BBA 45693

FLUORESCENCE OF CHLOROPHYLL IN PHOTOSYNTHETIC SYSTEMS

IV. INDUCTION OF VARIOUS EMISSIONS AT LOW TEMPERATURES

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(Received February 27th, 1968)

SUMMARY

The time course of fluorescence was measured at −196° in spinach chloroplasts, *Chlamydomonas reinhardi*, *Anacystis nidulans*, *Porphyridium cruentum* and *Porphyra yezoensis* and also at −70° in spinach chloroplasts. The three emissions, F684, F695 and F-l observed at −196° showed induction. The fluorescence intensities rose rapidly at the onset of illumination and then increased gradually to reach the final steady level. The induction of F695 was more marked than F684, as estimated by the relative magnitudes of the transient component of fluorescence to the total or by the values for "work integral". The kinetic analysis leads to the conclusion that C_{f695} (pigment emitting F695) is linked to photoreaction II, and that there is a return transfer of excitation energy from C_{f695} to C_{f684} (pigment emitting F684). In spinach chloroplasts, the value of the "work integral" of F695 at −196° was almost equal to that of F684 at room temperature, indicating that the size of the electron pool was not significantly changed by cooling. It was concluded, from the analysis of the behavior of F-l, that in the blue-green and red algae, on excitation of pigment system II, F-l is mainly excited through the excitation transfer from C_{f684} to C_{f-1}, and that in spinach chloroplasts and *C. reinhardi*, the transfer is less significant.

INTRODUCTION

The fluorescence spectra of photosynthetic pigments *in vivo*, as well as in subchloroplast particles obtained by detergent treatment, have been studied at liquid-nitrogen temperature[1–8]. Three forms of (probably) chlorophyll *a*, C_{f684}, C_{f695} and C_{f-1}, have been characterized by the emission of fluorescence F684, F695 and F-l, respectively, with peaks at 684 mμ, 695 mμ and 715–740 mμ. The relationship of these forms of chlorophyll *a* to the pigment systems of photosynthesis has been investigated on the basis of the action spectra for the three emissions[3] and the emission spectra of the fractionated subchloroplast particles[6–8]. It has been confirmed that C_{f684} and C_{f695} belong to pigment system II, whereas C_{f-1} seems to be contained in pigment system I.

Abbreviations: CMU, 3-(4′-chlorophenyl)-1,1-dimethylurea; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; BIMU, 5-bromo-3-isopropyl-6-methyluracil.

The induction of fluorescence of chlorophyll *a* in chloroplasts and algae[9–13] has been studied at room temperature, and a correlation has been established between the intensity of fluorescence and the rate of photoreaction II. These studies have supplied much information concerning the nature of photoreaction II and the electron flow in photosynthesis.

In our previous study[9], we introduced a quantity, the "work integral", which is defined as follows:

$$KI_a \int_0^s (F_s - F(t))\, dt/F_s$$

K is the fraction of absorbed quanta transferred to, or directly absorbed by, the fluorescent chlorophyll *a*. I_a is the amount of absorbed quanta. $F(t)$ is the fluorescence intensity at time t after onset of illumination, and F_s is the intensity at the steady state of fluorescence reached after sufficient time of illumination. The work integral can be calculated from the time course of fluorescence. Theory has shown that the work integral represents the number of electrons carried over through photoreaction II during the induction period. It furnishes, therefore, a method for estimating the amount, or pool size, of the electron carriers in the electron transport chain under the given experimental conditions. MALKIN AND KOK[10] and MALKIN[11] independently worked out a similar hypothesis and obtained a value of 0.65 for K in spinach chloroplasts[10].

Another measure for comparing the magnitude of induction of fluorescence is the ratio of initial (F_1) and transient (F_t) components of fluorescence[13]. The values of the ratio, $F_t/(F_1 + F_t)$, obtained under various experimental conditions were compared. The theoretical consideration, as will be described in detail (see below), shows that this quantity is highly useful as a tool for analyzing the sequence of energy transfer and photochemistry in the photosynthetic mechanism. In the present study this method was also utilized in analyzing induction of fluorescence at −196° with special reference to the problem of whether $C_{f_{684}}$ or $C_{f_{695}}$ is directly connected with photoreaction II (*i.e.*, photochemical step of system II.)

