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LIGHT-DEPENDENT DEVELOPMENT OF THERMOLUMINESCENCE, DELAYED EMISSION AND FLUORESCENCE VARIATION IN DARK-GROWN SPRUCE LEAVES

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SUMMARY

Thermoluminescence profiles of spruce leaves grown under various light or dark conditions were measured after excitation at a low temperature (−70 to −20 °C) by 1-min illumination with red light, and the following results were obtained. Mature spruce leaves showed five thermoluminescence bands at −30, −5, +20, +40 (or +35) and +70 °C (denoted as Z_v, A, B_1, B_2 and C bands, respectively), but dark-grown spruce leaves with a similar chlorophyll content showed only two bands, at −30 and +70 °C (the Z_v and C bands) and were devoid of the three other bands (the A, B_1 and B_2 bands). On exposure of the dark-grown leaves to continuous red light, the A, B_1 and B_2 bands were rapidly developed, and the development was accompanied by enhancement of delayed emission, fluorescence variation and the Hill activity (photoreduction of 2,6-dichlorophenolindophenol with water as electron donor). It was demonstrated that the dark-grown spruce leaves are devoid of the water-splitting system in Photosystem II, and that the latent water-splitting activity is rapidly photoactivated by exposure of the leaves to continuous red light. These results on the gymnosperm spruce leaves, in which greening proceeds in complete darkness, being independent of the development of the water-splitting system in light, were discussed in relation to previous observations on angiosperm leaves, in which both greening and the activity generation proceed in the light.

INTRODUCTION

In angiosperms, the development of photosynthetic apparatus is dependent on light. On illumination of etiolated angiosperm leaves with continuous light, protochlorophyllide is converted to chlorophyllide, which triggers rapid biosynthesis of chlorophylls and carotenoids to form grana. The activities of Photosystems I and II

Abbreviations: Cl_2Ind, 2,6-dichlorophenolindophenol; DPC, diphenylcarbazide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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are developed during this process of chlorophyll biosynthesis [1, 2]. Under intermittent flash illumination at intervals longer than a few minutes, however, the development of these photosystems proceeds incompletely. The chloroplasts developed under such intermittent illumination are capable of bringing about Photosystem-I reactions and Cl_2Ind (2,6-dichlorophenolindophenol) photoreduction (photosystem II reaction) with DPC (diphenylcarbazide) as an artificial electron donor but are incapable of bringing about Cl_2Ind photoreduction with water as the donor to evolve oxygen [3, 4]. In other words, the long interval flashes develop the reaction centers of both photosystems, but do not develop the water-splitting system. The water-splitting activity is rapidly generated, when these leaves flashed at long intervals are exposed to continuous light or short interval flashes [5–9]. This process, which may be called photoactivation of the water-splitting system, was studied previously by various approaches [6–10]. The results indicated that the process is composed of three consecutive photoreactions [10] and is accompanied by an enhancement of the delayed fluorescence and development of fluorescence variation and thermoluminescence bands [7, 10, 12, 13]. Mature wheat leaves emitted five thermoluminescence bands (denoted as Z_v, A, B_1, B_2 and C bands, respectively) at different temperatures, but the intermittently flashed leaves emitted only the Z_v and C bands. The remaining three (A, B_1, B_2 bands) were rapidly developed by exposure of the long interval flashed leaves to continuous red light. This indicated that these three bands are emitted from a structure(s) responsible for or closely related to the water-splitting activity to evolve oxygen [13]. Earlier literatures on thermoluminescence are reviewed in the previous paper [12].

As opposed to angiosperms, some photosynthetic apparatus such as light-harvesting chlorophylls are formed in complete darkness in gymnosperm leaves. The Hill activity to split water has been found by Oku et al. [14] for the chloroplasts prepared from the dark-grown spruce leaves, although the activity was not high. The present paper reports the thermoluminescence, delayed emission and fluorescence variation of light- and dark-grown spruce leaves. These measurements were made in order to see whether or not the close correlation found for angiosperms between the water-splitting activity and thermoluminescence bands, delayed emission or fluorescence variation, exists in the case of gymnosperms.

