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MULTIPLE-FLASH ACTIVATION OF THE WATER-PHOTOLYSIS SYSTEM IN WHEAT LEAVES AS OBSERVED BY DELAYED EMISSION

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SUMMARY

The chloroplasts from wheat leaves greened under intermittent illuminations (1 ms in duration) at long intervals (5 min) are capable of photoreducing DCIP (2, 6-dichlorophenolindophenol) with diphenylcarbazide as an electron donor but are incapable of photoreducing DCIP with water as the donor. On exposure of such intermittently illuminated leaves to flashes spaced at intervals of less than 10 s, the delayed light emission from the leaves was greatly enhanced in parallel with the generation of Hill activity. The mechanism of this photoactivation was studied by following the changes of the delayed emission from intermittently illuminated leaves exposed to short-interval flashes programmed in various ways. Analysis of the kinetic data indicated that the photoactivation involves three consecutive photoreactions with a rate-limiting dark reaction between them;

P–light $\rightarrow$ A_0–light $\rightarrow$ A_1–dark $\rightarrow$ A_2–light $\rightarrow$ A_3

in which P is a precursor convertible to A_0, the first intermediate with a longer lifetime of $t_{\frac{1}{2}} \approx 100$ s and A_3 is the final activated compound or state converted by short-interval flashes from A_0 through A_1 and A_2, two other intermediates with shorter lifetimes of $t_{\frac{1}{2}} \approx 0.4$ s and 5 s, respectively.

INTRODUCTION

The development of photosynthetic apparatus in the plastids of angiosperms depends on light. When dark-grown leaves are illuminated continuously with strong light, the development proceeds normally. Photoconversion of protochlorophyllide into chlorophyllide triggers biosyntheses of chlorophylls, carotenoids and proteins necessary for grana formation [1–3]. The electron transport from water to a Hill oxidant via Photosystem II appears after photosynthetic activities are developed in

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; *td*, dark interval between short-interval flashes in an assemblage; *Td*, dark interval between multiple-flash assemblages; *n*, number of short-interval flashes in an assemblage; *N*, number of multiple-flash assemblages given to intermittently illuminated leaves; *I*, delayed emission measured as total photon count; ΔI, increment of *I* after exposure of intermittently illuminated leaves to flashes or flash assemblages.

10–15 h [3–5]. Under intermittent illumination at intervals longer than a few minutes, however, the development proceeds differently.

More chlorophyll *a* relative to chlorophyll *b* is formed without lag to yield leaves with a higher *a*/*b* ratio [6]. The chloroplasts in such intermittently illuminated leaves partially greened by widely spaced flashes are capable of bringing about Photosystem I reactions and DCIP (2,6-dichlorophenolindophenol) photoreduction with diphenylcarbazide, an electron donor at a water side of Photosystem II [7], but are incapable of bringing about certain Photosystem II reactions such as oxygen evolution, fluorescence variation and DCIP photoreduction with water as the electron donor.

Recent observations by Remy [8] and Michel and Sironval [9] revealed that Photosystem II of these chloroplasts is not functional because of a deficiency of the water-splitting system, while the reaction centers of both photosystems have been built almost completely. In fact, a brief exposure of intermittently illuminated leaves to continuous light activated the latent sites to evolve oxygen [10] and to exhibit fluorescence variations [9]. The action spectrum for the activation showed a marked shoulder around 650 nm due to chlorophyll *b* absorption, which suggested that the electron transfer driven by Photosystem II is essential for the photoactivation [11]. Similar photoactivation has been found for dark-grown *Chlorella* or Mn-deficient *Anacystis* cells by Cheniae and Martin [12–15]. Their studies on activation of the oxygen evolution system by flashes at short intervals revealed that the activation is a multi-quantum process involving unstable intermediates. Based on computer analysis of the kinetic data on the photoactivation by repeated flashes with varied dark intervals, Radmer and Cheniae [16] proposed a mechanism for the photoactivation which involves two sequential photoevents. They correlated these photoevents to the oxidation of Mn^{2+} to Mn^{4+} through Mn^{3+} in the oxygen-evolving center of Photosystem II. Multiple photoacts have been also proposed for higher plants by Remy [8] and Inoue et al. [17, 18] to explain the photoactivation of intermittently illuminated leaves by flashes at shorter intervals. More recently, Phung-Nhu-Hung et al. [19] observed the ESR signal from Mn atoms in wheat chloroplasts and confirmed that Mn atoms are incorporated into one of the Mn pools in higher plant chloroplasts on photoactivation of the oxygen-evolving system.

