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STUDIES ON THE DELAYED LIGHT EMISSION IN SPINACH CHLOROPLASTS.

II. PARTICIPATION OF PRIMARY ELECTRON DONOR AND ACCEPTOR OF PHOTOREACTION II IN PRODUCING THE DELAYED LIGHT EMISSION

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SUMMARY

1. The fluorescence of chlorophyll *a* and the delayed light emitted at 0.1–1.2 msec after repeated flash light (duration, 0.5 msec) were simultaneously measured in isolated spinach chloroplasts. An extensive study was made of the initial rapid-rise component of delayed light emission which is closely related to the primary photoact in photosynthesis.

2. Electron donors of System II (ascorbate, hydroquinone, *p*-phenylenediamine and $MnCl_2$) decreased the magnitude of the initial rapid-rise component of delayed light without producing any significant change in the time course of fluorescence.

3. Treatment of chloroplasts with a high concentration of Tris buffer, known to block the electron transport somewhere between Photoreaction II and water, enhanced the initial rapid-rise component but suppressed the second slow-increase component of delayed light. Addition of the electron donors to the Tris-washed chloroplasts suppressed the enhanced initial rapid-rise component of delayed light emission.

4. These results indicate that the production of delayed light requires the oxidized form of the primary electron donor in addition to the reduced form of the primary electron acceptor of Photoreaction II.

INTRODUCTION

A close correlation between the mechanisms of delayed light emission and the primary photochemical energy conversion processes in photosynthesis has been suggested by several investigators[1–7]. The current tentative view on the mechanism of delayed light emission is that it originates from the reverse process of photosynthesis, *i.e.* recombination of oxidized and reduced photoproducts of Photoreaction II[8–11]. The capacity to ~~emit~~ the delayed light after a period of illumination is supposed to depend upon the amounts of the reduced form of primary electron

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

acceptor and the oxidized form of primary electron donor produced in the preceding light period[8].

In the previous study[12], we have shown that on illumination with intermittent light, the intensity of millisecond delayed light showed a complicated time course. There were at least two different phases of increase in delayed light emission corresponding to different stages of photosynthesis. At the onset of illumination, the intensity of delayed light increased rapidly and then slowly to attain a maximum which was followed by a gradual decrease to reach a steady-state level. It was inferred that the initial rapid-rise component is closely related to the electron transport reactions, whereas the second slow-increase component reflects an accumulation of high-energy intermediates of photophosphorylation. The latter component could be selectively eliminated on addition of uncouplers of photophosphorylation.

In the present work, we attempt to evaluate the possible roles of the primary electron acceptor, X, and donor, Y, of Photoreaction II in the mechanism of delayed light emission. The chlorophyll *a* fluorescence was also measured as an indicator of the oxidation-reduction state of X (refs. 13–15). Experiments were performed under conditions in which the redox states of X and Y were controlled by additions of inhibitors and/or artificial electron donors, as well as by treatments of chloroplasts which inhibited the electron transport reaction.

METHODS

Spinach chloroplasts were prepared according to the procedures described previously[12]. The chloroplasts were dispersed in a medium containing 0.05 M Tricine–NaOH buffer, pH 7.8, 0.4 M sucrose and 0.01 M NaCl. In each measurement, the chloroplasts were diluted with a medium containing 0.05 M Tricine–NaOH buffer, pH 6.5, 0.4 M sucrose and 0.01 M NaCl to give a final chlorophyll concentration of 2 μg/ml. The final pH was 6.8. The Tris treatment of chloroplasts was carried out according to the methods of YAMASHITA AND BUTLER[16].

The measurements of delayed light were made using the instrument described previously[12] with minor changes in the cycle of light and dark periods. One cycle was 1.8 msec in duration; 0–0.5 msec for excitation, 0.5–1.8 msec for dark. Delayed light was measured from 0.6 to 1.7 msec; that is, delayed light emitted during the period 0.1–1.2 msec after cessation of the excitation flash was measured. The delayed light emitted at a right angle to the excitation light was detected using a photomultiplier R-374 (Hamamatsu TV) with an attachment of a glass cut-off filter, V-R 65 (Toshiba) which transmits light with wavelengths longer than 650 nm.

Simultaneous measurement of fluorescence was made at the opposite side to that for measurement of delayed light in a way similar to that used by CLAYTON[11]. Between the sample and the photomultiplier R-236 (Hamamatsu TV), a glass cut-off filter V-R 65 and an interference filter with a transmission peak at 694 nm (Japan Vacuum Optics) were placed.