EXPERIMENTAL

Spruce seeds (*Picea abies* L.) were germinated and grown on moist vermiculite in darkness at 24 ± 1 °C, and 20 to 30-day old seedlings were harvested. The dark green needle-like leaves were picked from the seedlings and wrapped with moist tissue paper to be kept in a dark box at room temperature. The leaves from 40 seedlings were spread on moist filter paper and illuminated for activation with continuous red light from a projector with a 300 W incandescent lamp through a fan-cooled heat absorbing filter and a red glass filter (VR-63, Toshiba Kasei Co.). The light intensity on the surface of leaves was 28 $\mu W/cm^2$. The illuminated leaves were then kept in darkness for 10–30 min before measurements were begun.

Thermoluminescence from leaves was measured by the method reported previously [12, 13], which is briefly described below. 15 spruce leaves exposed to red light for a desired period for activation were arranged on moist filter paper (2.2×4.0 cm^2) and sandwiched between a heater and a transparent acrylic plate to be

mounted on an aluminum holder. The leaves on the holder were cooled in a Dewar bottle to the desired temperature below 0 °C, and illuminated for 1 min with red light (600 μW/cm^2) from a 300 W incandescent lamp through a red glass filter (VR-63) and a heat absorbing water layer (15 cm) and then rapidly cooled down to liquid nitrogen temperature. The sample was transferred with the Dewar bottle to the housing of a photon counter (Jasco model KC-200) equipped with a 30 Hz mechanical chopper and a red-sensitive photomultiplier (EMI 9659QB). The leaves were heated at a rate of 0.5 °C/s, and the photons emitted from the leaves during heating were counted through a red glass filter (VR-63). The temperature of the leaves was monitored with a copper-constantan thermocouple inserted between the sample and the acrylic plate. The digital photon count integrated over a period of 32 cycles of chopping was converted to an analogue signal to be recorded against temperature on an X-Y recorder.

In the measurement of fluorescence variation, about 10 sample leaves placed on a small metal plate (1.5×2.0 cm^2) were illuminated with blue light at an intensity of 450 μW/cm^2 from a 500-W Xe lamp through band-pass glass filters (Corning, 9782 and Toshiba Kasei, VB-46) and a heat absorbing water layer (5 cm). The fluorescence from the sample surface was focused on a photomultiplier (R-446, Hamamatsu TV Co.) through a red glass filter (VR-65). The slow fluorescence variation during 1 min after the onset of actinic illumination was recorded directly on a strip chart recorder. The light intensity or energy on the samples was measured with a Kipp and Zonen thermopile (model E-2) or with a Quantronics thermocouple (model 500).

Chloroplasts for the measurement of the Hill activity were prepared from the spruce leaves with Tris buffer containing polyethylene glycol (10 %), according to the method of Oku et al. [14]. The activity of Cl_2Ind photoreduction with water or DPC as electron donor and the chlorophyll concentration in the sample suspension were measured by the procedure described previously [8, 9].

RESULTS

Induction of thermoluminescence bands

Solid curves in Fig. 1 show the thermoluminescence profiles of light-grown mature leaves of spruce excited for measurement by 1-min illumination at three different temperatures. As reported previously [12], the profile was greatly dependent on the excitation temperature (denoted as T_{ex} throughout this paper). The profile obtained by excitation at $T_{ex} = -75$ °C shows a strong broad band at about +20 °C with a shoulder at 30–40 °C and two weak bands at −65 and +70 °C, respectively. Judging from the emission temperatures, the broad band is a composite of the B_1 and B_2 bands denoted previously [12]. The small band at −65 °C is the Z_v band whose emission temperature changes depend on the excitation temperature [12], and the low flat band around +70 °C is the C band emitted at this constant temperature. These assignments were made according to the previous nomenclature [12, 13] partially modified from that of Arnold [15].

The profile obtained at a higher excitation temperature of $T_{ex} = -45$ °C shows the variable Z_v band at a higher emission temperature of −25 °C, a strong B_2 band with a B_1 shoulder and a flat C band. At $T_{ex} = -20$ °C, a new strong band appeared around −5 °C and the B_1 and B_2 bands remarkably decreased in height.

