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TEMPERATURE DEPENDENCE OF DELAYED LIGHT EMISSION IN SPINACH CHLOROPLASTS

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SUMMARY

1. Delayed light of chlorophyll emitted at 0.1–3.9 ms after cessation of repetitive flash light was studied at temperatures between +40 and −196 °C in isolated spinach chloroplasts.

2. Induction kinetics of delayed light varied depending on temperature. It was found to be composed of two phases; one was an initial rapid rise followed by a rather fast decline to a low steady state level (fast phase), and the other was a slow increase after the initial rapid rise to the maximum followed by an insignificant slow decrease to a high steady state level (slow phase). The fast phase existed between −175 and 40 °C with the maximum at −40 °C, while the slow phase, between 0 and 40 °C with the maximum at 25 °C.

3. The intensity of delayed light at −175 °C was found to be less than one fiftieth that at 0 °C, and no delayed light emission was observed at −196 °C within experimental accuracy. This is in contrast to the results reported by Tollin, G., Fujimori, E. and Calvin, M. ((1958) Proc. Natl. Acad. Sci. U.S. 44, 1035–1047) in which the intensity of delayed light measured at −170 °C was about a half that at 0 °C.

4. The induction of delayed light measured at −96 °C was found to be significantly suppressed by the preillumination at −196 °C. This finding suggests that the primary photochemical event still survives at −196 °C without emission of delayed light.

5. Decay kinetics of delayed light after the flash excitation revealed the presence of at least two decay components. A slow decay component with a half decay time of several tens of milliseconds was observed at temperatures higher than 0 °C. A fast decay component with a half decay time of about 0.2 ms was observed at temperatures between −120 and 25 °C. The decay rate of this component was slightly retarded on cooling.

6. The System II particles derived from spinach chloroplasts with digitonin treatment showed a temperature dependence of delayed light similar to that of the chloroplasts. System I particles, on the other hand, scarcely emitted the delayed light at any temperature between 40 and −196 °C.

INTRODUCTION

Delayed light emission of chlorophyll emitted by various photosynthetic organisms is in close relation to the primary photochemical reaction of photosynthesis [1–4]. In green plants, the delayed light emitted at milliseconds after the excitation flash originates from pigment System II presumably through a reverse process of the primary photochemical reaction [5–10].

In a previous study [10] in System II particles in which the phosphorylation reaction was uncoupled from the electron transport, the induction of delayed light measured at 0.1–1.8 ms after the repetitive excitation flash was found to be inversely related to the induction of chlorophyll *a* fluorescence. These experimental results lead us to a mechanism of delayed light in which the delayed light originates through a recombination reaction between the reduced primary electron acceptor and the oxidized primary electron donor of Photoreaction II.

Tollin and Calvin [11] and later Tollin et al. [2] investigated temperature dependence of delayed light in spinach chloroplasts and *Chlorella*. In their experimental results, the delayed light measured at 1.5–10 ms after the flash had the maximum at about −40 °C, and the yield of delayed light at −170 °C was about a half that at 0 °C. Between −130 °C and −170 °C, they did not find any temperature-dependent change in yield and decay rate of delayed light.

In the present study, the temperature dependence of the delayed light was investigated under the excitation with the repetitive red flash light (0.9 ms in duration having wavelengths longer than 600 nm). It will be shown that, in contrast to the results of Tollin et al. [2, 11], no delayed light emission was observed in isolated spinach chloroplasts at temperatures lower than −175 °C in an experimental accuracy. The temperature-dependent changes in the shape of the induction of delayed light during repetitive illumination were also investigated.

MATERIALS AND METHODS

Chloroplasts (Class 2) were obtained from spinach leaves in a medium containing 0.4 M sucrose, 0.01 M NaCl and 0.05 M phosphate buffer, pH 7.8, as described previously [8]. System I particles and System II particles were prepared using digitonin according to the method of Ohki and Takamiya [12]. In measurements of delayed light and fluorescence, the chloroplasts or the particles were suspended in 0.025 M phosphate buffer, pH 7.8, 0.2 M sucrose, 0.005 M NaCl and 10% polyethyleneglycol (average molecular weight, 4000). Polyethyleneglycol was added to reduce the strong light scattering due to ice cracks below the freezing point of the medium. Chlorophyll concentration, measured according to Arnon [13], was 2–3 μg chlorophyll per ml in the measurements except for otherwise stated.