The delayed light emission from intermittently illuminated leaves was measured in a previous study [20], which demonstrated a remarkable enhancement of the emission during activation. This indicated the possibility of studying the activation mechanism more precisely and easily by measuring the delayed emission from intermittently illuminated leaves after illumination under various conditions without isolating chloroplasts for activity measurements. Such measurements of emission were conducted in the present study on the intermittently illuminated leaves exposed to single or multiple flashes spaced at short intervals in order to analyze the process of photoactivation in higher plant leaves based on the method previously used by Cheniae and Martin [13] and Radmer and Cheniae [16] for the kinetic analysis of the photoactivation in algal cells. An assemblage of multiple flashes (the number denoted as n) spaced at short intervals (denoted as td) was given repeatedly at long intervals (Td) to intermittently illuminated leaves and, after exposure to a certain number (denoted as N) of such assemblages, a single flash was given to the leaves for measuring the delayed emission in total photon count (denoted as I) by a sensitive photon-counting technique.

EXPERIMENTAL

Preparation of intermittently illuminated leaves

Intermittently illuminated leaves were prepared by the previous procedure [11, 17] which is briefly described below. Seven-day old etiolated leaves were harvested, cut into segments 5 cm long from apexes, and subjected to intermittent illuminations (1 ms light+5 min dark) with xenon flashes. The light intensity on the surface of leaves was 5.7×10^4 ergs/cm^2 per flash, and the temperature was 24 ± 1 °C. After 24 h of illumination with about 300 flashes, the pale green, intermittently illuminated leaves were harvested and kept in darkness in a refrigerator before use.

Activation by short-interval flashes

The intermittently illuminated leaves were then activated by subjecting them to flash illumination with varied dark time between the flashes. Twenty segments of intermittently illuminated leaves arranged close together on moist filter paper (3.7×4.0 cm^2) were sandwiched between a thin acrylic plate (8×8 cm^2) and a glass plate (8×8 cm^2), and illuminated at a saturating intensity [17] with xenon flashes (half-peak width $= 2\,\mu$s) from a stroboscope (MSK-1A, Sugawara Co.). The intervals of illumination were programmed in various ways with an external trigger and timer system. The energy per flash on sample leaves was 2.1×10^2 ergs/cm^2, which was measured with a Quantronics thermocouple model 500 equipped with a control unit, model 503.

Photon counting of delayed emission

The intermittently illuminated leaves activated with the short flashes were kept in darkness for a few minutes, during which the delayed emission arising from the flashes for activation decayed completely. The leaves were then illuminated with a single, 6 ms xenon flash (flash energy, 4.3×10^5 ergs/cm^2 per flash) from another stroboscope set in a photon-counting device. The photons emitted from the leaves were measured through a red glass filter (VR-63, Toshiba Kasei Co.) with a photon counter (Jasco. model KC-200) equipped with a 30-Hz mechanical chopper and a red-sensitive photomultiplier (EMI 9659QB) cooled with cold nitrogen gas. The measurement was started 120 ms after the onset of the actinic flash and continued until the emission decayed (several minutes). The count of photons in every 4/30 s was converted to an analogue signal with a rate meter and recorded on a strip chart against time. The total photon count (denoted as I) after the start of measurement was estimated from the area of the decay curve.

Activity measurement

Chloroplasts were prepared from sample leaves with 0.05 M Tricine buffer (pH 7.5) containing 0.4 M sucrose and 0.01 M $MgCl_2$. The Hill activity and the photoreduction of DCIP with diphenylcarbazide as electron donor were measured as described previously [11].