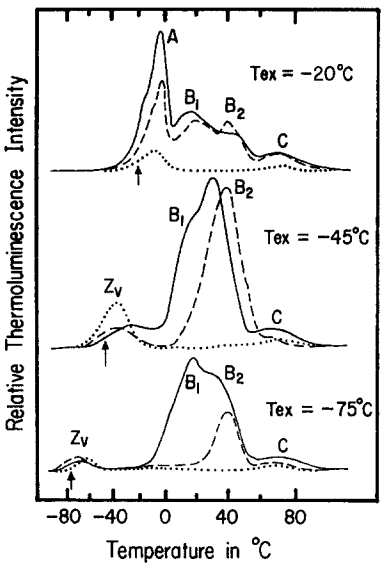

Fig. 1. Thermoluminescence profiles of spruce leaves measured at three different excitation temperatures of −75, −45 and −20 °C (T_{ex}); mature leaves (solid curves), dark-grown leaves (dotted curves) and 180-min illuminated dark-grown leaves (broken curves). The excitation was made by 1-min illumination with strong red light (≧630 nm, 600 μW/cm^2) at the respective temperature. The dark-grown leaves were illuminated for 180 min with red light (≧630 nm, 28 μW/cm^2) at room temperature for full activation of the latent water-splitting system. The arrows indicate the excitation temperature.

The strong band is considered as the A band found previously for spinach leaves by excitation at $T_{ex} = -10$ °C. The Z_v band could hardly be recognized because of this strong A band in the same temperature region. The B_2 band was located at +40 °C which is slightly higher than the emission temperature of the B_2 band found for spinach and wheat leaves [12, 13]. These observations on spruce leaves are in good agreement with those made on angiosperm leaves [12], except for the slight differences in emission temperature.

The dark-grown spruce leaves were deeply green and contained a considerable amount of chlorophylls *a* and *b* (0.9 μg/mg fresh weight) which is about 25 % of the content in mature leaves (3.5 μg/mg fresh weight). In spite of this high content of chlorophylls, their thermoluminescence profiles were remarkably different from those of mature leaves as seen from the dotted curves shown in Fig. 1. The profiles are composed of a distinct Z_v band and a very weak C band but completely devoid of the A, B_1 and B_2 bands. The Z_v band was emitted at a little higher temperature than the excitation temperature as in the case of mature leaves but its height obtained at $T_{ex} = -45$ °C was much larger than that of mature leaves.

The dark-grown spruce leaves were illuminated continuously with red light (≧ 630 nm, 28 μW/cm^2) at room temperature. Fig. 2 shows the effect of this continuous illumination on the thermoluminescence profile. The curves on the left side of the figure are the data obtained at $T_{ex} = -45$ °C. A broad B_2 band appeared after a brief exposure (2 min) of the leaves to continuous light, and this band was intensified progressively during prolonged illumination until 60 min. However, further illumination until 180 min did not change the profile much, although the Z_v band was lowered

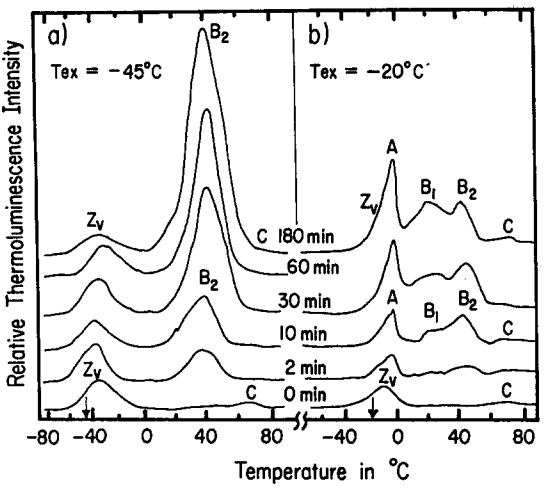

Fig. 2. Development of thermoluminescence bands during continuous illumination of dark-grown spruce leaves with red light (≧630 nm, 28 μW/cm^2) at room temperature. The profiles measured by excitation at $T_{ex} = -45$ °C are shown on the left side, and those at $T_{ex} = -20$ °C on the right side. The illumination times on activation shown in the middle are in common for these two types of profiles. The arrows indicate the excitation temperature.

