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INDUCTION OF CHLOROPHYLL *a* FLUORESCENCE IN ISOLATED SPINACH CHLOROPLASTS AT LIQUID NITROGEN TEMPERATURE

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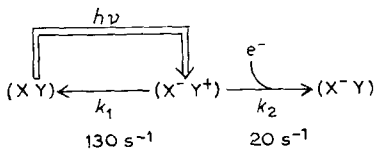
SUMMARY

1. The induction of chlorophyll *a* fluorescence was measured at 690 nm in isolated spinach chloroplasts at liquid N_2 temperature. The effects of preillumination of the chloroplasts with varied durations of flashes were investigated. When the degree of fluorescence induction was represented by the extent of the variable component of fluorescence, a 10-μs flash at a saturating intensity eliminated 13 % of the induction, and a 20-ms flash eliminated 50 %.

2. An oxidant, ferricyanide, markedly decreased the fluorescence yield at the steady-state level but little at the initial rise level.

3. The dark recovery of the fluorescence induction at liquid N_2 temperature was found to be slow and only partial. The maximum recovery was reached after 20 min of dark time.

4 The following scheme is presented for the reaction at the reaction center of System 2 at liquid N_2 temperature.



X and X^- are the primary electron acceptors in the oxidized and reduced forms, respectively. Y and Y^+ are the primary electron donors in the reduced and oxidized forms, respectively. The (X Y) state is converted to (X^- Y^+) by consuming the excitation energy. The (X^- Y^+) state formed is degradated either to (X Y) with loss of energy or to (X^- Y) with reduction of Y^+ by a secondary electron donor. It is suggested that all the reaction centers are in the (X^- Y) state at the maximum fluorescence yield. Kinetic analysis indicates that the rate constants of Reaction 1 and 2 at liquid N_2 temperature are 130 s^{-1} and 20 s^{-1}, respectively, and the life time of the (X^- Y^+) state is 4.5 ms.

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

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INTRODUCTION

The fluorescence yield is known to change depending on the photosynthetic reactions of chloroplasts. Kautsky *et al.*[1] proposed a mechanism in which the fluorescence yield is inversely related to the rate of electron flow through the photochemical reaction. The same idea was used in the analyses of fluorescence induction in isolated chloroplasts[2-4]. An estimation was made for the size of the electron pools which are in the oxidized form in the dark and in the reduced form in the light. Duysens and Sweers[5] and also Butler and Bishop[6] proposed another mechanism in which the fluorescence yield depends on the amount of the reduced form of the primary electron acceptor. This is more general in validity than the former. The former mechanism can be deduced from the latter when the reaction between water and Photoreaction 2 is not rate determining.

At liquid N_2 temperature, two fluorescence bands at 685 nm and 695 nm of pigment System 2 show induction[7], a gradual increase from the rapidly attained initial rise level to the maximum steady-state level. This fact suggests that the photoreaction occurs even at liquid N_2 temperature. However, on cooling the chloroplasts from room temperature to liquid N_2 temperature, the induction kinetics becomes much faster at intermediately low temperatures, and then slower on further cooling[7] Similar findings were also reported by Thorne and Boardman[8] and Malkin and Michaeli[9]. These findings may suggest that the mechanism used for the analysis of fluorescence induction at room temperature[2-4] cannot be directly applied to the fluorescence induction at liquid N_2 temperature.

In the present study, the effects of flash illumination upon the fluorescence induction at liquid N_2 temperature were investigated, and a new scheme is proposed to interpret the fluorescence induction at the low temperature.

METHODS

Spinach chloroplasts were prepared from market spinach leaves according to a method described previously[7]. The chloroplast suspension containing 0.3 μg chlorophyll per ml was placed in a cuvette, 1 cm square and 0.1 cm thick, made of aluminum and glass plates. The cuvette was immersed in liquid N_2, and then was held just above the liquid N_2 layer.