The cuvette was made of a copper plate of 1 mm thickness with a hole of 1 cm × 2 cm in surface area sandwitched by two transparent polymethyl methacrylate resin plates of 1 mm thickness.

The cuvette containing the chloroplast or the particle suspension was warmed in water (20–40 °C), or cooled in cold nitrogen gas or liquid nitrogen, to desired temperature, which was attained within less than 20 s. For the measurements of delayed light and fluorescence, the cuvette was moved into Dewar vessel having quartz windows, the temperature of which was previously adjusted to the desired one. The temperature of the sample was monitored with a calibrated gold–constantan platinum thermocouple placed in the cuvette. The sample in the cuvette was renewed for each measurement.

The delayed light and the fluorescence were measured with a modified Becquerel type phosphoroscope as described previously [8]. A cycle of excitation and measurement was 5.7 ms; 0–0.9 ms for excitation, 0.9–5.7 ms for darkness. The time from fully open to fully closed was 0.1 ms. The delayed light was measured 1.0–4.8 ms (0.1–3.9 ms after cessation of 0.9 ms illumination). The excitation light was obtained from a 500 W xenon arc lamp (Ushio Electric Co.) through optical filters, Hoya HA-50 and Toshiba V-R 60. This combination of the light source and the filters produced red light having wavelengths between 600 and 800 nm which did not excite luminescence of quartz nor poly(methyl methacrylate) resin at the low temperatures. The intensity of excitation light was 17 000 ergs · cm^{-2} · s^{-1} (with the sector stopped) at the surface of the cuvette. The delayed light emitted at the side opposite to the exitation light was detected by a photomultiplier, Hamamatsu TV R-374 (S-20 photoresponse) with an optical filter, Toshiba V-R 65. With this combination of the photomultiplier and the filter, light with wavelengths between 650 and 850 nm was detected. In the measurement of fluorescence, excitation light was obtained from a tungsten lamp (Kondo Electric) operated at DC 10 V, 7 A combined with an interference filter with a transmission peak at 480 nm (Japan Vacuum Optics) and optical filters, Corning 9782 and Hoya HA-50. Intensity of the excitation light was 130 ergs · cm^{-2} · s^{-1} at the surface of the cuvette. The surface of the cuvette was set at an angle of 45 degrees to the excitation light. The fluorescence emitted behind the cuvette at right angles to the excitation light was detected by a combination of the photomultiplier and the filter the same as in the measurement of delayed light emission. The signal from the photomultiplier was directly fed into a storage type oscilloscope (Hitachi, V-018) or was amplified after averaged by an RC combination (time response, 0.1 s) and recorded on a strip chart servo recorder (SPH-4, Riken Denshi).

RESULTS

Temperature dependence of delayed light emission

Induction kinetics of delayed light emission varied depending on temperature (Fig. 1). At 17 °C the intensity of delayed light rapidly increased from zero level to an initial rise level designated as I, and then, after showing a slight but distinct trough, increased slowly to the peak, P, which was followed by a slow decline to its final steady state level, S, if a longer illumination was applied. This is a typical induction pattern of delayed light at room temperature [8, 14]. At 1 °C, the level of initial rise was slightly higher than that at 17 °C, while the second increase became very slow. As a result, a rather fast decline after I was clearly seen. At −14 °C, there appeared a high initial rise followed by a fast decline to a low steady state level. The slow increase phase was not observed. The initial rise level was further elevated at −45 °C. On further cooling, i.e. to −95 and −131 °C, the I level was depressed. At the temperatures below −14 °C, the induction mode was scarcely changed except for a deviation in the rate of decline from the I level to the S level; half time of the decline

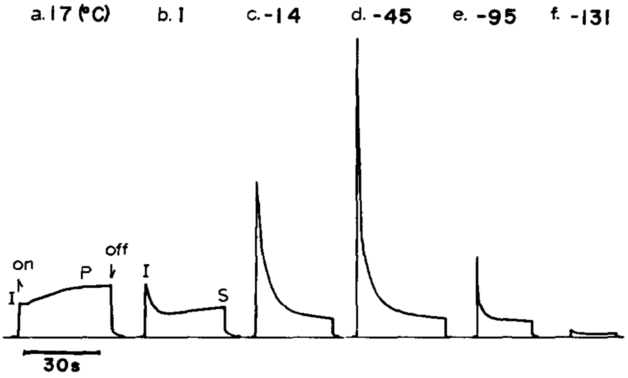


Fig. 1. Induction of delayed light emission in spinach chloroplasts at various temperatures. Temperature: a, 17 °C; b, 1 °C; c, −14 °C; d, −45 °C; e, −95 °C; f, −131 °C.

was 3.3 s at −14 °C, 1.0 s at −45 °C and 2.0 s at −131 °C.