appreciably during the illumination. The B_1 band was barely seen as a shoulder on this strong B_2 band. Similar leaves under development, when observed by excitation at $T_{ex} = -20$ °C, showed the appearance of the A, B_1 and B_2 bands comparable in relative heights, as seen from the profiles shown on the right side of Fig. 2. The Z_v band was masked with the A band after 2 min of illumination, and the C band was very weak. The profile obtained after 180 min of continuous illumination was very similar to that of mature leaves excited at the same temperature of $T_{ex} = 20$ °C. The two top curves in Fig. 2 of 180-min illuminated leaves observed at $T_{ex} = -45$ °C and −20 °C are reproduced as broken curves in Fig. 1. The close similarity between 180-min illuminated and mature leaves at $T_{ex} = -20$ °C described above is shown by the solid and broken curves on top of the figure. At the excitation temperature of $T_{ex} = -45$ °C, however, the broken curve shows a strong B_2 band but a very weak shoulder or none as the B_1 band, while the solid curve shows a distinct shoulder on the strong B_2 band at a slightly lower temperature as compared with the B_2 band on the broken curve. This shift of the B_2 band might be due to the effect of overlapping of the B_1 band. The profile of the 180-min illuminated leaves measured by excitation at $T_{ex} = -75$ °C (broken curve on the bottom of Fig. 1) shows the above difference more distinctly. The B_1 band could not be recognized at all on the broken curve, while the B_1 band appeared as a peak with a shoulder of the B_2 band on the solid curve of mature leaves.

Induction of delayed emission and fluorescence variation

The curves in Fig. 3 show the decay of delayed emission observed for dark-grown spruce leaves exposed to continuous red light for different periods. The dark-grown leaves before the exposure (the curve at the left end, $t = 0$) showed weak emission at the beginning of observation (120 ms after the onset of actinic flash of

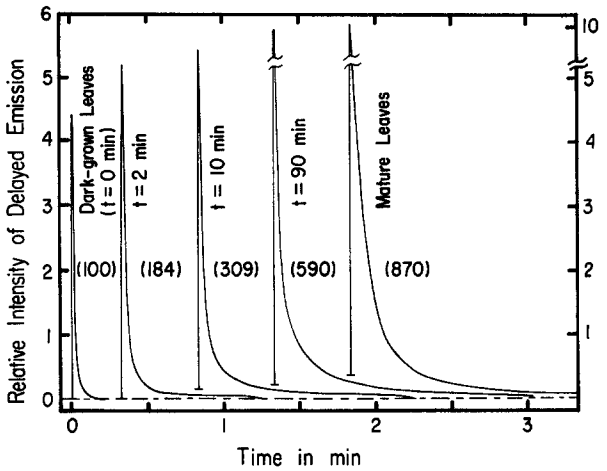

Fig. 3. Changes of the delayed emission from dark-grown spruce leaves during continuous illumination with red light (≧630 nm, 28 μW/cm^2) as compared with the emission from mature leaves; t stands for the illumination time, and the figures in parentheses indicate relative areas (total photon count) under the decay curves measured from 120 ms after actinic 6-ms Xe flash. The area for dark-grown leaves was taken as 100. The preilluminated dark-grown leaves were kept in darkness for 10 min before measurements.

6-ms duration) which decayed rapidly to almost zero within 10 s. The emission was remarkably enhanced by exposure of the dark-grown leaves to continuous red light, as seen from the curves measured after 2, 10 and 90 min of illumination. The emission at the beginning of observation was intensified 2 times and the total photon count (the area under the decay curve shown in a relative unit with parentheses on each curve) was increased about 6 times by 90 min of continuous illumination. The total count of 90-min illuminated leaves was not widely different from that of mature leaves. It may be also seen from these curves that the exposure to continuous light increased the life time as well as the intensity. It was demonstrated in the previous study [11] that the decay curve is composed of a few component curves with different life times. The longer tailing with increasing exposure time suggests that the components with longer life times contributed to the increase of the total count. Similar intensification was found previously [11] when intermittently flashed wheat leaves were exposed to continuous light.