For excitation of fluorescence, 475-nm monochromatic light was obtained from a Bausch and Lomb grating monochromator combined with blue optical filters, Hoya HA-50 (Hoya Glass) and 9782 (Corning). The fluorescence emitted on the same side of the excitation was collected by a lens, passed through an interference filter with a transmission peak at 690 nm, and detected by a photomultiplier, R-374 (Hamamatsu TV). The signal of the photomultiplier was amplified and recorded on a strip chart servo recorder (Riken Denshi, SPJ-2), or fed to a memory oscilloscope (Hitachi, V-018) and photographed.

In the study of the effects of flash illumination on the fluorescence induction, a xenon flash lamp (Ushio, UF-580) was used to obtain a short strong flash light of 10 μs duration Longer flashes were obtained from a 500-W short arc xenon lamp (Ushio, UXL-500S) combined with a mechanical shutter (Seikokoki Co.). Ultraviolet and infrared light was removed by using optical glass filters HA-50 and U-V39 (Toshiba).

RESULTS

Fig. 1 shows the effects of a 10-μs flash upon the fluorescence induction at liquid N_2 temperature. It was noted that only a part of the induction was eliminated by the flash illumination, although the flash intensity employed was strong enough to produce maximum elimination of the fluorescence induction; one tenth of this flash intensity produced the same effect. This is in marked contrast to the result obtained at room temperature where flash illumination completely eliminated the induction if 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) was present[4]. Preillumination of cooled chloroplasts with 5 successive flashes produced further suppression of the fluorescence induction.

For quantitative presentation of the effects, the magnitude of the variable component of fluorescence after preillumination, F_v, divided by that of the control experiment, F_v(control), was taken as the degree of fluorescence induction, f_v.

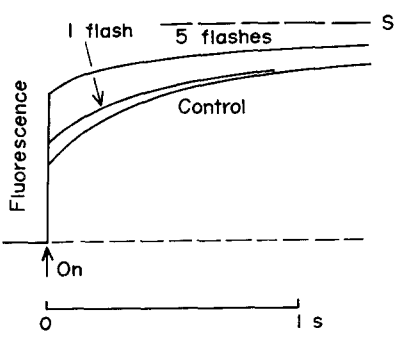
$$f_v = F_v/F_v(\text{control})$$

When there is no elimination of the fluorescence induction, f_v is equal to 100 %. When there is a complete elimination, f_v is equal to 0 %. Thus, the value for f_v changes between 0 and 100 % depending on the conditions of preillumination.

Fig. 1 indicates that after a 10-μs flash illumination f_v was 87 %, and after five repeated flashes f_v was 50 %.

Fig. 2 shows the fluorescence induction after preillumination with 17-ms, 60-ms and 1-s flashes. The elimination of fluorescence induction depended on the flash duration.

Fig. 3 shows the relationship between the flash duration and the degree of fluorescence induction. It was tested for each flash duration so that the flash intensity was high enough to produce the maximum effectiveness. The result in Fig. 3 suggests that a short flash with duration between 10 μs and 1 ms leaves 87 % of fluorescence



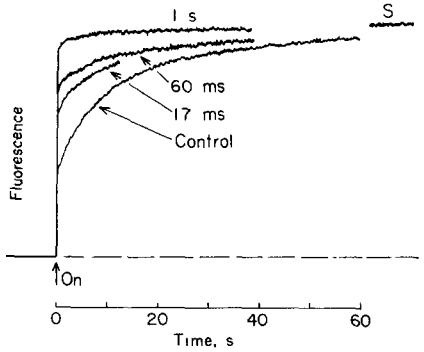


Fig. 1. Effects of a 10-μs flash on fluorescence induction at −196 °C. Chloroplasts were chilled in the dark and then illuminated with flash light. After kept in the dark for 30 s, chloroplasts were illuminated with the excitation light to measure the fluorescence induction. Excitation light, 475 nm (20-nm half-bandwidth), 450 ergs $cm^{-2} \cdot s^{-1}$. Intensity of flash light, 30 000 ergs cm^{-2} · per flash.

Fig. 2. Effects of long flashes on fluorescence induction. Experimental procedures were the same as in Fig 1. Excitation light, 475 nm (10-nm half-bandwidth), 30 ergs · $cm^{-2} \cdot s^{-1}$. Flash durations were 17 ms, 60 ms and 1 s. Intensity of flash light 500 000 ergs · $cm^{-2} \cdot s^{-1}$.

