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STUDIES ON THE DELAYED LIGHT EMISSION IN SPINACH CHLOROPLASTS

I. NATURE OF TWO PHASES IN DEVELOPMENT OF THE MILLISECOND DELAYED LIGHT EMISSION DURING INTERMITTENT ILLUMINATION

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SUMMARY

The time course of changes in delayed light emission measured at 1.2 msec (average) after each excitation flash during intermittent illumination of spinach chloroplasts showed a complicated time-dependent change. In this time course, the existence of at least two components was found. One which developed slowly during illumination was selectively and thoroughly eliminated by addition of the uncouplers methylamine or a low concentration of carbonylcyanide 3-chlorophenylhydrazone (CCCP), or by uncoupling treatments of chloroplasts such as EDTA washing, sonic oscillation of chloroplasts and addition of a low concentration of Triton X-100. This component was found to be completely suppressed by addition of inhibitors of electron transport, 3-(4'-chlorophenyl)-1,1-dimethylurea (CMU), 3-(3',4'-dichlorophenyl)-1 1-dimethylurea (DCMU) and *o*-phenanthroline. A suspending medium of pH 6.0 was optimal for the development of the component.

The other component which developed rapidly at the onset of illumination was suppressed by the inhibitors CMU, DCMU, *o*-phenanthroline and high concentrations of CCCP. The highest yield of this component was observed at pH 4.

The mechanism of the delayed light emission was discussed on the basis of the assumption that it is ultimately excited by a reverse reaction of the electron flow through Photoreaction II. It was concluded that the former, slowly developing component of delayed light emission is related to the amounts of accumulated high-energy intermediate or state of photophosphorylation, and the latter, fast-developing component to the amounts of reduced and oxidized products formed during illumination by Photoreaction II.

INTRODUCTION

Since the discovery of delayed light emission in green plants by STREHLER AND ARNOLD[1], many investigators have studied the phenomenon in relation to the primary

Abbreviations: CCCP, carbonylcyanide 3-chlorophenylhydrazone; CMU, 3-(4'-chlorophenyl)-1,1-dimethylurea; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea.

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processes in photosynthesis. Delayed light emission was ubiquitously found in photosynthetic organs of various classes of photosynthetic organisms; namely, unicellular and thallophytic algae, leaves and chloroplasts of higher green plants and intact cells and chromatophores of photosynthetic bacteria[1–8].

It is now generally accepted that the delayed light in higher plants and algae is emitted from chlorophyll *a* in pigment System II[1,3,6,9,10] through transition from the lowest excited singlet state to the ground state[2,3,5]. Kinetic studies have shown that several phases of decay in intensity of the delayed light emission occur, the most rapid so far known having a half-decay time of about 1 msec, and another having a much longer half-decay time[4,5,11–14]. The fast-decaying component which will be denoted here as millisecond delayed light, behaved differently from the longer-lived components[4,5,12,13,15] and seems to be closely connected with the quantum conversion process of photosynthesis[5]. This millisecond delayed light emission is known to be enhanced by addition of artificial electron acceptors and suppressed by inhibitors of photosynthetic electron transport or uncouplers of photophosphorylation[15]. On the basis of these findings, a suggestion has been made that the millisecond delayed light bears a close relation to photophosphorylation[15]. However, the difference between the suppressive effects of inhibitors and uncouplers on delayed light emission still remains unclear.

It is known that the development of the capacity for delayed light emission shows an induction at the beginning of illumination[10,11,16]. Kinetic analysis of such changes, however, has been carried out only with respect to the longer-lived components of delayed light[16].

MATERIALS AND METHODS

Isolated spinach chloroplasts were used as test material throughout the present work. Fresh spinach leaves were mildly ruptured in a Waring blendor, using a sucrose–NaCl–phosphate medium containing 0.4 M sucrose, 0.01 M NaCl, 0.05 M phosphate buffer, pH 7.8. The homogenate was filtered through eight layers of cheese cloth. The filtrate was centrifuged at 1500 × *g* for 5 min and the precipitate obtained was suspended in the sucrose–NaCl–Tricine medium containing 0.4 M sucrose, 0.01M NaCl, 0.05 M Tricine–NaOH buffer, pH 7.8. The suspension was centrifuged at 200 × *g* for 1 min to remove coarse debris and then chloroplasts were collected by further centrifugation at 1500 × *g* for 5 min. The precipitate formed (chloroplasts) was resuspended in the sucrose–NaCl–Tricine medium.