RESULTS

Chloroplasts prepared from the intermittently illuminated wheat leaves used in the present study showed no activity of DCIP photoreduction with water as electron

donor but an appreciable activity (20 ± 4 μmol DCIP reduced/mg chlorophyll per h) with diphenylcarbazide as the donor. On illumination of these leaves with continuous light or with short-interval flashes, the activity of DCIP photoreduction with water as the donor was rapidly generated and, simultaneously, the delayed emission in total photon count (I) from the leaves was greatly enhanced. The delayed emission from completely activated leaves was approx. 10 times the emission from intermittently illuminated leaves before activation.

Curves A and B in Fig. 1 show the time courses of enhancement of emission and activation of the Hill activity, respectively, during illumination of intermittently illuminated leaves with repeated single flashes at 2-s intervals. Both Hill activity and emission enhancement increased linearly in the early stage of activation (< 100 flashes) and then both gradually levelled off. This provides the experimental basis that the increment (ΔI) of emission can be used as a measure of photoactivation in the early stage [20]. After about 200 flashes, essentially no further increase of emission was observed. However, the Hill activity continued to increase up to about 500 flashes.

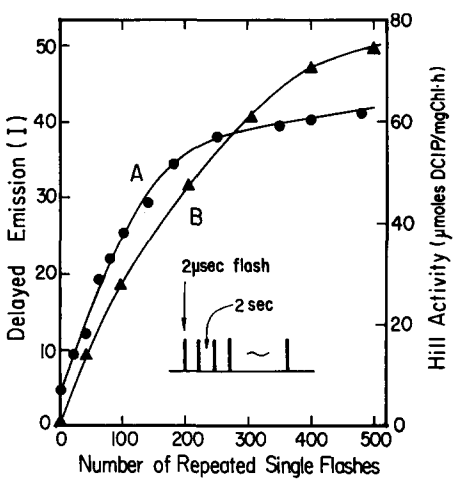


Fig. 1. Delayed emission (I, curve A) and the Hill activity (curve B) during photoactivation of intermittently illuminated leaves by repeated single flashes at 2-s intervals. A 6-ms flash was given to the sample leaves after exposure to these repeated flashes in order to count the photons (I) of delayed emission between 120 ms and 5 min. The Hill activity was measured for the chloroplasts isolated from the intermittently illuminated leaves after exposure to flashes.

The close parallelism between activation and emission increment is clearly demonstrated in Fig. 2 where the activities and increments after exposure to 100 single flashes are plotted against the dark interval between successive single flashes. These activities (open circles) and increments (solid circles) plotted in a suitable relative scale in the figure follow the same curve with a maximum at 2 s. The curve shows a steep drop of activity or emission at shorter intervals and a gradual drop at longer intervals. The shortest interval above which neither activation nor enhancement was observed was about 20 s. This provided the basis for further experiments in which the interval (Td) of 30 s was set between successive flash assemblages.

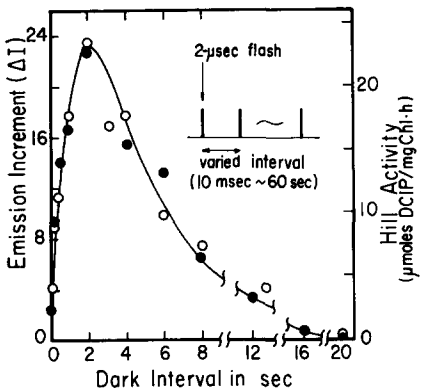


Fig. 2. Effect of dark interval on the degree of photoactivation as estimated from the increment (ΔI) of delayed emission (solid circles) and from the Hill activity (open circles). One hundred single flashes were given to intermittently illuminated leaves at uniform intervals. The interval was varied between 10 ms and 60 s to see the effect of dark interval.