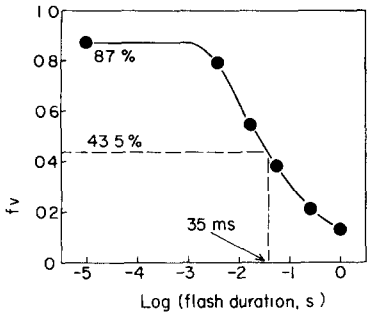


Fig. 3. Relationship between flash duration and the degree of fluorescence induction. f_v Intensity of flash light was 500 000 ergs · cm^{-2} · s^{-1} for flash durations longer than 1 ms, and 30 000 ergs · cm^{-2} per flash for a 10-μs flash. The flash duration was about 20 ms for f_v 50 %, and was about 35 ms for f_v 43.5 %.

induction. With flashes longer than 1 ms the fluorescence induction is further eliminated. The flash length for the half-elimination was about 20 ms.

The results presented in Fig. 3 may suggest that the elimination of the fluorescence induction by the flash consists of two phases; one is produced by a flash with a duration which is shorter than 1 ms. The magnitude of this phase is independent of the flash duration. This amounts to 13 % of the fluorescence induction. Another phase of elimination appears when a flash with longer than a 1-ms duration is used. This phase attains 87 % of the fluorescence induction. Since this phase depends on the flash length, a dark reaction which occurs during the flash time must contribute to the appearance of the phase. The half-elimination time for the latter phase is estimated to be 35 ms, which is the time for the degree of fluorescence induction 43.5 % (*i.e.* one-half of 87 %).

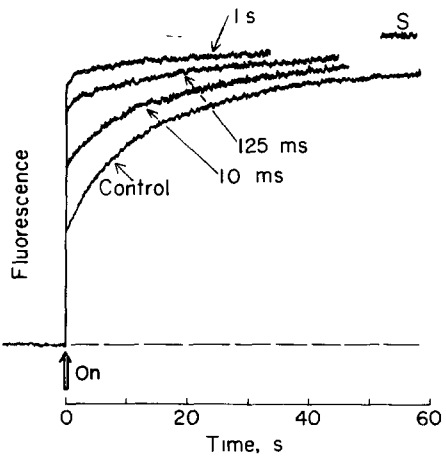
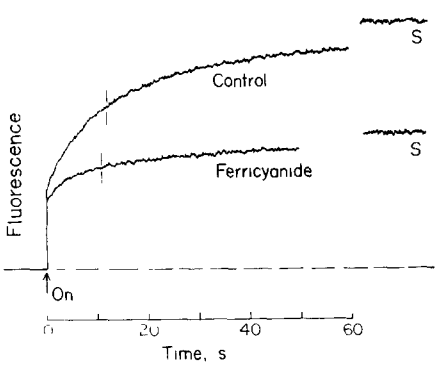


Fig. 4 Effects of 10-ms, 125-ms and 1-s flashes on fluorescence induction. Experimental procedures were the same as in Fig 1. Intensity of flashes was controlled by means of neutral density filters so that the amounts of total incident light quanta per flash remained the same, 5000 ergs cm^{-2} per flash Excitation light 475 nm (10-nm half-bandwidth) 30 ergs cm^{-2} s^{-1}.

Another flash experiment was planned in which the intensity of the flash light was controlled so that the amounts of total incident light quanta per flash were adjusted so that they were the same. Fig. 4 shows that also in this case the elimination of the fluorescence induction depended on the flash duration. This suggests that the light quanta are inefficiently used in the photochemical reaction when they are served in a short time.

Boardman and Thorne[10] first discovered the effect of ferricyanide in changing the yield of chlorophyll *a* fluorescence at liquid N_2 temperature. Okayama and Butler[11] elucidated that the fluorescence emitted from System 2 depends on the redox potential of the medium. Fig. 5 indicates that the oxidizing condition depressed the variable component but the initial component of fluorescence was only slightly depressed. At ferricyanide concentrations high enough to produce the maximum depression, there still remained a part of the variable component, the amount of which was almost equal to the initial rise component. This finding is in accordance with the observation of Okayama and Butler[11]. The remaining portion of the variable fluorescence seemed to follow a time course similar to that of the control. The half-increase time of the variable component was 12 s in the control and 11 s in the presence of 20 μM ferricyanide.