When dark-incubated mature leaves are exposed to strong light, the fluorescence from the leaves varies in intensity, showing a few maxima, during the first 10 or 20 s of illumination, until it reaches a stationary level. This phenomenon of fluorescence variation has been investigated repeatedly for angiosperm leaves since the early observation by Kautsky [16]. Mature spruce leaves showed similar variations as seen from the top curve in Fig. 4. The intensity reached a maximum about 1 s after the onset of actinic light and then decayed gradually to a stationary level within 30–60 s. A shoulder was found around 4–6 s during this decay. By contrast, dark-grown spruce leaves did not show such marked variations. The curve on the bottom of the figure shows an instantaneous rise of intensity to a level, and the later variation on the level did not exceed 4 %.

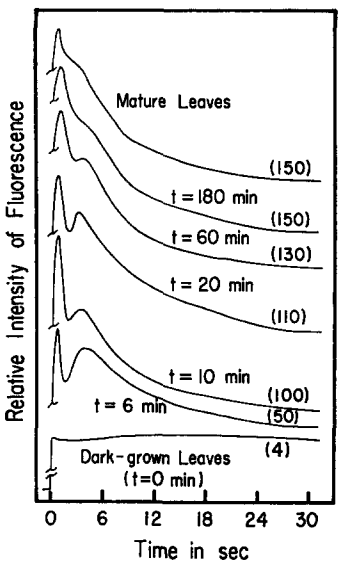

Fig. 4. Fluorescence variations observed for dark-grown spruce leaves illuminated for different periods with red light (≧630 nm, 28 $\mu W/cm^2$) as compared with the variation observed for mature leaves; *t* stands for the illumination time, and the figures in parentheses indicate the height of the first highest peak above the stationary level relative to the stationary level which was taken as 100. The pre-illuminated dark-grown leaves were kept in darkness for 30 min before measurements. The curves do not share a common base line, but show the relative shape of variations.

Exposure of the dark-grown leaves to continuous light changed the time course remarkably. After a brief exposure for 6 min, a sharp maximum appeared at 1 s, and a broad second maximum appeared around 4 s before the curve approached the stationary level. The degree of variation estimated as the first peak height above the stationary level relative to the height of the stationary level taken as 100 % (given in parentheses on each curve) increased from 4 to 50 % after the first 6 min of continuous illumination and further increased to 150 % during the prolonged illumination until 180 min. The second maximum was clearly observed in the early stage but obscured in the later stage, probably because of the effect of the intensified first peak tailing over the second maximum. These results are in agreement with those obtained for intermittently flashed wheat leaves exposed to continuous light [11].

Induction of the Hill activity

A suspension of the chloroplasts prepared from the dark-grown spruce leaves under dim green safe light showed an appreciable activity of Cl_2Ind photoreduction (about 200 μmol/mg chlorophyll per h) in the presence of DPC as artificial electron donor, but a very low activity of 40 μmol/mg chlorophyll per h in its absence. About 40 % of the activity in the absence of DPC was resistant to DCMU. This implies that the Hill activity via Photosystem II with water as electron donor was as low as 25 μmol/mg chlorophyll per h, which is roughly 10 % of the activity in the presence of DPC. When the dark-grown leaves were exposed to continuous red light before the preparation of chloroplasts, the activity with water as the donor was remarkably