In the next experiment, intermittently illuminated leaves were illuminated repeatedly at this long interval of 30 s (*Td*) with an assemblage of double, triple or quadruple flashes ($n = 2, 3$ or 4) at short uniform intervals (*td*). The short interval between successive flashes in each assemblage was varied between 10 ms and 10 s in order to see the effect of this interval. The degrees of enhancement after exposure to 25 assemblages are plotted against *td* in Fig. 3, which indicates similar bell-shaped curves with a maximum around 0.5 s for these multiple-flash assemblages. It may be deduced from these data that at least two photoreactions are necessary for this enhancement. The steep drop of enhancement at shorter intervals indicates that a dark

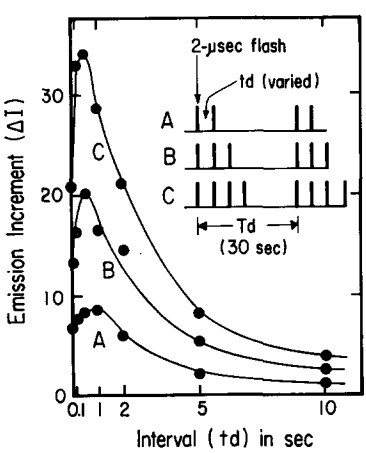


Fig. 3. Effect of dark interval (*td*) between successive flashes in a multiple-flash assemblage. Assemblages of double (curve A), triple (B) and quadruple (C) flashes were given repeatedly for 25 (*N*) times to intermittently illuminated leaves. The interval (*td*) between successive flashes in each assemblage was varied between 10 ms and 10 s, and the interval (*Td*) between assemblages was kept constant at 30 s except for the quadruple flash assemblage at $td = 10$ s, in which the *Td* interval was programmed to be 40 s.

period of 0.5 s is required for the intermediate (A_1 in the scheme shown later) formed on the first photoreaction to be converted to another intermediate (A_2) sensitive to the follow-up flash for the second photoreaction, and the gradual drop at longer intervals indicates that this photosensitive intermediate (A_2) decays within the dark interval of 30 s.

The number (n) of short interval flashes in an assemblage was next varied at fixed intervals of $td = 0.5$ s and $Td = 30$ s in order to see the effect of n on the degree of enhancement. In the experiment, approx. 100 flashes in total ($N \times n$) were given to intermittently illuminated leaves at different n numbers. The average increments per flash ($\Delta I/nN$) calculated from the data are shown as a function of n by open triangles on curve A in Fig. 4 which indicate a more gradual increase of the average increment at larger n numbers, starting from zero at $n = 1$. Solid circles on curve B show the increments per assemblage as a function of n which was caluculated from the data on curve A. This calculation per assemblage indicates progressively more efficient enhancement with increasing n number. At large n numbers, however, the enhancement per assemblage rises almost linearly. In other words, addition of a flash raises the enhancement to the same extent at larger n numbers. These data indicate the presence of some intermediate whose concentration increases progressively at small n numbers (< 3–4) and becomes constant at large n numbers (> 3–4).

In the next experiment, the long interval (Td) between successive assemblages was varied, keeping the flash number (n) and the short interval ($td = 0.5$ sec) between flashes in the assemblage constant in order to see the effect of the long interval (Td) on

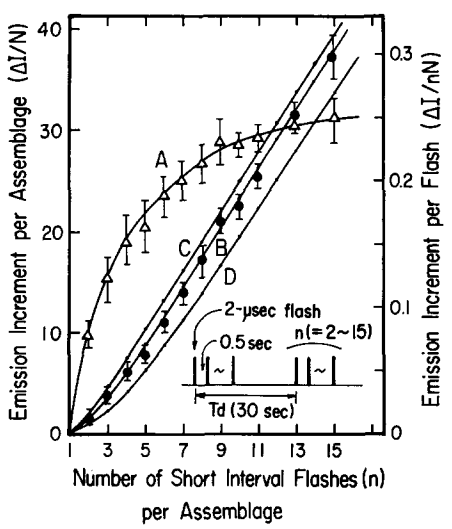


Fig. 4. The emission increment ($\Delta I/nN$) per flash (open triangles on curve A) and the increment ($\Delta I/N$) per assemblage (solid circles on curve B) plotted as a function of n (the flash number in each assemblage). Intermittently illuminated leaves were exposed to multiple flash assemblages with varied n number so as to give approx. 100 flashes in total ($N \times n$), and the increments per flash and per assemblage, $\Delta I/nN$ and $\Delta I/N$, were calculated from the increment ΔI, by the 100 flashes. Curves B, C and D with dots are the theoretical curves obtained by assuming $\alpha = 0.29$ and $f = 0.5$, 0.7 and 0.3, respectively. Bars for triangles and circles indicate the errors calculated from two separate experiments.