Sonic treatment of chloroplasts suspended in the sucrose–NaCl–phosphate medium was carried out in a sonic oscillator (Toyo Riko Co., 20 kcycles/sec) operated at middle power. The temperature was kept at about 0° throughout this treatment. EDTA treatment of the chloroplasts was carried out according to the method of HIND *et al.*[17].

For the measurement of the delayed light, the chloroplast suspension was diluted with the sucrose–NaCl–Tricine medium, unless stated otherwise, to approximately 2 μg chlorophyll per ml. In experiments in which the pH dependence of the delayed light emission was investigated, the reaction medium consisted of 0.4 M sucrose, 0.01 M NaCl and 0.05 M 2-(*N*-morpholino)ethane sulfonic acid buffer for pH levels

between 3.8 and 5.5; for pH levels between 6.0 and 9.0, 0.05 M Tricine buffer was used instead.

The delayed light was measured with a Becquerel-type phosphoroscope (Narumi Co.), in which the excitation of the chloroplasts and the measurement of delayed light were performed alternately by the use of a rotating sector. A cycle of excitation, and measurement was 3.6 msec in duration; 0–1.3 msec for excitation, 1.3–3.6 msec for dark. The delayed light was measured in the middle of the dark period, *i.e.* 1.5–3.5 msec; in other words, the average intensity of the delayed light emitted during the period from 0.2 to 2.2 msec (average, 1.2 msec) after cessation of excitation light was measured.

The source for excitation light was a 500-W high pressure mercury lamp (Ushio Electric Co.). To eliminate ultraviolet and infrared light, optical filters, Toshiba U-V39, Hoya HA-50 and Hoya B-460, were placed in the light path. The light intensity, at the surface of the cuvette, was $1.2 \cdot 10^5$ ergs/cm^2 per sec as measured with the sector stopped. (The actual light intensity during continuous rotation of the sector, *i.e.* average through alternating light and dark period, was $3.4 \cdot 10^4$ ergs/cm^2 per sec.) The delayed light emitted from the sample placed in a 1 cm × 1 cm × 4 cm (height) four-side transparent quartz cuvette at a right angle to the excitation light was filtered through an optical filter, Toshiba V-R65, transmitting wavelengths longer than 650 nm, and detected by a photomultiplier, Hamamatsu TV R-374. The signal of the photomultiplier, square wave in form, was amplified and rectified by an RC combination with a time constant of 0.5 sec and recorded on a strip chart servo recorder.

Measurements were performed at room temperature (approx. 20°) under aerobic conditions.

RESULTS

Time course of changes in millisecond delayed light emission during illumination

Fig. 1 shows the changes in intensity of the millisecond delayed light emission by chloroplasts during intermittent illumination at pH 7.8. At the onset of excitation, the intensity of the millisecond delayed light increased rapidly at first (O–I). Then followed a slower phase of increase to attain a maximum level (P), which, in turn, was followed by a slow decline towards a steady level (S). The initial rise (O–I) of

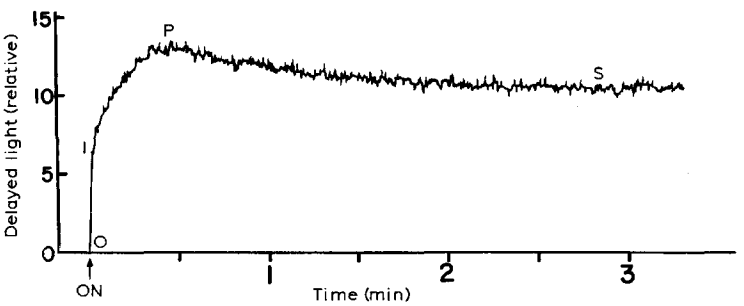


Fig. 1. Time course of changes in delayed light emission during intermittent illumination of spinach chloroplasts. Chloroplasts were excited by an intermittent illumination of 278 cylces/sec with flashes of 1.3 msec duration. The delayed light was measured at a period of 0.2–2.2 msec after cessation of each flash.

delayed light was usually completed within 0.5 sec of excitation. The time required to attain the maximum intensity (P) was 20–30 sec under the standard conditions of the experiment.