METHODS

Spinach chloroplasts were prepared as usual in a sucrose–phosphate medium (0.4 M sucrose, 0.05 M phosphate, 0.01 M NaCl, pH 7.8), washed once and resuspended in the same medium. Algal cells and thalli were cultured autotrophically. *Anacystis nidulans* were grown in KRATZ–MYERS' C medium[14] at 37° under illumination with 4000-lux incandescent lamp light with or without a red cut-off filter (50% transmittance at 650 mμ). Following JONES AND MYERS[15], the cells cultured in white light and red light will be designated as W-cells and R-cells. The ratio of the contents of chlorophyll *a* and phycocyanin was calculated on an absorbance basis according to the method of JONES AND MYERS[15]. W-cells contained almost the same amounts of chlorophyll *a* and phycocyanin, and in R-cells the amount of chlorophyll *a* was one-quarter that of phycocyanin. *Porphyridium cruentum* was grown in KOCH'S[16] medium at 25° under illumination with 2000-lux incandescent lamp light. *Chlamydomonas reinhardi* was grown in SAGER–GRANICK'S[17] medium at 25° under illumination of 4000-lux fluorescent lamp light. *Porphyra yezoensis* was grown in synthetic sea water

under fluorescent lamp light. This material was supplied by the courtesy of Yamamoto Nori Research Laboratory.

Young cells of unicellular algae were collected by centrifugation and suspended in the respective culture media. The concentration of the chloroplasts or algal cells in the sample to be measured was controlled to absorb less than 20 % of the incident light at the absorption peak. The cuvette was 2 mm in thickness and 2 cm × 2 cm in surface area. The sample was cooled in the dark to —70° or —196° with a dry ice–alcohol mixture or liquid N_2, respectively.

The excitation light was furnished by a Bausch and Lomb grating monochromator (20-mμ bandwidth) equipped with an infrared-absorbing filter, IRO-1A (Toshiba), or by a combination of two blue filters, V-B46 (Toshiba) and B460 (Hoya Glass). The transmittance of the filter combination was 32 % at 470 mμ (peak), 16 % at 415 mμ and 515 mμ and lower than 1 % at wavelengths longer than 580 mμ. The source of the excitation light was a tungsten lamp. The fluorescence from the same side which was illuminated was analyzed, using another Bausch and Lomb grating monochromator equipped with a red cut-off filter, V-R65 (Toshiba). The fluorescence intensity was detected by a photomultiplier, 7102 (RCA) (spectral response, S-1) cooled with solid CO_2 or R136 (Hamamatsu TV) (spectral response, S-10) operating at room temperature. The signal was amplified and recorded on a strip chart servo recorder (response time, approx. 0.2 sec), or traced on an oscilloscope and photographed. The emission spectra of fluorescence were measured by the method described previously[3]. The fluorescence spectra were not corrected for the spectral sensitivity of the equipment used, except as otherwise stated.

RESULTS

The fluorescence of chlorophyll *a* in spinach chloroplasts was found to exhibit induction at a temperature as low as —196°, a finding which indicates that even at this low temperature there occurs a photoreaction connected with the fluorescent forms of chlorophyll *a* in question. Fig. 1 shows the time courses of fluorescence measured at this temperature at the wavelengths 684 mμ, 695 mμ and 735 mμ, corresponding to the peaks of the three emissions, F684, F695 and F-l. After the onset of illumination, the fluorescence intensity showed an instantaneous rise, followed by a gradual increase to reach a final steady level. We will designate the first part as the initial rise component of fluorescence, F_1, and the second as the transient component of fluorescence, F_t. The induction of F-l was found to be less marked than that of the other two emissions. As shown in Fig. 1, the intensities of F684 and F695 at —196° increased faster at the commencement of the transient phase immediately following the initial rise, but took a longer time to reach the steady level as compared with that of F684 at room temperature.