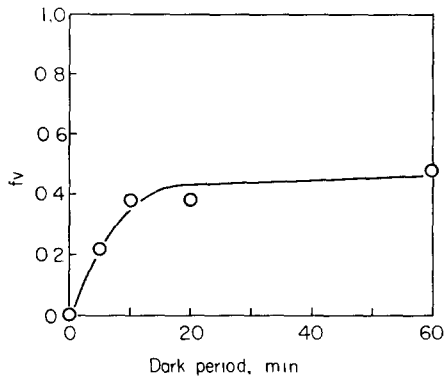


Fig 5 Effects of ferricyanide on fluorescence induction Excitation light 475 nm (10-nm half-bandwidth), 30 ergs cm^{-2} s^{-1}. Concentration of ferricyanide, if added, 20 μM. This concentration was high enough for the maximum depression of fluorescence yield

Fig. 6 Time course of dark recovery of fluorescence induction at −196 °C Chloroplasts were chilled in the dark, then illuminated with the excitation light for 60 s After the required length of dark period, the exitation light was again turned on to measure the recovery of the fluorescence induction. The extent of recovery was expressed by the degree of fluorescence induction, f_v Excitation light 475 nm (20-nm half-bandwidth), 450 ergs · cm^{-2} s^{-1}.

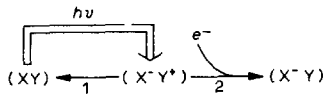
Fig. 6 shows the time course of dark recovery of the fluorescence induction. A maximum recovery of 50 % in f_v was attained after 20 min of the dark period, which remained unchanged even after 60 min of dark time. The rate of the dark recovery is very slow when compared to that at room temperature, in which 20 min is long enough for complete recovery[3].

DISCUSSION

The characteristic features of the fluorescence induction at liquid N_2 temperature are as follows: (1) The fluorescence induction is markedly retarded, as compared to that at −70 °C (refs 7–9). (2) The flash illumination only partly eliminates the fluorescence induction. (3) DCMU has little effect on the fluorescence induction[7]. (4) The steady-state level of fluorescence depends on the reduced form of cytochrome b_{559} present before the excitation[11]. (5) The dark recovery of fluorescence induction is partial and very slow. A reaction scheme is presented by which these characteristics of fluorescence induction at liquid N_2 temperature can be interpreted.

The states of reaction centers are represented as a pair of the reduced and/or oxidized form of the primary electron acceptor and donor, such as (X Y), (X^- Y) and (X^- Y^+). X and X^- are, respectively, the oxidized and reduced forms of the primary electron acceptor. Y and Y^+ are, respectively, the reduced and oxidized forms of the primary electron donor.

The following is a scheme for the reactions at liquid N_2 temperature



A light quantum absorbed by a photosynthetic unit and transferred to a reaction center in the (X Y) state converts (X Y) to (X^- Y^+). Reaction 1 is a direct recombination of positive and negative charges, and Reaction 2 is a reduction of Y^+ by a secondary electron donor

The occurrences of these dark reactions at liquid N_2 temperature are justified as in the following consideration. If Reaction 2 does not occur, it is inevitable to assume that the (X^- Y^+) state should correspond to the maximum steady-state level of the fluorescence yield. In this case, a partial elimination of fluorescence induction by the flash (Fig. 1) is possible only when Reaction 1 is rather fast. On the other hand, the very slow dark recovery of fluorescence induction (Fig. 6) is possible only when Reaction 1 is very slow Thus, in the absence of Reaction 2, no scheme can explain both these findings.

If Reaction 2 occurs at liquid N_2 temperature, the (X^- Y) state should be due to the maximum fluorescence yield. In this case a partial elimination of the fluorescence induction by the flash is possible only in the presence of Reaction 1. The slow dark recovery of fluorescence induction is also interpreted in this scheme as a very slow conversion of (X^- Y) to(X Y); however, this is not shown in the scheme.