Preliminary experiments showed that the time course of the millisecond delayed light emission described above was not reproduced unless the sample had been kept in the dark for several minutes after the preceding measurement. With a short dark interval after cessation of the preceding illumination, the level of the initial rapid rise at the onset of the second illumination was higher than that obtained without preillumination, and the maximum intensity (P), which remained unchanged, was reached in a shorter period of illumination than in the first case without preillumination.

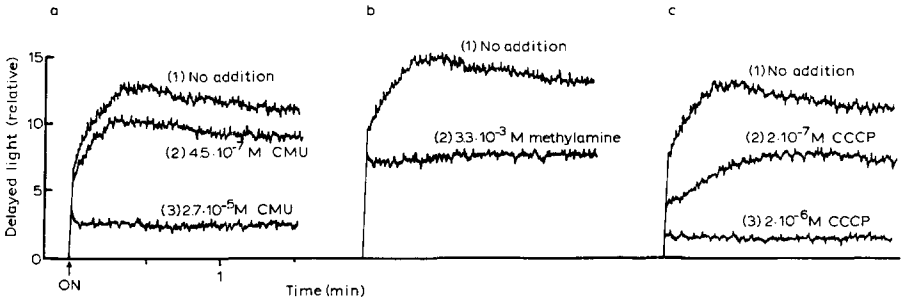


Fig. 2. Effects of CMU, methylamine and CCCP on time course of changes in delayed light emission. (a) CMU: 1, no addition; 2, $4.5 \cdot 10^{-7}$ M; 3, $2.7 \cdot 10^{-5}$ M. (b) Methylamine: 1, no addition; 2, $3.3 \cdot 10^{-3}$ M. (c) CCCP: 1, no addition; 2, $2 \cdot 10^{-7}$ M; 3, $2 \cdot 10^{-6}$ M. Conditions for determination of delayed light were the same as in Fig. 1.

Effects of electron transport inhibitors

Inhibitors of the electron transport, such as 3-(4'-chlorophenyl)-1,1-dimethylurea (CMU), 3-(3',4-dichlorophenyl)-1,1-dimethylurea (DCMU) and *o*-phenanthroline, affected the delayed light emission in a similar manner.

As shown in Fig. 2a, CMU at $4.5 \cdot 10^{-7}$ M suppressed both the initial rise and the subsequent slow increase of millisecond delayed light emission. At this concentration of CMU, the maximum intensity level was attained earlier than in the control without the poison. With higher concentrations of CMU, inhibition of the initial rapid rise became more apparent. In the presence of $2.7 \cdot 10^{-5}$ M CMU, the initial rapid rise was suppressed to about one-half in magnitude, while the second slow increase was completely abolished. These findings were also reproduced using DCMU ($3 \cdot 10^{-6}$ M) and *o*-phenanthroline ($4 \cdot 10^{-4}$ M) as poisons.

Effects of methylamine and carbonylcyanide 3-chlorophenylhydrazone (CCCP)

On addition of methylamine, a potent uncoupler of photophosphorylation[18], there was a significant inhibition of the second slow increase of the millisecond delayed light emission without any marked suppression of the initial rapid rise (Fig. 2b). With 0.1 mM of methylamine, the second slow increase was still observed, but its rate was markedly suppressed and the time to attain the maximum level was increased. The intensity of the maximum level attained also decreased at this concentration of methylamine. At 3.3 mM, methylamine completely eliminated the second slow

increase, while it still had little effect on the initial rapid rise. This fact suggests that only the second slow increase, but not the initial rapid rise, of the millisecond delayed light is closely related to the mechanism of phosphorylation.

CCCP, another potent uncoupler of photophosphorylation[19], at a concentration of $2 \cdot 10^{-7}$ M, suppressed the magnitude of the second slow increase of the millisecond delayed light emission. The time required to attain the maximum was also increased by addition of the uncoupler (Fig. 2c). However, in the presence of a higher concentration of CCCP (*e.g.* $2 \cdot 10^{-6}$ M), not only a complete elimination of the second slow increase occurred but also a significant suppression of the magnitude of the initial rapid rise, just as was the case with CMU (Fig. 2a). It should be noted that a similar suppressing effect of high concentrations of CCCP was also observed with chloroplasts in which the rapid rise had been suppressed by addition of a high concentration of CMU (*e.g.* $1 \cdot 10^{-5}$ M). Since CCCP at higher concentrations (*e.g.* $2 \cdot 10^{-6}$ M) is also known to inhibit the electron transport in chloroplasts[20], the observed effect may be a result of affected electron transport.