Signals from these two photomultipliers were amplified independently, and were recorded on a dual trace strip chart servo recorder (Riken Denshi Co., SPH4).

The intensity of excitation light at the surface of the cuvette was $1.0 \cdot 10^5$ ergs/cm^2 per sec measured with the sector stopped and $2.8 \cdot 10^4$ ergs/cm^2 per sec with the sector rotating.

The sample was placed in a 1 cm × 1 cm × 4 cm four-side transparent quartz cuvette. Measurements were performed at room temperature (20°) under aerobic conditions.

RESULTS

Effects of electron donors of System II

Fig. 1 shows the time courses of delayed light and fluorescence emitted from chloroplasts during intermittent illumination. The intensity of delayed light showed a complicated time course at pH 6.8 as was described previously[12]. At the onset of illumination, the intensity of delayed light increased rapidly to a certain level (I) and then slowly to reach a maximum (P) which was followed by a gradual decrease to a steady-state level.

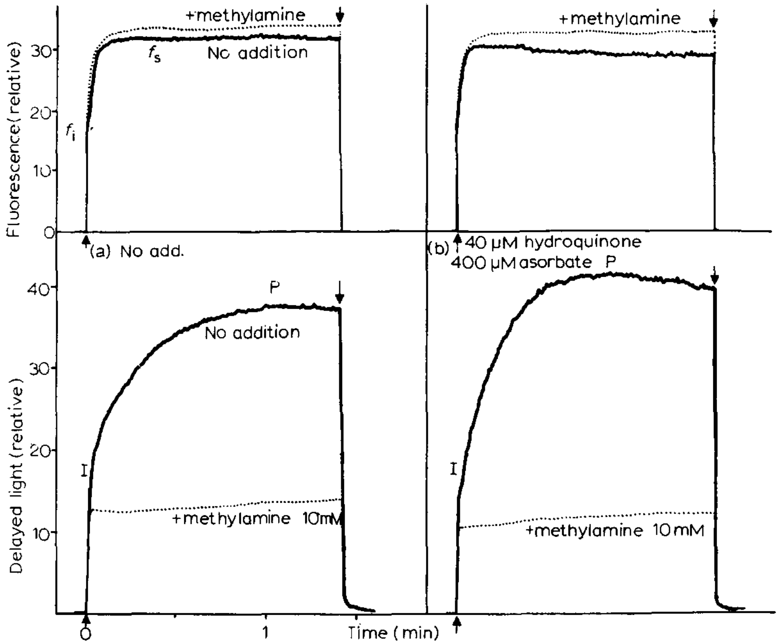

Fig. 1. Effects of methylamine and electron donors on time courses of fluorescence and delayed light emission. The chloroplast suspension was illuminated by repeated flashes of 0.5 msec duration with 556 cycles/sec. Fluorescence was measured during the excitation period, and delayed light was measured in the dark from 0.1 to 1.2 msec after cessation of each flash. Arrows indicate onset (↑) and cessation (↓) of intermittent illumination. Upper figures: fluorescence. Lower figures: delayed light emission. (a) ———, no addition; ······, 10 mM methylamine. (b) ———, 40 μM hydroquinone and 400 μM ascorbate; ······, 40 μM hydroquinone, 400 μM ascorbate and 10 mM methylamine.

The fluorescence yield rose at the onset of excitation instantaneously to a certain level, f_i, and then increased slowly to reach a maximum steady level, f_s (refs. 14, 15). The fluorescence increase took a shorter time than the delayed light in attaining the maximum level.

As shown by the dashed lines in Fig. 1, the second slow-increase component of the delayed light was eliminated by addition of an uncoupler, methylamine[17], but the initial rapid-rise component remained unchanged. In contrast, methylamine caused only a slight change in the time course of fluorescence.

Addition of a low concentration of hydroquinone (40 μM) with sodium ascorbate (400 μM) increased the rate and magnitude of the second slow-increase component (Fig. 1b), whereas it had little effect on the initial rapid-rise component. In the presence of methylamine in the reaction medium, the addition of the reductants decreased the magnitude of the delayed light emission. The time course of fluorescence, however, was only slightly affected by additions of the electron donors.