Since DCMU, which inhibits the dark oxidation of X^- by a secondary electron acceptor[5], has no effect on the fluorescence induction at liquid N_2 temperature[7], the reaction must be blocked at the low temperature. This is the reason why the reaction is neglected in the reaction scheme.

Rate constants of the reactions and the life time of the (X^- Y^+) state can be estimated from the experimental results with short and long flashes (Fig 3), on the assumption that the degree of fluorescence induction, f_v, is equal to the fraction of the reaction centers in the (X Y) state before the onset of excitation light. On illumination with a 10-μs flash of saturation intensity all the reaction centers in the chloroplasts are converted to (X^- Y^+). In the following dark period, 87 % of reaction

centers are reconverted back to $(X\ Y)$, and 13 % to $(X^-\ Y)$. This means that the ratio of the rate constants of Reactions 1 and 2 is equal to 6 7 (*i.e.* 87/13).

A further elimination of fluorescence induction was obtained upon the longer flashes Since the intensity of long flashes is so high that the proportion of the reaction centers in the $(X\ Y)$ state is negligibly small during the flash period, the reaction occurring during the flash period is the conversion of $X^-\ Y^+)$ to $(X^-\ Y)$. At the end of the flash period, a part of the reaction centers is in the $(X^-\ Y)$ state, and the other part is in the $(X^-\ Y^+)$ state which in the subsequent dark period degrades to $(X\ Y)$ or $(X^-\ Y)$ with the ratio of 6.7 . 1. It is deduced from this analysis that the time of f_v 43.5 % (Fig. 3), *i.e.* 35 ms, corresponds to the half-time for the conversion of $(X^-\ Y^+)$ to $(X^-\ Y)$ On the assumption that the reactions are of the first order, the rate constant of Reaction 2 is estimated to be 20 s^{-1}, and thus that of Reaction 1 is 130 s^{-1}. The life time of $(X^-\ Y^+)$ estimated from these rate constants is 4.5 ms.

Reaction 1 is not a physiologically profitable process, since it is a loss of energy. The presence of this reaction at liquid N_2 temperature lowers the apparent quantum efficiency of the conversion of $(X\ Y)$ to $(X^-\ Y)$, and this will be the reason for the retardation of fluorescence induction. At room temperature Reaction 2 must be much faster than Reaction 1, and thus, Reaction 1 is practically negligible. This will correspond to the finding by Forbush and Kok[4] that a short flash completely eliminated the fluorescence induction at room temperature when the dark oxidation of X^- by the secondary electron acceptor is inhibited in the presence of DCMU. The difference in contribution of Reaction 1 at the different temperatures will be due to a much lower temperature dependence of Reaction 1 as compared to that of Reaction 2.

It should be noted that Reaction 1 is not the same as the slow back reaction that was assumed to interpret the dark recovery of fluorescence induction at room temperature in the presence of DCMU[12]. The latter reaction will probably be the reaction of $(X^-\ Y)$ to $(X\ Y)$ with oxidation of X^- by a secondary electron donor

The primary electron acceptor and donor X and Y, have not been chemically identified. A light-induced absorbance change at 682 nm designated as chlorophyll a_{II} by Doring *et al.*[13,14] and P680 by Froyde *et al.*[15] possibly corresponds to the oxidation and reduction of Y. The dark recovery time of the absorbance change at liquid N_2 temperature was reported to be 4.5 ms[15], which shows a good correspondence to the life time of Y^+ estimated in the present study.

Okayama and Butler[11] suggested in the experiment of redoxy titration of low temperature fluorescence that the secondary electron donor was cytochrome b_{559} at liquid N_2 temperature This will be explained in the above scheme to indicate that the conversion of $(X^-\ Y^+)$ to $(X^-\ Y)$ is blocked when this cytochrome is in the oxidized form. The finding by Vermeglio and Mathis[16] that a flash illumination oxidized only a part of cytochrome b_{559} at liquid N_2 temperature is compatible with the above-mentioned scheme. The fact that even with a saturating amount of ferricyanide a part of the variable fluorescence component remained suggests the presence of another secondary electron donor. A probable scheme is that a part of the reaction centers is linked to cytochrome b_{559}, and the other part to the unknown electron donor.