Effect of methyl viologen

The development of the millisecond delayed light emission was changed by the addition of methyl viologen, which is known to act as an artificial electron carrier mediating a pseudocyclic electron transport in isolated chloroplasts (Fig. 3). The second slow increase becomes so fast that it cannot be readily discriminated from the initial rapid rise. However, the two phases can be distinguished as separate parts when examined in an expanded time scale (Fig. 3, lower figure). It will be seen that no

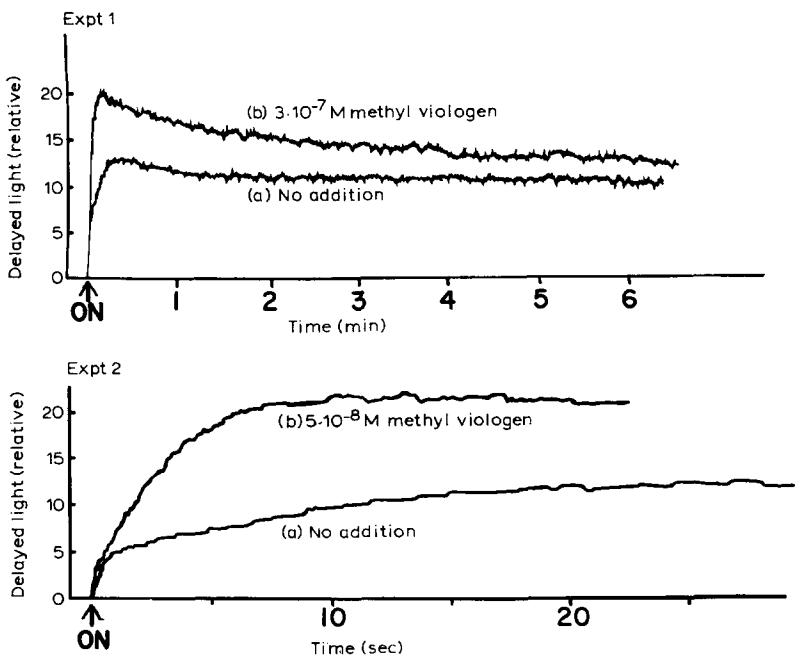


Fig. 3. Effect of methyl viologen on the time course of changes in delayed light emission. Concentration of methyl viologen, Expt. 1: (a) no addition; (b) $3 \cdot 10^{-7}$ M; Expt. 2: (a) no addition; (b) $5 \cdot 10^{-8}$ M. Conditions for determination of delayed light were the same as in Fig. 1.

appreciable change in magnitude of the initial rapid rise was caused by the addition of methyl viologen. Methyl viologen at a concentration of $3 \cdot 10^{-7}$ M stimulated the second slow increase and shortened the time required to attain the maximum level to 10 sec as compared with 20 sec in the control without methyl viologen. Such enhancement in intensity of delayed light emission was also observed at the steady level subsequent to the peak of emission. The extent of stimulation of height of the peak level caused by the addition of methyl viologen was usually 1.5–4-fold at the intensity of excitation light used ($1.2 \cdot 10^5$ ergs/cm^2 per sec). Under these experimental conditions, a concentration of 10^{-7} M of methyl viologen was found to be optimal for the enhancement of the peak level. It has to be noticed that the effect of methyl viologen depends on the intensity of excitation light used. At intensities below $8 \cdot 10^3$ ergs/cm^2 per sec, addition of methyl viologen did not enhance but decreased the height

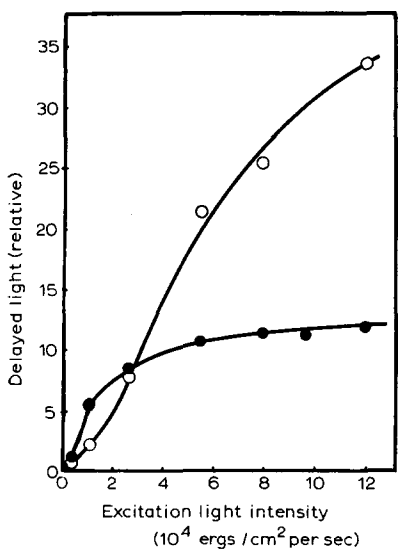


Fig. 4. Effect of intensity of excitation light on delayed light emission. Intensity of delayed light at the maximum peak in the time course was plotted against the intensity of excitation light. ●, no addition; ○, in the presence of $4.5 \cdot 10^{-7}$ M methyl viologen.