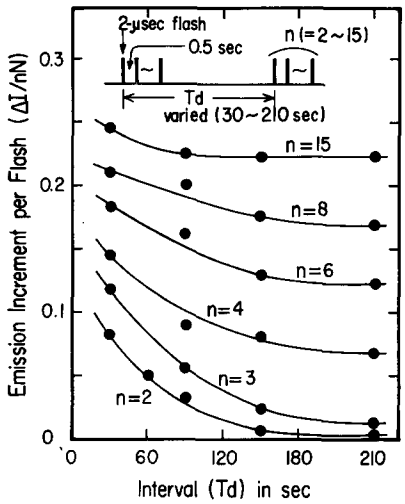


Fig. 5. Effect of the dark interval (*Td*) between assemblages on the enhancement of emission. Multiple flash assemblages with a fixed *n* number at a dark interval of $td = 0.5$ s were given to intermittently illuminated leaves repeatedly at a varied uniform interval of *Td* between assemblages. The total flash number ($N \times n$) given to the leaves was kept to be about 50, and the increment ($\Delta I/nN$) per flash was calculated from the data.

the average increment per flash ($\Delta I/nN$) such as shown by curve A in Fig. 4 for the case of $Td = 30$ s. The result shown in Fig. 5 indicates deactivation during the long dark interval. The decay of enhancement was greater and faster at smaller *n* numbers with increasing *Td*. With double or triple flashes, for example, the enhancement decayed practically to zero at $Td = 210$ s, while the decay with 15 flashes in the assemblage was only 7%. The mechanism of this decay process will be discussed later.

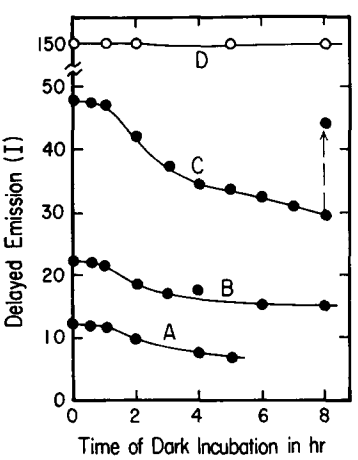


Fig. 6. Effect of dark incubation on the emission (ΔI) from intermittently illuminated leaves after exposure to continuous light for a short period (1, 2 and 5 min for curves A, B and C, respectively) and from mature leaves (curve D). The sample leaves were subjected to the emission measurement at intervals of 30 min or 1 h during the dark incubation.

A different type of deactivation, which proceeds much more slowly, can be observed when the intermittently illuminated leaves exposed to continuous light for a few or several minutes were incubated in darkness for hours. During this dark incubation, the delayed emission was measured at intervals of 30 min or 1 h. Curves A, B and C in Fig. 6 obtained for the samples illuminated for 1, 2 and 5 min, respectively, show nearly the same trend of deactivation. The emission did not appreciably decay during 1 h of dark incubation but, later, decayed slowly to a level about 30 % lower than that before the dark incubation.

Re-illumination of the incubated sample for curve C with continuous light enhanced the emission to the original level as shown by an arrow on the curve. Exchange of the atmosphere in the dark incubation to nitrogen did not affect these decay curves. Mature leaves incubated similarly as a control did not undergo such deactivation as shown by curve D. It was thus confirmed that this type of deactivation during dark incubation is little, unless the incubation time exceeds 1 h. The longest period in the experiment of Fig. 5 was 87.5 min ($n = 2$, $N = 25$, $Td = 210$ s), and the degree of deactivation during this period as estimated from the data in Fig. 6 was less than 15 %.