These findings suggest that the induction kinetics of delayed light is composed of at least two phases; one is a rapid initial rise followed by a rather fast decline to a low steady state level (fast phase), and the other is a slow increase after the I level to the maximum followed by a slow decrease to a high steady state level (slow phase). The above-mentioned experimental results indicate that the induction at temperatures lower than 0 °C is only due to the fast phase, while that at temperatures higher than 0 °C consists of the fast and slow phases.

Analysis of the induction of delayed light at room temperature in the previous studies [8–10, 14] indicates that the fast phase responds to the state of the primary electron donor and acceptor of Photoreaction II and that the slow phase represents an accumulation of the high energy state of photophosphorylation. It may be concluded, therefore, that the high energy state is not accumulated at the low temperature, or if it is, it has no effect on the delayed light emission.

An uncoupler of photophosphorylation, gramicidin S [15], which has been shown to eliminate the slow phase of delayed light induction [10], had little effects on the induction at temperatures below 0 °C. This experimental result is expected, since the slow phase does not exist at the low temperature.

The intensities of delayed light measured at the I and the S levels were plotted against temperature (Fig. 2). The I level was low at room temperature and was maximum at about −40 °C. A discontinuous increase in the I level on cooling was observed at −3 °C at which the medium freezed. At −40 °C, the I level was ten times as high as that at 0 °C. At −175 °C, it was less than one-fiftieth that at 0 °C. On the other hand, the steady state level, S, responded to temperature in a different manner. The S level was maximum around 25 °C, was at a very low level at temperatures between −30 and −90 °C, and became lower on further cooling.

The result that the intensity of delayed light at −175 °C was lower than one-fiftieth that at 0 °C is in contrast to the work by Tollin et al. [2], in which the intensity of delayed light at −170 °C measured between 1.5 and 10 ms after the flash light was about a half that at 0 °C.

The temperature-dependence curves shown in Fig. 2, however, do not represent an intimate relationship between the temperature and the delayed light emission,

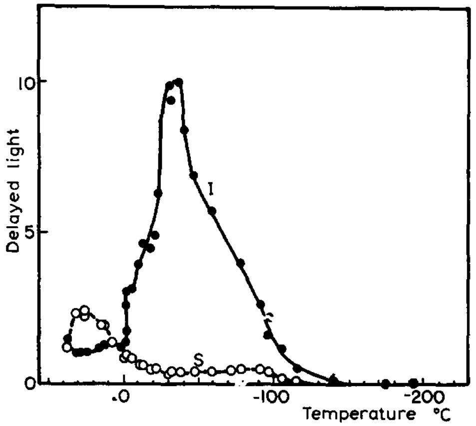


Fig. 2. Temperature dependences of the initial rise (I) and steady state (S) levels of induction of delayed light. Intensities of delayed light at I and S levels were plotted against temperature. ●-●, I level. ○-○, S level.

because absorption of incident light and self-absorption of emitted delayed light are markedly increased due to formation of ice cracks at temperatures below the freezing point of the medium. To eliminate these effects, the intensity of delayed light was divided by the fluorescence intensity. It should be noted here that according to Lavorel [5, 16] the measured intensity of delayed light is the rate of regenerating the excitation energy through the mechanism of delayed light emission multiplied by the fluorescence yield. Thus, the above-mentioned treatment of delayed light emission produces a temperature dependence of the rate of regenerating excitation energy.