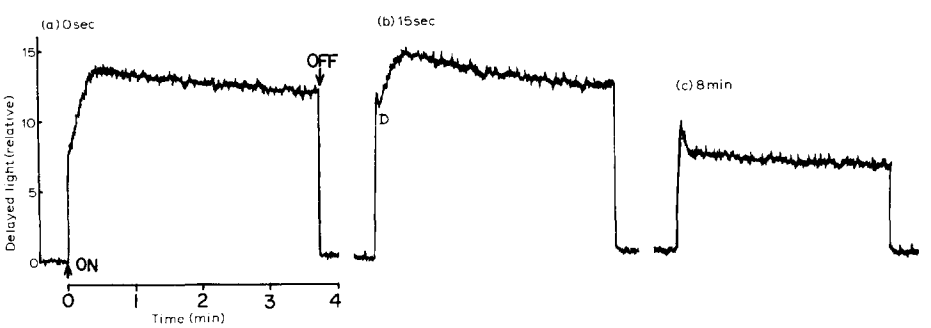


Fig. 5. Effect of sonication of chloroplasts on the time course of changes in delayed light emission. Duration of sonication: (a) 0 sec; (b) 15 sec; (c) 8 min. Conditions for determination of delayed light were the same as in Fig. 1.

of the peak level of the millisecond delayed light emission (Fig. 4). The stimulatory effect of the electron acceptor described above agrees with the observations of previous investigators made on the steady state levels of millisecond delayed light emission[13,15].

Effects of sonication of chloroplasts

GRESSEL AND AVRON[21] showed that photophosphorylation was uncoupled from electron transport when the chloroplasts were sonicated. Fig. 5 shows the time courses of the millisecond delayed light emission in chloroplasts which had been subjected to sonication for different periods of time. The treatment selectively suppressed the second slow increase of emission, while the magnitude of the initial rapid rise was not suppressed but slightly increased by the treatment. In the case of the chloroplasts sonicated for 15 sec, a small but distinct depression (designated as D in the figure) was noticed between the initial rapid rise and the second slow increase to the maximum level (Fig. 5b). After more prolonged sonication, the second slow increase disappeared. In chloroplasts which had been sonicated for 8 min or longer, the initial rapid rise was followed immediately by a gradual decrease towards the steady level of emission (Fig. 5c). Longer sonication resulted in a slight decrease in magnitude of emission, but did not change the general pattern of the time course any further. Once the steady level of emission had been reached in such chloroplasts after some period of illumination, a subsequent dark interval of several minutes was needed to reproduce the same pattern of time course having an initial spike as shown in Fig. 5c.

The effects of the inhibitors and uncouplers on the time course of the millisecond delayed light emission in sonicated chloroplasts are shown in Fig. 6.

With sonicated chloroplasts, methylamine (10^{-3} M) was found to be without effect. On the other hand, CMU ($2.5 \cdot 10^{-4}$ M) and a high concentration of CCCP ($2 \cdot 10^{-5}$ M) showed marked effects. Addition of CMU did not affect the initial rapid rise but significantly enhanced the rate of decrease immediately following the initial rise, and, eventually, a markedly suppressed steady-state level of emission was attained. In the presence of CCCP ($2 \cdot 10^{-5}$ M), both the magnitudes of the initial rapid rise and the steady-state level were markedly suppressed and the spike-like figure in the initial part of the time course was not appreciable.