DISCUSSION

The enhancement of delayed emission measured for leaves exposed to repeated single flashes or multiple-flash assemblages indicated that the enhancement requires at least two photoevents which take place sequentially. This result is in qualitative agreement with the previous observation by Cheniae and Martin [13] on the activation of Mn catalyst in algal cells as well as with the mechanism of photoactivation with the two-quantum process proposed previously by Radmer and Cheniae [16] based on computer analysis of the effect of dark interval on the activation with repeated flashes or flash pairs. The curves in Figs 2 and 3 obtained at various dark intervals indicated that a dark interval optimal for the activation is about 1 s. According to the mechanism proposed by these authors [13, 16] and by ourselves [17] in the previous paper, this interval is the time required for an intermediate (A_1) produced from A_0 with a flash to be converted by the next flash to a final active state (A_3);

$$A_0 \xrightarrow{\text{light}} A_1 \xrightarrow{\text{dark}} A_2 \xrightarrow{\text{light}} A_3$$
$$\xleftarrow[\text{dark}]{}$$

The decay half-times of the intermediates A_1 and A_2, estimated from the experiment with repeated flashes (the curves in Figs 2 and 3) were 0.3–0.5 and 4–6 s, respectively. The former lifetime is in approximate agreement with the lifetime (0.2 s) determined by Cheniae and Martin [13] from the data on algal cells, but the latter lifetime is about 3 times longer than the lifetime (1.6 s) determined on algal cells. It is interesting to note that the lifetime of A_2 is much longer in higher plants as compared with that in algae. An interval (*Td*) of 30 s placed between flash assemblages in the present experiment was appropriate to make A_1 and A_2 decay completely back to the state of A_0.

The assumptions made in the above mechanism to account for the photoactivation of the Hill reaction were as follows; (i) a small fixed number (α) out of a

large number of A_0 sites is converted to A_2 through A_1 by the photoreaction with a flash followed by the dark reaction of 0.5 s (zero order photoreaction), (ii) a fixed fraction (f) of A_2 sites is converted by a flash to A_3 (first order photoreaction) and (iii) the intermediates A_1 and A_2, decay back to A_0 during the dark interval (Td) of 30 s.

Based on these assumptions, the enhancement yields, $y_1, y_2, y_3, \ldots\ldots\ldots$ and y_n, of A_3 by the first, 2nd, 3rd, $\ldots\ldots\ldots\ldots$ and nth flashes, respectively, in the assemblage and the total enhancement (ΔI) by the assemblage are expressed as follows;

$$y_1 = 0 \times f = 0$$
$$y_2 = \alpha f$$
$$y_3 = [\alpha + \alpha(1-f)]f = (2-f)\alpha f$$
$$y_4 = [1+(2-f)(1-f)]\alpha f$$
$$y_5 = [1+(1-f)+(2-f)(1-f)^2]\alpha f$$
$$\ldots\ldots\ldots\ldots\ldots\ldots$$
$$y_n = [1+(1-f)+(1-f)^2+\ldots\ldots\ldots$$
$$\ldots\ldots\ldots\ldots+(1-f)^{n-4}+(2-f)(1-f)^{n-3}]\alpha f$$
$$\Delta I = y_1+y_2+y_3+\ldots\ldots\ldots\ldots+y_n$$

The yield, y_1, is zero because A_2 has not been formed on illumination with the first flash. The number of A_2 sites increases progressively with increasing flash number, since not all of the A_2 sites formed from A_0 are converted with a flash to A_3.

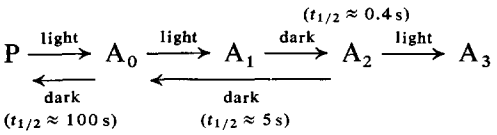
With a large number (n) of flashes in each assemblage, however, a steady state is attained such that the number of A_2 sites converted with a flash to A_3 is identical to the number of A_2 sites newly formed with the flash from A_0. Under this condition, we can approximate the above equation for y_n to be

$$y_n = \alpha f \frac{1}{1-(1-f)} = \alpha$$

This equation indicates the linear rise of ΔI at large n numbers shown by curve B in Fig. 4. The value of α, estimated from the slope of the linear rise of ΔI, was 0.29 counts. At smaller n numbers, the increment y_i, by additional flash is lower than α, and increases gradually with an increasing n number to this upper limit of $\alpha = 0.29$. Curves B, C and D with dots in Fig. 4 are the theoretical curves calculated by assuming the f value to be 0.5, 0.7 and 0.3, respectively. The experimental data shown by solid circles are on curve B for $f = 0.5$, which is not widely different from $f = 0.4$ estimated previously from activity data [17]. Such agreement of the experimental data with the theoretical curve confirmed that two photoreactions with one dark reaction are involved in the photoactivation in higher plant leaves, as in the mechanism of photoactivation in Mn-deficient algal cells previously proposed by Cheniae and Martin [13] and by Radmer and Cheniae [16].