The effects of other electron donors of System II[16,18,19] were also investigated. Addition of *p*-phenylenediamine (18 μM) with ascorbate (400 μM) or $MnCl_2$ (500 μM) produced results similar to those on addition of the hydroquinone–ascorbate couple in both delayed light and fluorescence. $MgCl_2$, on the other hand, only slightly stimulated the initial rapid rise. Therefore, the action of $MnCl_2$ in suppressing the initial rapid rise does not seem common to divalent cations. Sodium ascorbate at a higher concentration (10 mM) slightly decreased the initial rapid-rise component, and enhanced the second slow-increase component by a factor of three. Sodium dithionite decreased the delayed light emission of both the rapid-rise and the slow-increase components. In the presence of previously added methylamine, all the above-mentioned electron donors suppressed the magnitude of the remaining delayed light emission. In contrast, the time course of fluorescence was not affected by addition of the electron donors.

Addition of 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosynthetic electron transport, completely eliminated the second slow-increase in the time course of delayed light, but only slightly suppressed the initial rapid-rise component. The delayed light showed, after the initial rapid rise, a gradual decline to a steady-state level (Fig. 2).

A further decrease in magnitude of the initial rapid rise of delayed light was

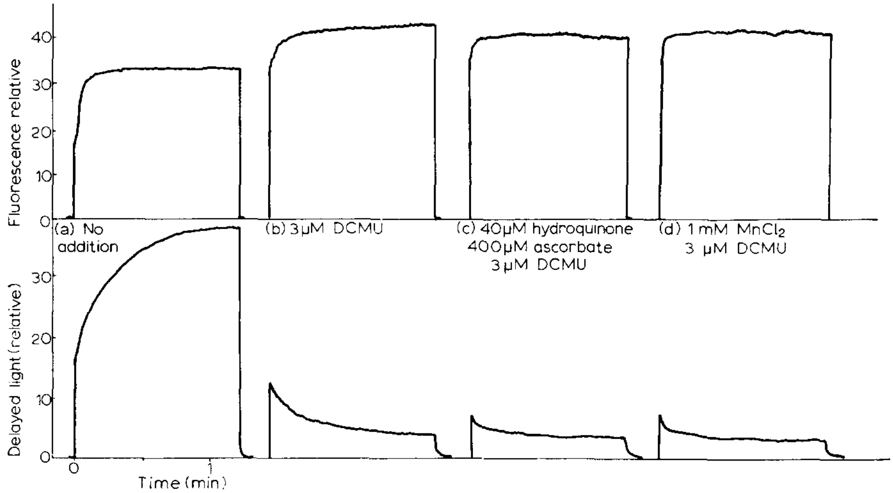

Fig. 2. Effects of electron donors on time courses of fluorescence and delayed light emission in the presence of DCMU. Upper figures: fluorescence. Lower figures: delayed light emission. (a) No addition. (b) 3 μM DCMU. (c) 40 μM hydroquinone, 400 μM ascorbate and 3 μM DCMU. (d) 1 mM $MnCl_2$ and 3 μM DCMU. Measurements of fluorescence and delayed light emission were the same as in Fig. 1.

induced by addition of the electron donor system, such as the hydroquinone–ascorbate couple or Mn^{2+} (Figs. 2c and 2d). These reductants also caused an acceleration of the decline after the attainment of the initial rise.

In contrast to these marked alterations in the time course of delayed light emission, the time course of fluorescence was essentially unaffected by additions of the reductants, although the addition of DCMU caused a definite increase in rate of the second increase and in the steady-state level of fluorescence (Fig. 2).

Similar effects were found in other electron donors, *p*-phenylenediamine and a high concentration of ascorbate, with respect to suppressing the delayed light emission and in producing only insignificant effects on the fluorescence yield. $MgCl_2$ (1 mM) had little effect both on delayed light and fluorescence in the presence of DCMU.

Table I shows the concentrations of these reductants for 50 % suppression of the magnitude of the initial rapid rise of the delayed light in the presence of DCMU. These concentrations of electron donors listed in Table I lie in the same concentration range in which these reductants serve as electron donors of System II[16, 18, 19].

TABLE I

EFFECTIVENESS OF ELECTRON DONOR SYSTEMS IN DECREASING DELAYED LIGHT EMISSION

The initial rapid-rise level was measured in the presence of 6 μM DCMU.

Additions	*Concentration required for 50 % suppression*
Sodium ascorbate	18.5 mM
Phenylenediamine (+ sodium ascorbate, 300 μM)	20 μM
Hydroquinone (+ sodium ascorbate, 300 μM)	28 μM
$MnCl_2$	40 μM

In summing up the above-mentioned results, additions of the electron donors suppresses the initial rapid-rise component of delayed light.

Effects of Tris treatment

The time courses of delayed light emission and fluorescence were examined in the Tris-washed chloroplasts (Fig. 3).