Effect of EDTA treatment

JAGENDORF AND SMITH[22] have demonstrated that washing of chloroplasts with

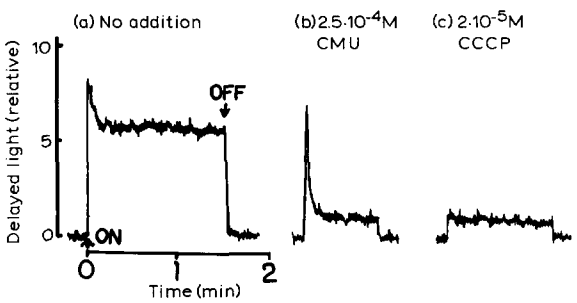


Fig. 6. Effects of CMU and CCCP on the time course of changes in delayed light emission in sonicated chloroplasts. Chloroplasts which had been sonicated for 20 min were used with: (a) no addition; (b) $2.5 \cdot 10^{-4}$ M CMU; (c) $2 \cdot 10^{-5}$ M CCCP. Conditions for determination of delayed light were the same as in Fig. 1.

EDTA results in an uncoupling of photophosphorylation. As shown in Fig. 7, EDTA treatment of chloroplasts caused a selective and complete suppression of the second slow increase of emission. The time course obtained was similar in pattern to those obtained with sonicated chloroplasts. Also in this case, methylamine only had an insignificant effect whereas CMU and CCCP markedly affected the time course; the modes of action of these poisons were similar to those observed above with sonicated chloroplasts.

Effects of Triton X-100

Low concentrations of Triton X-100 are known to cause uncoupling of photophosphorylation in isolated chloroplasts with concomitant stimulation of the rate of electron transport[23]. Addition of the detergent (final concentration, 0.007 %) to the chloroplasts during illumination caused a decrease in the steady-state level of millisecond delayed light emission (Fig. 8a). When the detergent had been added in the dark before illumination, there was a suppression of the second slow increase while the initial rapid rise was affected to a lesser extent. The second slow increase was completely eliminated on addition of 0.06 % of the detergent (Fig. 8b). The suppressed steady state level of emission in this case was not affected by further addition of 3.3 mM methylamine.

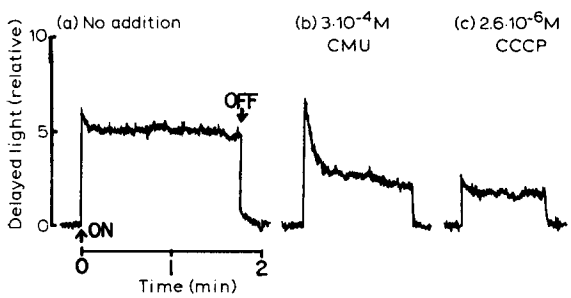


Fig. 7. Effects of CMU and CCCP on the time course of changes in delayed light emission of EDTA-treated chloroplasts. (a) No addition; (b) $3 \cdot 10^{-4}$ M CMU; (c) $2.6 \cdot 10^{-6}$ M CCCP. Conditions for determination of delayed light were the same as in Fig. 1.

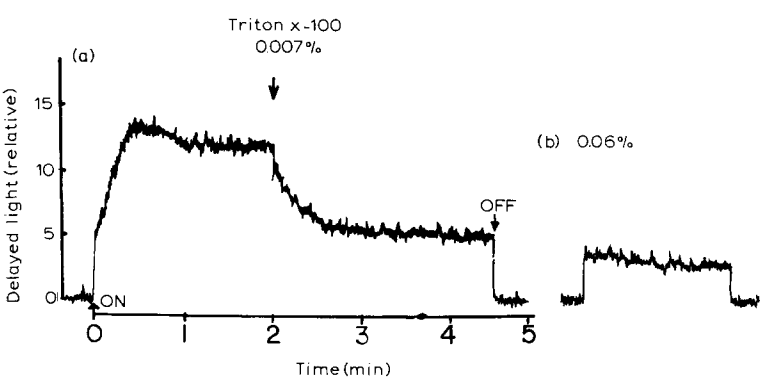


Fig. 8. Effects of Triton X-100 on the time course of changes in delayed light emission. (a) Triton X-100 (final concentration 0.007 %) was injected into the reaction mixture at the indicated time; (b) 0.06 % Triton X-100 was injected 1 min before the onset of illumination. Conditions for determination of delayed light were the same as in Fig. 1.

Effect of pH

The time course of the millisecond delayed light emission varied markedly with the pH of the suspending medium (Fig. 9), as has been noticed by ARTHUR AND STREHLER[4] using lyophilized chloroplasts.