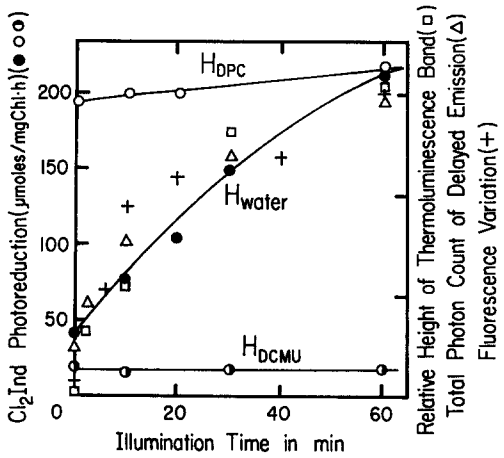

Fig. 5. Development of the Hill activity during illumination of dark-grown spruce leaves with red light (≧630 nm, 28 μW/cm²); solid circles on curve H_{water} and open circles on curve H_{DPC} show the activities of Cl_2Ind photoreduction with water and with DPC, respectively, as electron donor, and half solid circles on curve H_{DCMU} indicate the activity resistant to 50 μM DCMU in the absence of DPC. Squares, triangles and crosses along curve H_{water} show the relative heights of the B_2 band in the thermoluminescence profiles measured at $T_{ex} = -45$ °C in Fig. 2, the total photon counts of delayed emission in Fig. 3 and the degree of fluorescence variation estimated from the relative height of the first highest peak above the stationary level in Fig. 4, respectively, which were plotted in appropriate relative scales.

enhanced as shown by solid circles on curve H_{water} in Fig. 5. During 60 min of illumination, the activity increased to 200 μmol/mg chlorophyll per h which is approximately equal to the activity with DPC before this continuous illumination. The activity with DPC as the donor, on the other hand, did not increase much during the continuous illumination (curve H_{DPC}). The chloroplasts prepared from the fully activated dark-grown leaves showed nearly the same activity regardless of the presence or the absence of DPC. The activity resistant to DCMU did not change during the induction, as shown by curve H_{DCMU}. The Hill activity of the chloroplasts prepared from mature leaves was 150 μmol/mg chlorophyll per h, which is 75 % of the activity developed by continuous illumination of dark-grown leaves. This slightly lower activity of mature chloroplasts seems to be due to the higher content of light-harvesting chlorophylls as compared with the dark-grown chloroplasts. Attempts to observe the activation by direct illumination of isolated chloroplasts were unsuccessful.

The relative heights of the B_2 band observed at $T_{ex} = -45$ °C in Fig. 2 and the total photon counts of delayed emission in Fig. 3 are plotted in Fig. 5 as open squares and triangles, respectively. These squares and triangles plotted in appropriate relative scales roughly follow the curve of H_{water} for the Hill activity. This indicates that these three phenomena are closely related to each other and may originate from the same structure responsible for splitting water to evolve oxygen. The crosses in the same figure indicate the changes of fluorescence variation as estimated as the relative height of the first peak above the stationary level. The peak-height change shows a similar trend of development during the activation. These three properties of thermoluminescence, delayed emission and variable fluorescence, once induced in the leaves,

were fairly stable and did not decay in darkness. The thermoluminescence, fluorescence variation and the Hill activity were sustained on the levels of about 70 % of the original activities after 24 h of dark incubation at room temperature, although the delayed emission decayed to about 30 % after the incubation.

DISCUSSION

The thermoluminescence profiles of mature spruce leaves showed five bands (Z_v, A, B_1, B_2 and C) emitted at different temperatures, and the relative heights of these bands were greatly dependent on the excitation temperature. The A band was stronger at higher excitation temperatures than the B_1 and B_2 bands, and the B_1 and B_2 bands were stronger at lower excitation temperatures. These results agree with those obtained in the previous similar observations on wheat leaves [13]. The profile of dark-grown spruce leaves showed the Z_v and C bands, but the other three bands of A, B_1 and B_2 were absent in the profile. The C band in the dark-grown leaves was very weak and intensified slightly by illumination, whereas this band in the profile of the intermittently flashed wheat leaves was stronger [13]. Since the C band decreased in height during greening of etiolated wheat leaves, we considered this band not related to photosynthetic activities. The Z_v band was gradually lowered during 180 min of illumination to about one half the original height which is close to the height in the profile of mature leaves.

The A, B_1 and B_2 bands, which were absent in the profiles of dark-grown spruce leaves, were developed rapidly when the leaves were continuously illuminated (Fig. 2). The B_1 band appeared in the profiles observed at higher excitation temperatures but could not be recognized in the profiles at lower excitation temperatures. In the case of intermittently flashed wheat leaves exposed to continuous light [13], the B_1 and B_2 bands appeared simultaneously, regardless of the excitation temperature. We had, therefore, previously considered these bands to originate from a same site. However, their relative height being greatly dependent on the excitation temperature on illuminated spruce leaves, indicated that these bands could arise from different sites. It may be inferred that at least two different structures responsible for these two bands are developed by the activation with continuous light, but that the one responsible for the B_1 band is less excited at lower temperatures in the case of spruce leaves.