YAMASHITA AND BUTLER[16] showed that washing of the chloroplasts with 0.8 M Tris buffer, pH 8.0, resulted in a selective inhibition of the electron transport at the oxygen-evolving side of Photoreaction II. In these chloroplasts artificial electron carriers such as those employed above served as electron donors of System II instead of water[16]. Therefore, in Tris-washed chloroplasts the accumulation of a high level of Y^+, the oxidized form of the primary electron donor, Y, is expected under the illumination.

The Tris treatment of chloroplasts was found to produce a marked increase in magnitude of the initial rapid rise of delayed light emission, and an almost complete suppression of the second slow increase (Fig. 3a). This enhanced initial rapid-rise component was not affected by the addition of methylamine.

On addition of the hydroquinone (40 µM)–ascorbate (400 µM) couple, the time course of delayed light restored the original pattern observed in the untreated chloroplasts. The initial rapid rise was markedly suppressed and the second slow increase reappeared (Fig. 3c). Further addition of methylamine eliminated the recovered slow-increase component, indicating that also under these circumstances the second slow increase was connected to the phosphorylation system. $MnCl_2$ was also effective,

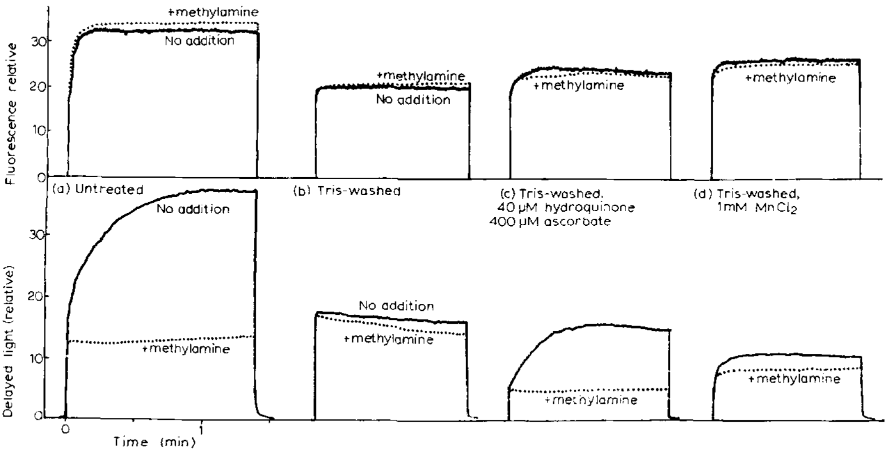

Fig. 3. Effects of Tris treatment of chloroplasts on time courses of fluorescence and delayed light emission. Upper figures: fluorescence. Lower figures: delayed light emission. (a) Untreated, no addition. (b) Tris-washed, no addition. (c) Tris-washed, 40 µM hydroquinone and 400 µM ascorbate. (d) Tris-washed, 1 mM $MnCl_2$. Dashed lines in (a), (b), (c) and (d), further addition of 10 mM methylamine. Measurements of fluorescence and delayed light emission were the same as in Fig. 1.

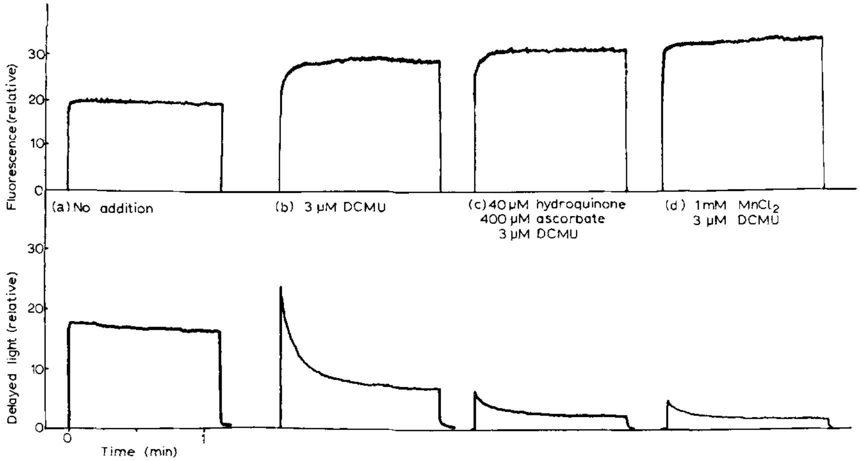

Fig. 4. Effects of Tris treatment on time courses of fluorescence and delayed light emission in the presence of DCMU. Upper figures: fluorescence. Lower figures: delayed light emission. (a) Tris-washed, no addition. (b) Tris-washed, 3 µM DCMU. (c) Tris-washed, 40 µM hydroquinone, 400 µM ascorbate and 3 µM DCMU. (d) Tris-washed, 1 mM $MnCl_2$ and 3 µM DCMU. Measurements of fluorescence and delayed light emission were the same as in Fig. 1.

although to a lesser extent than the hydroquinone–ascorbate couple, in decreasing the initial rapid rise and restoring the second slow increase of the delayed light (Fig. 3d).