Fig. 3a shows the temperature dependence of the intensities of delayed light

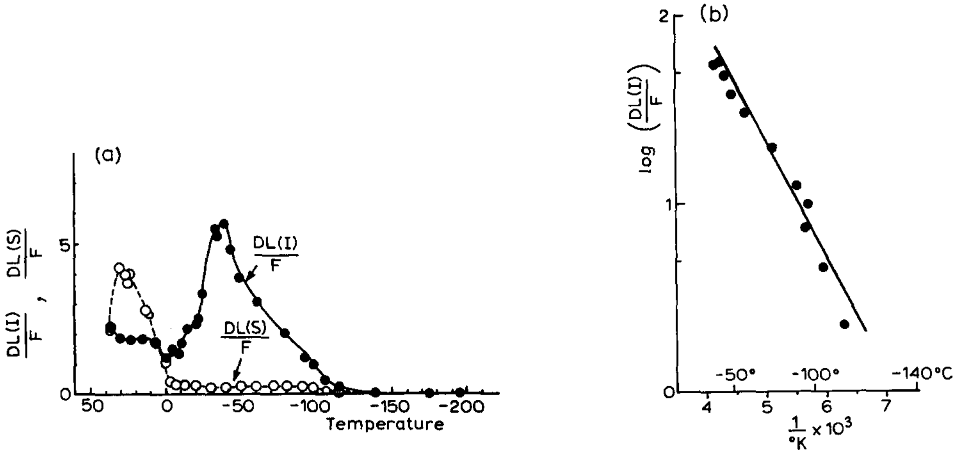


Fig. 3. (a) Temperature dependence (°C) of delayed light corrected for the changes in the fluorescence intensity. Values for DL(I)/F and DL(S)/F (see the text) were calculated from data in Fig. 2. ●-●, DL(I)/F; ○-○, DL(S)/F. (b) Arrehenius plot of DL(I)/F. Values for DL(I)/F over the temperature range from −40 to −120 °C in (b) were plotted in logarithmic scale against 1/°K.

at the I and S levels divided by the fluorescence intensity designated as DL(I)/F and DL(S)/F, respectively. No discontinuous point was observed at the freezing point.

In Fig. 3b logarithms of DL(I)/F at the temperatures between −30 and −120 °C were plotted against the reciprocal of the absolute temperature. An apparent activation energy for the generation of excitation energy calculated from the inclination of the curve was 0.12 eV (2.8 Kcal/mole).

Effects of preillumination at −196 °C

Effects of preillumination with red light at −196 °C on the induction of delayed light at −96 °C were investigated (Fig. 4). After cooled to −196 °C the chloroplasts were illuminated for 30 s with the excitation light, and then were warmed in the dark to −96 °C in several minutes and the induction of delayed light was measured. In the induction of delayed light after the preillumination, the I level was suppressed to 30% of the control, while the S level was a little diminished (Fig. 4b).

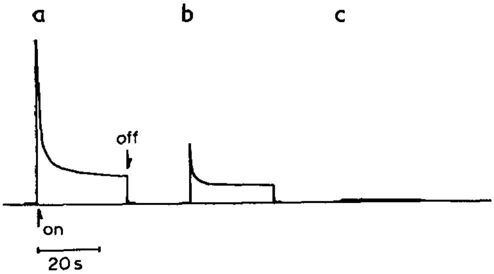


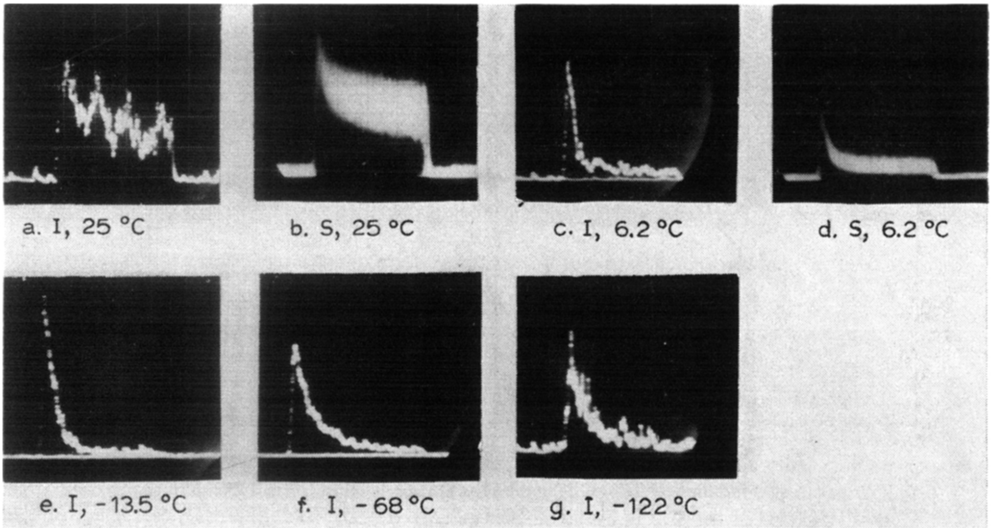
Fig. 4. Effects of preillumination at −196 °C on the induction of delayed light at −96 °C. a, Frozen in the dark without preillumination. b, Frozen in the dark, and then preilluminated for 30 s at −196 °C, and warmed to −96 °C. c, Frozen in the light, and then preilluminated for 30 s at −196 °C, and warmed to −96 °C.