In order to compare the time courses of the three emissions, the early part of the transient phase was measured with an oscilloscope. In some of these experiments, the time courses of F684 and F695 were measured at 682 mμ and 698 mμ, respectively, so as to diminish the mutual overlapping of F684 and F 695. The transient components of fluorescence of the three emissions exhibited similar time courses. The time required for fluorescence to rise to the half-level of F_t (half-rise time) was measured. It was

identical for F684, F695 and F-1 (Table I). The relationship of the three emissions in half-rise time did not change with the intensity of the excitation light.

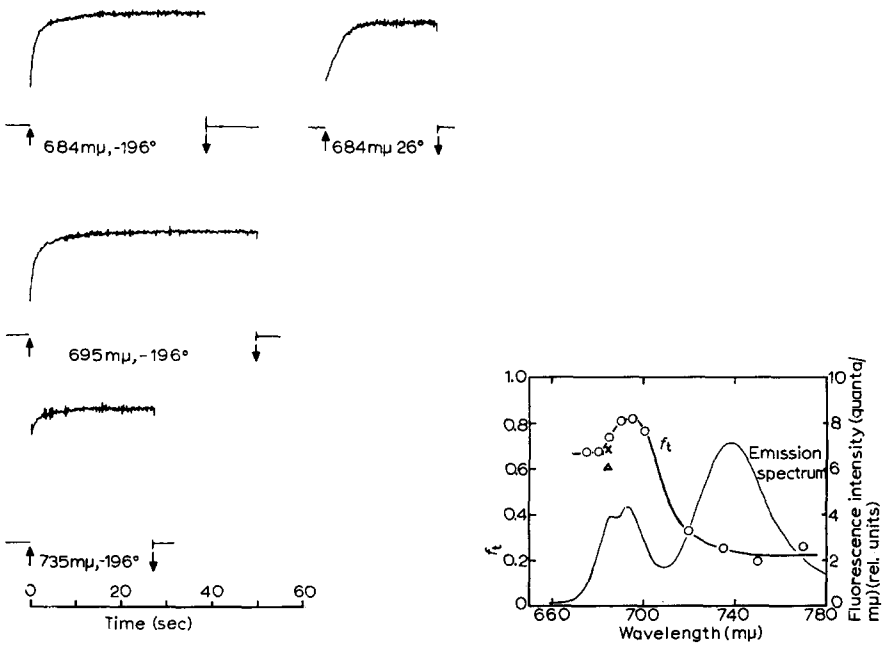


Fig. 1. Time courses of fluorescence in spinach chloroplasts at −196° and 26°. Excitation light, 475 mμ, 0.37 nEinstein/cm²·sec. Fluorescence was measured at 684 mμ, 695 mμ and 735 mμ with a 7.5-mμ band width at −196°, and at 684 mμ with a 15-mμ band width at 26°.

Fig. 2. Relative magnitudes of transient components of fluorescence, $f_t = F_t/(F_1 + F_t)$, at various wavelengths in spinach chloroplasts at −196°, −70° and 18°. Excitation light, 475 mμ, 0.37 nEinstein/cm²·sec. Fluorescence was measured with a 10-mμ band width. The tracing of the time course of fluorescence on the oscilloscope was photographed, and F_1 and F_t were measured. Steady level of fluorescence was measured 5 min after onset of each illumination. (1) f_t; o, −196°; △, −70°; ×, 18°. (2) Thin line represents emission spectrum of spinach chloroplasts at −196°. The spectrum is corrected for the spectral sensitivity of the equipment.