The steady-state level of the fluorescence yield was greatly suppressed by the Tris treatment of chloroplasts as was reported by YAMASHITA AND BUTLER[16]. Most of the variable portion of fluorescence was lacking (Fig. 3b). On addition of electron donors, the variable part of fluorescence reappeared, resulting in a marked increase in level of the steady-state fluorescence (Figs. 3c, 3d). However, in contrast to the case of delayed light emission, methylamine was found to be almost without effect on the recovered induction of fluorescence.

Addition of DCMU to the Tris-washed chloroplasts stimulated the initial rapid-rise component of delayed light emission and suppressed the steady-state level (Fig. 4). Among all the conditions employed in the present study, this produced the highest level of the initial rise. Addition of electron donors to this preparation suppressed the delayed light emission in both the initial and the steady-state levels. The obtained time courses were almost the same as those in the untreated chloroplasts with addition of DCMU and electron donors.

The steady-state level of fluorescence was elevated by the addition of DCMU, indicating that there is still a slow electron transport through Photoreaction II in Tris-washed chloroplasts. Further addition of electron donors to this system induced a higher steady-state level of fluorescence.

DISCUSSION

In our previous study[12] on the millisecond delayed light emission, two components were found in the time course of the delayed light during intermittent illumination of the chloroplasts. One was the initial rapid rise at the onset of illumination, and the other was the slow increase following the initial rise. It was suggested there that the initial rapid-rise component should be a direct reflection of the rate of the reverse reaction of Photoreaction II, *i.e.* the rate of recombination of the reduced primary electron acceptor, X^-, and the oxidized primary electron donor, Y^+. The slow-increase component should be related to an accumulation of the high-energy state of phosphorylation. In view of this hypothesis, it is expected that the initial rapid-rise component is controlled by changing the oxidation–reduction levels of the primary electron donor and acceptor (X and Y) of Photoreaction II.

The levels of accumulation of X^- were estimated from the fluorescence yield of chlorophyll *a*[13–15]. The experimental results showed that at the steady level of fluorescence, almost all of X was reduced under illumination except for the case in Tris-washed chloroplasts with no addition.

The primary electron donor Y is oxidized by Photoreaction II, and reduced by dark reactions. Water is the source of electrons in the normal chloroplasts. Artificial electron donors such as hydroquinone and Mn^{2+} are more efficient electron sources than water in reducing Y^+. Thus, the accumulation of Y^+ must be lower in the presence of electron donors. The suppression of the initial rapid-rise component of delayed light on the addition of electron donors discovered in this study must have resulted from the decreased level of Y^+.

The Tris treatment of chloroplasts inhibits the reaction somewhere between Y and water[16]. Y^+ is accumulated in the light. Addition of artificial electron donors that are known to reduce Y^+ in Tris-washed chloroplasts[16] suppresses the accumulation of Y^+. The hypothesis described above suggests that the higher the oxidized level of Y, the stronger the intensity of the delayed light emission. This situation is consistent with the experimental results that the initial rapid-rise component was enhanced in Tris-washed chloroplasts and that the rapid-rise component was suppressed by addition of electron donors.

DCMU inhibits the dark oxidation of X^-, and consequently X^- accumulates in the light. Under these conditions, Photoreaction II cannot oxidize Y to Y^+ because of the lack of the electron acceptor X. The experimental results of the inhibitory effects of DCMU on the initial rapid-rise component can, thus, be explained.

On addition of DCMU to Tris-washed chloroplasts, the reduction of Y^+ and oxidation of X^- are inhibited. This must be the best situation for emission of the delayed light. Experimentally, the initial rapid rise of delayed light was largest under these conditions.

In summary, all experimental results in this study can be interpreted by the mechanism that the recombination of the oxidized form of the primary electron donor Y^+, and the reduced form of the primary electron acceptor, X^-, plays a cardinal role in producing the delayed light emission.

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