In the sample that was cooled to −196 °C in the light and illuminated for 30 s at −196 °C, and then warmed to −96 °C in the dark, the yield of delayed light was almost negligible (Fig. 4c).

These results indicate that the primary photochemical events that are needed to produce the delayed light at −96 °C occur even at −196 °C at which temperature no delayed light was emitted, and that the events are irreversible at temperatures between −196 °C and −96 °C.

Decay kinetics of delayed light emission

Decay kinetics of delayed light emission was measured at various temperatures. Traces in Fig. 5 were obtained at 0.5 or 30 s after the onset of repetitive illumination, at which time the induction of delayed light attained to the I level or the S level, respectively. In this experiment gramicidin S was added to minimize the slow phase of induction. At 25 °C, the decay kinetics of delayed light was composed of two dominant components with half decay time of about 0.2 ms and of several tens of milliseconds. This fast-decay component seems to correspond to the 200-μs component of delayed light reported by Zankel [17]. At 6 °C, the fast-decay component was enhanced and the slow-decay component was very small. At −13, −68 and −122



a. I, 25 °C

b. S, 25 °C

c. I, 6.2 °C

d. S, 6.2 °C

e. I, -13.5 °C

f. I, -68 °C

g. I, -122 °C

Fig. 5. Dark decay of delayed light at the I level and the S level at various temperatures. Delayed light at 0.1–3.7 ms after the cessation of the excitation light was measured. The I and S levels represent the decay kinetics measured after 0.5 s and 30 s of intermittent illumination, respectively. a, I level, 25 °C; b, S level, 25 °C; c, I level, 6.2 °C; d, S level, 6.2 °C; e, I level, −13.5 °C; f, I level, −68 °C; g, I level, −122 °C. Time base in horizontal axis was 1 ms/division. Scales in longitudinal axis: a, b and g, 0.02 V; c and d, 0.05 V; e, 0.1 V; f, 0.2 V. Chlorophyll concentrations were 6 μg/ml for a–d, and 2 μg/ml for e–g, respectively. 9 μM gramicidin S was added in every case.

°C, almost all the delayed light observed was due to the fast-decay component. The half-decay time of the fast-decay component was 0.17 ms at 6 °C, 0.23 ms at −13 °C, 0.38 ms at −68 °C and 0.32 ms at −122 °C.

Temperature dependences of the fast and slow components in the decay kinetics and of the fast and slow phases in the induction of delayed light lead to a conclusion that the fast phase of the induction consists of only the fast-decay component and that the slow phase might consists of both the slow- and the fast-decay components. According to Clayton [18] and Wraight [19], the induction of the slow-decay component follows a time course similar to that of fluorescence induction. On the other hand, the fast decay-component is inversely related to the fluorescence induction kinetics [10].

Delayed light emission in the System I and System II particles

Temperature dependence of delayed light was also examined in the System I particles and the System II particles. At room temperature, the induction of delayed light in the System II particles showed a high initial rise level on onset of illumination which was followed by a rather fast decline to the low steady state level during illumination (see Fig. 1 in ref. 10). The System I particles, on the other hand, emitted a very low intensity of delayed light at room temperature [10]. Temperature dependence of the I level in these particles was shown in Fig. 6. In the System II particles, the temperature dependence was similar to that of chloroplasts except that the intensity in the System II particles was about 2–3 times as high as that of chloroplasts on a chlorophyll content basis. On the other hand, the System I particles scarcely