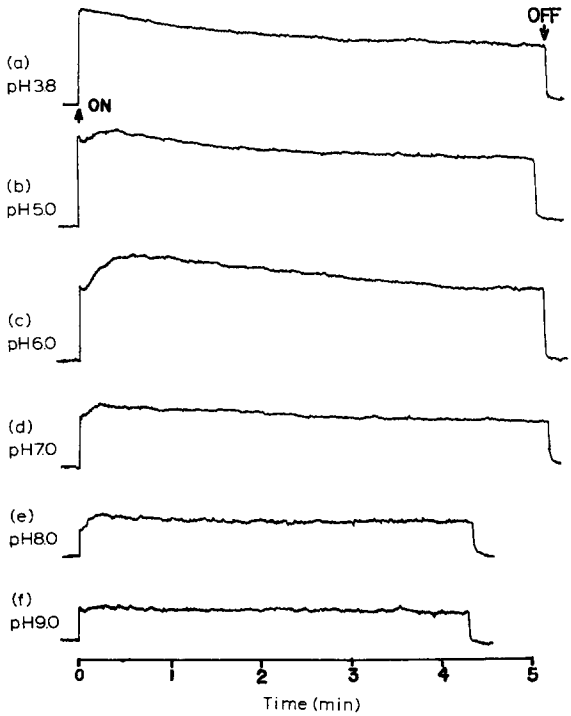


Fig. 9. pH dependence of the time course of changes in delayed light emission. Reaction media used were: for pH 3.8 to 5.0, 0.05 M 2-(*N*-morpholino)ethane sulfonic acid buffer–0.4 M sucrose–0.01 M NaCl; for pH levels between 6.0 and 10.0, 0.05 M Tricine buffer–0.4 M sucrose–0.01 M NaCl. The pH values of the media in (a), (b), (c), (d), (e) and (f) were 3.8, 5.0, 6.0, 7.0, 8.0 and 9.0, respectively. Conditions for determination of delayed light were the same as in Fig. 1.

The initial rapid rise and the second slow increase responded differently to the pH of the suspending medium. The initial rise became larger with decreasing pH of the medium. The magnitude of the initial rapid rise observed at pH 3.8 was 5 times as large as that at pH 9.0. A similar effect of varied levels of pH on the initial rapid rise of the delayed light emission was also observed with sonicated and EDTA-treated chloroplasts. In both cases, there was no second slow increase of the delayed light emission at any level of pH tested. On the other hand, the second slow increase in the untreated chloroplasts was most distinct at pH 6, and was suppressed at either higher or lower levels of pH. At pH 3.8 and pH 9.0, there was no detectable second slow increase of the delayed light emission.

DISCUSSION

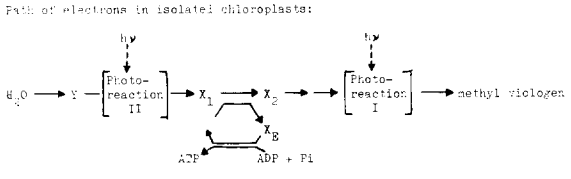
The results obtained in the present study indicate that the time course of the delayed light measured in spinach chloroplasts 0.2–2.2 msec after each repeated

flash of excitation light consisted of at least two components overlapping each other; namely, a first component which showed a rapid rise at the onset of illumination and a second one which increased slowly to attain a peak and then decreased slowly to a steady-state level. The second component was eliminated selectively by the addition of uncouplers or by various treatments of chloroplasts known to uncouple photophosphorylation from photosynthetic electron transport. An enhancement of the second component was observed on addition of methyl viologen which is known to increase the accumulation of the high-energy intermediate, or state, of photophosphorylation through acceleration of the rate of electron transport. Inhibitors of electron transport such as CMU, DCMU and *o*-phenanthroline also eliminated the second component, which can be interpreted as a result of suppression of the accumulation of the high-energy intermediate, or state, of photophosphorylation caused by the inhibition of electron transport in the presence of these poisons. The pH responses of the second component are also accounted for by this view, since the same pH range as that for enhancement of the second component of delayed light emission is known to be the most favorable for the accumulation of the high-energy intermediate X_E which can be estimated by the capacity of the preilluminated chloroplasts to induce ATP formation in the subsequent dark period with ADP and P_i[24]. It can be concluded, therefore, that the second slow component of the millisecond delayed light emission observed during actinic illumination with intermittent light is a reflection of the accumulation of the high-energy intermediate, or state, of photophosphorylation coupled with the electron transport in isolated chloroplasts.