This mechanism of activation does not, however, account for the deactivation shown in Fig. 5 during the dark interval (Td) longer than 30 s between assemblages. The deactivation proceeded to a great extent with double or triple flashes in the assemblage, and is different from the deactivation shown in Fig. 6, which required at

least 1 h of dark incubation to be observed. The greater deactivation with a smaller number of flashes in the assemblage in Fig. 5 strongly suggests the decay of some intermediate which decays more slowly than A_1 and A_2; these intermediates decay completely within 30 s. Considering these, the author is tempted to assume another photoreaction for the formation of A_0 from its precursor, P;



P is the precursor accumulated in intermittently illuminated leaves and is converted by light to A_0. A_0 is stable for more than 30 s in darkness but gradually decays back to P during the prolonged interval of 210 s in the experiment of Fig. 5. The pool for A_0 is filled up rapidly by a small number (n) of flash assemblages when the interval (Td) is much shorter than 210 s or when the number (n) of short interval flashes in each assemblage is large. When the dark interval is longer than 30 s, a steady state may be established on a lower A_0 level, particularly when the number (n) of short interval flashes in the assemblage is small. At a lower A_0 level comparable to α, we cannot assume the zero order photoreaction for the conversion of A_0 to A_2 through A_1. The yield of A_2 per flash may be less than α which will decrease the yield of the whole process as shown in Fig. 5.

The mechanism of photoactivation proposed above involves three photoreactions taking place sequentially with a rate-limiting dark reaction and three intermediates with different lifetimes. This mechanism is compatible with the scheme proposed previously by Cheniae and Martin [12, 13] or by Radmer and Cheniae [16] to account for the photoactivation of the latent oxygen-evolving system in Mn-deficient *Anacystis* cells and in dark-grown *Chlorella* cells, although their mechanism involves two photoreactions. In the scheme of Radmer and Cheniae [16], Mn^{2+} adsorbed to the latent oxygen-evolving site is oxidized by two sequential photoreactions driven by Photosystem II into Mn^{4+} through Mn^{3+} to yield an active center for oxygen evolution. They inferred that the rate-limiting dark reaction(s) are the diffusion of Mn^{2+} to the latent site in Mn deficiency and/or the transfer of electrons from the primary electron acceptor of Photosystem II to Photosystem I. Judging from the experimental conditions and the lifetimes of the intermediates, Mn^{2+} adsorbed to the latent site, which is the starting state in Radmer's scheme, appears to correspond to the intermediate A_0, of the longer lifetime, and Mn^{3+} produced after the first photoreaction may correspond to the intermediate A_2, of the shorter lifetime. This is then converted to Mn^{4+} by the second photoreaction to yield the final activated state. Considering these, the third light-requiring process proposed in the present study for higher plant leaves may be adsorption of Mn atoms to the latent site. It seems quite reasonable to assume that the adsorption requires a conformational change of protein which is induced by light absorption. The light requirement for conformational changes has been demonstrated in the inactivation of electron transfer at the water side of Photosystem II by the treatment with Tris [21], and in the adsorption of a certain chemical to Photosystem II particles (unpublished). More recently, Yamashita and Tomita [22] reported a related phe-

nomenon that the incorporation of Mn atom into Tris-treated chloroplasts requires light.

Considering these together with the recent observation by Phung-Nhu-Hung et al. [19] that the photoactivation of wheat leaves involves the incorporation of Mn atoms into chloroplasts, the photoactivation in higher plant leaves is probably the activation of Mn catalyst involving its valency change as proposed by Radmer and Cheniae [16] for Mn-deficient algal cells and also by Cheniae and Martin [12] for dark-grown algal cells. Conformational changes of its protein moiety by light may be necessary for the center to undergo the valency change of the Mn atom. Measurements from different approaches are awaited to identify the intermediate states assumed in the above scheme. One of such measurements may be the thermoluminescence from intermittently illuminated leaves which will be reported elsewhere [18].

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