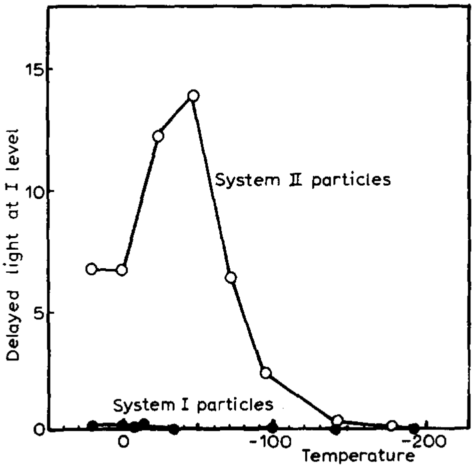


Fig. 6. Temperature dependence (°C) of delayed light at the I level of the induction in the System I and the System II particles. ●-●, the System I particles; ○-○, the System II particles. Chlorophyll concentrations were adjusted to 29 μg/ml in both cases.

emitted the delayed light at any temperature tested. These results indicate that the delayed light originates only from System II at low temperature as well as at room temperature [6, 10].

DISCUSSION

It is found in the present study on the temperature dependence of delayed light emission that the fast phase of the induction of delayed light exists at temperatures between −175 and 40 °C with the maximum at −40 °C, and the slow phase, between 0 and 40 °C with the maximum at 25 °C. The investigation on the decay kinetics of delayed light emission suggests that the fast phase corresponds to the change in intensity of the fast-decay component with a half decay time of 0.17–0.38 ms. This component may be the same as the 0.2-ms decay component of Zankel [17]. The induction kinetics of the fast-decay component (the fast phase) can be explained by the change in the state of the reaction center under intermittent illumination in which excitation energy is generated by the recombination reaction between the electron in the primary electron acceptor and the hole in the primary electron donor of Photoreaction II (ref. 10). The slow-decay component having a half decay time of several tens of milliseconds showed a different temperature dependence and seems to show a different induction mode from those of the fast-decay component [18, 19]. Both the fast and slow-decay components seem to be included in the slow phase of the induction which reflects the accumulation of the high energy state for the phosphorylation.

Below −40 °C the yield of the fast-decay component was significantly decreased on cooling, while its decay rate was only slightly affected. The apparent activation energy was 0.12 eV. This value may suggest a contribution of a (metastable) state of reaction center with an energy level less than 0.12 eV below that of the lowest excited singlet state of a bulk chlorophyll molecule (1.8 eV). The process requiring such low activation energy may be the regeneration of chlorophyll excitation energy in the recombination reaction between the separated positive and negative charges in the reaction center or may be a transfer of excitation energy from the reaction center to a bulk chlorophyll. A further investigation is required to solve the question what is the (metastable) state of reaction center.

On cooling the chloroplasts from 0 to −40 °C, the yield of the fast-decay component was increased more than 3 times, although the decay rate of the component was only slightly varied. At this temperature range, some of the electron transport reaction near Photoreaction II is inhibited [20]. According to Zankel [17], the yield of 0.2 ms decay component depends on the state of the precursor, S, of the oxygen evolving reaction which has been proposed in the model of Kok et al. [21]. It will be possible that the conversion of S state is somehow blocked at −40 °C resulting in the increase in the yield of delayed light emission.

The decay rate of the fast-decay component was found to be less sensitive to the temperature than its yield. Its half decay time was between 0.17 and 0.38 ms over the temperature range from 6 to −122 °C, while the yield of delayed light markedly varied. This result suggests that the degradation reaction of the reaction center state, which state regenerates the excitation energy in chlorophyll, requires a very low activation energy. Effect of the preillumination at −196 °C on the induction of delayed light at −96 °C also suggests the existence of such degradation reaction having very low activation energy. These results are in consistent with the results which showed the activity of the Photoreaction II even at −196 °C (refs 22–24).

At room temperature, the fluorescence and the delayed light are both emitted mainly from pigment System II (refs 3, 6, 7, 10, 25). The fluorescence of pigment System I appears at 735 nm when the chloroplasts are cooled to −196 °C (refs 26–29). The fluorescence yield amounts to several times higher than that at 694 and 684 nm. According to Boardman et al. [28], the yield of fluorescence at 735 nm at −196 °C in the System I particles derived from spinach chloroplasts by using digitonin was more than 40 times as high as that at 20 °C. However, the experimental result in Fig. 6 shows that the yield of delayed light is very low in the System I particles at any temperature tested. Even if the difference in sensitivity of the photomultiplier at 684 and 735 nm is considered, the yield of delayed light in the System I particles is negligibly small as compared with that of the System II particles. These findings suggest that the absence of delayed light in the System I particles at room temperature is not a reflection of the low fluorescence yield of pigment System I.

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