The initial rapid rise of the delayed light emission (the first component) was little affected by the addition of uncouplers or by the uncoupling treatments, but markedly suppressed by the addition of electron transport inhibitors such as CMU, DCMU and *o*-phenanthroline, as well as higher concentrations of CCCP, thereby indicating a participation of the electron transport in this component.

It is now generally accepted that the delayed light emitted by chloroplasts originates mostly from the actively functioning pigment system II[1,3,6,9,10], and several investigators have suggested that it is due to the back reaction of Photoreaction II[8,9,16,25]. We would also assume that the regeneration of excited chlorophyll, although physically not quite obvious as yet, is caused by a back reaction of Photoreaction II, namely, the oxidation of the reduced primary electron acceptor X_1^- by the oxidized primary electron donor Y^+ that are produced by Photoreaction II during the illumination. The scheme of reaction will be formulated as follows:

Path of electrons in isolated chloroplasts:



Reactions of Photoreaction II:

$$Y\ Chl\ X_1 \xleftarrow{h\nu} Y\ Chl^*\ X_1 \longrightarrow Y^+\ Chl\ X_1^-$$

(1) (2)

In the above scheme, Chl and Chl* represent the ground and excited states of the fluorescent chlorophyll in pigment System II, respectively, and X_2 represents the redoxy substance receiving electrons from X_1 in the electron transport chain. The delayed light emission could be understood as a result of the reverse reactions of Reactions 2 and 1.

The second slowly increasing component of delayed light could be interpreted to reflect an increase in the level of X_1^- in the dark period subsequent to each excitation flash. A reverse transfer of electrons from the pool of electron acceptor X_2 to X_1 may be driven by the high-energy intermediate, or state, of photophosphorylation accumulated during illumination. This accumulation of the high-energy intermediate requires a high intensity of illumination light. The enhancing effect of methyl viologen on this component of the delayed light emission was only observed at high intensities of excitation light. The suppressing effect of methyl viologen observed at low intensities of excitation light may reflect the decrease of X_1^- caused by accelerations of the electron transport reaction.

The initial rapid rise (the fast component) of delayed light emission was little affected by the addition of uncouplers or by uncoupling treatment, but markedly suppressed by addition of inhibitors. These facts cannot be interpreted by regarding this component of delayed light emission solely as a reflection of the amounts of X_1^-, since these electron transport inhibitors (except for CCCP) have been shown to increase the steady level of X_1^- (refs. 26, 27). In the presence of CMU, illumination would cause a rapid reduction of X_1 to X_1^- with concomitant oxidation of Y to Y^+, and X_1^- would remain at a high level because its reoxidation by X_2 is blocked by the presence of the inhibitor. The level of Y^+, however, does not remain high since it is reduced by an electron carrier next to Y. This reaction is not affected by these inhibitors. Consequently, the production of Y^+ by the forward Reaction 2 is suppressed due to a lack of the electron acceptor X_1. Therefore, the amount of electron acceptor available for the reversed reaction of Reaction 2 (*i.e.* Y^+) becomes less as the illumination proceeds, and thus, the intensity of delayed light emission decreases. The occurrence of the spike-like figure in the initial part of the time course of the delayed light especially in uncoupled chloroplasts in the presence of CMU could be understood as a reflection of the changes taking place during this process.

High concentrations of CCCP showed a different mode of suppression on the fast component in the time course of the millisecond delayed light emission; the suppression in this case might also be due to an inhibition of the electron transport in Photoreaction II and this is in accord with the different inhibitory action of CCCP from that of CMU on the electron transport chain[20].

Another finding was that the intensity of the rapid component of delayed light emission became enhanced at low pH and showed a midpoint at pH 6.5, whereas the intensity of fluorescence did not show such a response towards pH (unpublished data). This seems to offer some relevant information concerning the nature of the delayed light emission although the actual mechanism of the effect of pH on delayed light emission still remains to be elucidated.

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