-adapted chloroplasts (which are predominantly in State S_1) [8], the third flash induces about 3 times as much of this component as the second one; the first one induces little or none [2]. At longer times however, e. g. at 40 ms after the last flash, the intensity of luminescence of isolated chloroplasts, in contrast to that of *Chlorella* [7, 8], is not or only little flash-number dependent. Although the extra positive charge generated by the second flash is stored in the chain, it is apparently stabilized in a time much shorter than 40 ms, so that it contributes only little to luminescence at 40 ms. Our results indicate that this stabilization is progressively inhibited on lowering the temperature below 0 °C, since then luminescence at a time as long as 40 ms is still strongly dependent on the S-state in which the flash was given. At −35 °C the flash-number dependence of luminescence at 40 ms strongly resembles that of the 200-μs component at room temperature. We concluded from Fig. 2 that at −40 °C the decay by secondary reactions of the luminescence precursor was so much inhibited that the luminescence decay is mainly governed by recombination with Q^-. The temperature dependence of delayed fluorescence in the temperature region between 0 and −40 °C was similar to that obtained in earlier studies [19, 20]. In these studies, however, the preillumination dependence of delayed fluorescence at low temperature was not taken into consideration.

NH_4Cl, when applied at a high concentration, is reported to inhibit O_2 evolution, but not the oxidation of artificial electron donors like hydroxylamine [21]. This indicates that it affects that part of the electron transfer chain that can accumulate four positive charges and can use these charges to oxidize water. This conclusion is supported by the measurements of luminescence (Figs 3—5 and [2]). In addition, the flash dependence of 40-ms luminescence at room temperature indicates that in the presence of NH_4Cl the oxidizing side of Photosystem 2 can still accumulate four positive charges, of which the first three are stabilized but the fourth charge, which normally disappears in the reaction that gives O_2, is not; apparently it decays at least partly by a recombination reaction with Q^-, giving an intense emission of luminescence. As stated above, the results obtained when NH_4Cl was added after the first or second flash indicate that the conversion of S_2 to S_3 is also affected.

The temperature dependence of luminescence suggests that Q^- is the electron donor in the luminescence reaction at low temperature. Such a reaction of Q^- should have its reflection in the fluorescence behaviour at low temperature. One would expect that after a flash a fluorescence decay would accompany the reoxidation of Q^- due to luminescence. Such a fluorescence decay, with kinetics comparable to those of luminescence (Fig. 2), has indeed been observed (at −52.5 °C) by Joliot and Joliot [22]. In accordance with what one would expect on basis of the luminescence measurements, the amplitude of this fluorescence decay was larger with preilluminated than with dark-adapted chloroplasts [22]. It appears that the dependence of the rise kinetics of fluorescence at −40 °C [22, 23] upon the number of preilluminating flashes cannot be explained in this way, because these kinetics are almost equal after one and after two flashes preillumination [22], which was not the case for the luminescence intensity.

Addition of DCMU between preillumination and cooling did not abolish the preillumination effect upon luminescence at −35 °C. This indicates that DCMU does not strongly enhance the rate of decay of States S_2 and S_3. This agrees with an earlier observation than an addition of DCMU only little affects the preillumination effect upon fluorescence induction at −40 °C [9]. The inhibition of O_2 evolution of chloroplasts by DCMU when added after preillumination, as observed by Rosenberg et al. [15], therefore appears to be due to some inhibitory rather than destabilizing effect of DCMU on the donor side (see also [18]). The acceleration of the deactivation of States S_2 and S_3 in the conditions of Renger [17] and Bouges-Bocquet et al. [16], i.e. with a non-saturating concentration of DCMU added before preillumination, may have been due to a back reaction between positive charge in the non-inhibited centers with Q^- from inhibited centers. This would indicate that such a back reaction is not restricted to oxidant and Q^- belonging to independent, separate, chains. The observation, made by Bennoun [24], that the kinetics of reoxidation of Q^- in the presence of DCMU are second order with respect to the concentration of Q^- is in agreement with this assumption.

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