One of the authors [14] has reported that the chloroplasts isolated from dark-grown spruce leaves possess a considerable Hill activity coupled with oxygen evolution, which seems to conflict with the present result of a very low activity. This discrepancy was, however, found to be due to the light condition during the preparation of chloroplasts. In the present experiment, all the procedures were carried out under dim green safe light, whereas some procedures were carried out in room light in the previous experiment. An experiment made in the present study demonstrated that weak room light of the order of 100 $\mu W/cm^2$ in intensity is strong enough to activate the chloroplasts in the dark-grown spruce leaves. The very slight Hill activity with water as the donor found for dark-grown chloroplasts in the present study might be due to the inevitable exposure (about 15 min) of leaves to the dim green light during preparation of chloroplasts.

By contrast, a high activity of Cl_2Ind photoreduction with DPC as electron donor was found for dark-grown spruce chloroplasts, and this activity did not change

during the continuous illumination. These data indicate that the reaction center of Photosystem II is completely developed in darkness, whereas the oxygen-evolving activity is absent. The appreciable amount of chlorophylls present in the dark-grown leaves further indicates that the apparatus necessary for harvesting light energy has been equipped with the reaction center in darkness. This contrasts with etiolated angiosperm leaves in which both reaction centers and light-harvesting chlorophylls are not developed at all in darkness. Their formation depends completely on light. Flashes given intermittently to etiolated wheat leaves brought about the development of both photosystems with the accumulation of chlorophylls. However, the chloroplasts isolated from such flashed leaves were not able to photoreduce Cl_2Ind at all to evolve oxygen and did not show the full activity of Cl_2Ind photoreduction even in the presence of DPC. This may indicate that Photosystem II in such chloroplasts is not complete in the structure responsible for the accepting of electrons from DPC in addition to the complete deficiency of the oxygen-evolving system. Photosystem II in dark-grown spruce leaves seems therefore more complete in structure than in the chloroplasts of flashed angiosperm leaves. Except for this difference in their affinity with DPC, the chloroplasts from the dark-grown gymnosperm leaves were very similar to those from flashed angiosperm leaves in the characteristics of thermoluminescence, delayed emission and fluorescence variation, and their activities were latent unless activated by continuous light.

The Hill activity with water as electron donor for oxygen evolution was rapidly generated by exposure to continuous light, accompanied by the development of thermoluminescence, delayed emission and fluorescence variation, and a close parallelism was found between these properties and the Hill activity. It was concluded previously from similar parallelism found during the activation of flashed angiosperm leaves by continuous light that these three phenomena originate from the same active site responsible for splitting water to evolve oxygen. The absence of fluorescence variation and the weak delayed emission in the dark-grown spruce chloroplasts devoid of the oxygen-evolving activity is in agreement with the adopted mechanism for fluorescence variation [17] and with the results obtained by Bertsch et al. [18] and by Itoh et al. [19] that the delayed emission was very weak in the *Scenedesmus* mutant lacking Photosystem II and that the ms component of delayed emission was affected by the oxidation-reduction state on the water side of Photosystem II. The absence of three thermoluminescence bands in the profiles of the chloroplasts devoid of the oxygen-evolving activity and their rapid development during activation by continuous light strongly suggest that the positive holes responsible for the emission of these bands are stored in the oxygen-evolving system.

Attempts were made to photoactivate isolated inactive chloroplasts directly. However, no significant activity has been observed yet after continuous illumination of isolated chloroplasts, although the thermoluminescence profile of illuminated chloroplasts showed some changes suggesting the activation. Improvements in the isolation procedure will allow us to isolate the spruce chloroplasts in an intact state for activation study. The manganese catalyst in the oxygen-evolving system, the activation of which has been proposed by Cheniae and Martin [20–23] to be involved in the process of photoactivation, may be studied with such chloroplasts in relation to the thermoluminescence bands, delayed emission and fluorescence variation.

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