The primary electron acceptor, X, corresponds to the Q of Duysens and Sweers[5]. However, whether it is equal to C550 (ref 17), a light-induced absorbance

change at 550 nm, is still obscure

Thorne and Boardman[8] have inferred that the retardation of fluorescence induction is due to a lowered quantum efficiency of the photochemical reaction at liquid N_2 temperature. This idea is in appearance similar to that proposed in this study, in which, however, the quantum efficiency of the photochemical reaction itself is not affected by the cooling.

Butler *et al* [18,19] tried to explain the retardation of fluorescence induction at liquid N_2 temperature by a model in which the fluorescence was quenched not only by X but also by Y^+. A recent personal communication of Butler reports that he has further improved the model and has reached a scheme which is similar to ours

ADDENDUM

During revision of this article, a paper by Joliot and Joliot[20] was published, in which they studied the effects of flash illumination on the flurorescence induction -40 to -70 °C. They introduced a new quencher in addition to the Q of Duysens and Sweers[5] to interpret the finding that the flash illumination could not completely eliminate the fluorescence induction at these temperatures. If the reaction scheme including the back reaction of the photochemical reaction as we are proposing here is employed, it does not seem necessary to assume this type of two-quencher model. The oscillation of fluorescence yield in their data after repeated flashes is also explained, if the temperature dependences of the reaction between the primary and the secondary electron donors vary depending on the state of the secondary electron donor

ACKNOWLEDGEMENTS

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REFERENCES

1 Kautsky, H . Appel, W. and Amann, H. (1960) *Biochem. Z.* 332, 277–292
2 Murata, N , Nishimura, M. and Takamiya, A (1966) *Biochim. Biophys Acta* 120, 23–33
3 Malkin, S and Kok, B. (1966) *Biochim. Biophys Acta* 126, 413–432
4 Forbush, B and Kok, B. (1968) *Biochim Biophys. Acta* 162, 243–253
5 Duysens, L N. M. and Sweers, H E. (1963) in *Studies on Microalgae and Photosynthetic Bacteria* (Japanese Society of Plant Physiologists, eds), pp 353–372, University of Tokyo Press, Tokyo
6 Butler, W L and Bishop, N. I. (1963) in *Photosynthetic Mechanisms of Green Plants* (Kok, B. and Jagendorf, A. T., eds), pp. 91–100, National Academy of Sciences, Washington, D C.
7 Murata, N (1968) *Biochim Biophys. Acta* 162, 106–121
8 Thorne, S. W and Boardman, N. K (1971) *Biochim. Biophys Acta* 234, 113–125
9 Malkin, S. and Michaeli, G. (1972) in *Proceedings of the 2nd International Congress on Photosynthesis Research* (Forti, G., Avron, M and Melandri, A. eds), pp 149–168, Dr W. Junk N V. Publishers, The Hague
10 Boardman, N K. and Thorne, S W. (1969) *Biochim Biophys Acta* 189, 294–297
11 Okayama, S and Butler, W. L (1972) *Biochim Biophys. Acta* 267, 523–529
12 Bennoun, P (1970) *Biochim Biophys Acta* 216, 357–363
13 Döring, G., Stiehl, H H and Witt, H. T. (1967) *Z. Naturforsch.* B22, 639–644

14 Doring, G., Renger, G., Vater, S. and Witt, H. T. (1969) *Z. Naturforsch.* B24, 1139–1143
15 Froyde, R A., Chance, B. and DeVault, D (1971) *Biochim Biophys. Acta* 226, 103–112
16 Vermeglio, A. and Mathis, P. (1973) *Biochim. Biophys. Acta* 292, 763–771
17 Knaff, D. B and Arnon, D. I. (1969) *Proc. Natl. Acad. Sci. U S.* 63, 963–969
18 Butler, W. L., Visser, J. W. M. and Simons, H. L. (1973) *Biochim. Biophys. Acta* 292, 140–151
19 Butler, W. L. (1972) *Proc. Natl. Acad. Sci. U.S* 69, 3420–3422
20 Joliot, P. and Joliot, A. (1973) *Biochim. Biophys. Acta* 305, 302–316