TABLE I

TIME REQUIRED FOR FLUORESCENCE TO RISE TO HALF LEVEL OF TRANSIENT FLUORESCENCE, HALF-RISE TIME ($t_{1/2}$), IN F684, F695 AND F-1 IN SPINACH CHLOROPLASTS AT −196°

Excitation light	*Light intensity (nEinstein/cm²·sec)*	*Fluorescence measurement*			$t_{1/2}$ *(sec)*
		Emissions	*λ (mμ)*	*Band width (mμ)*	
475 mμ	0.23	F684	684	7.5	0.4
		F695	695	7.5	0.5
		F-1	735	7.5	0.4
470 mμ*	2.4	F684	682	5.0	0.08
		F695	698	5.0	0.08
		F-1	735	5.0	0.08

* Blue light with filter combination (see METHODS).

For the purpose of obtaining an appropriate measure for comparing the magnitude of induction for each emission, the initial rise component and the transient component of fluorescence were measured at —196° at various wavelengths from the oscilloscope traces of fluorescence intensity. Fig. 2 shows the wavelength dependence of relative magnitude of F_t to the total, *i.e.*, $F_t/(F_1 + F_t)$. We will designate $F_t/(F_1 + F_t)$ as f_t. Considering the mutual overlapping of F684 and F695, f_t is approx. 0.85 for F695 and 0.65 for F684.

As will be seen in Fig. 2, essentially similar values of f_t were obtained throughout the wavelength range from 740 to 770 mμ. Since at 770 mμ there is no significant overlapping of the satellite bands of F684 and F695, the value of f_t, approx. 0.2, obtained at this wavelength obviously represents the real induction of F-1. The same value of f_t obtained at 740 mμ, therefore, indicates that the induction measured at this wavelength is not a mere artifact due to the overlapping of the satellite bands of F684 and F695, but, in fact, reflects the induction of F-1 itself.

Another fact to be noticed in Fig. 2 is that the values of f_t for F684 and F695 at —196° (0.65 and 0.85, respectively) were comparable to that obtained for F684 at room temperature (0.70).

The values for work integrals at room and liquid-N_2 temperatures are shown in Table II. The values measured at 684 mμ and 735 mμ were, respectively, four-fifths and one-third that obtained at 695 mμ, as expected from f_t's and the identity of time courses of F_t of the three emissions. The ratio of the values for F684, F695 and F-1 remained almost the same throughout the three experiments as shown in Table II. The values for work integrals of F684 and F695 were comparable to that of F684 at room temperature (0.05 equiv/chlorophyll *a*, assuming $K = 0.65$ according to MALKIN AND KOK[10]). These findings indicate that the size of electron pool associated with photoreaction II is not significantly changed by cooling to —196°. However, in calculating the values for the work integrals at 26° and at —196° in Table II, the values for K and I_a at room temperature were used. If I_a and K change on cooling,

TABLE II

VALUES FOR WORK INTEGRAL OF FLUORESCENCE, $KI_a\int_0^S(F_s - F(t))dt/F_s$, IN SPINACH CHLOROPLASTS MEASURED AT 684 mμ, 695 mμ AND 735 mμ.

Band width of measurement, 10 mμ. Values are presented in relative units, the value at 695 mμ in each experiment taken as 100. Throughout the calculation of work integral, values for I_a and K at room temperature were used. Excitation light, 475 mμ. The three experiments were performed with different chloroplast samples.

Expt. No.	*Excitation light intensity (nEinstein/cm²·sec)*	*Work integral (rel. units)*			
		—196°			26°
		684 mμ	*695 mμ*	*735 mμ*	*684 mμ*
1	0.37	79	100	30	83*
2	0.37	80	100	41	112
3	0.23	77	100	27	128
Av.	—	79	100	33	107

* The absolute value for the work integral at 26° measured in this experiment was 0.05 equiv/chlorophyll *a* on $K = 0.65$ according to MALKIN AND KOK[10].

the values thus obtained for the work integrals at —196° would deviate somewhat from their true values.

To relate the induction phenomena to the two photoreactions, a pre-illumination experiment was designed at —196°. Prior to the illumination with excitation light, chloroplasts were pre-illuminated with 710-mμ light absorbed by pigment system I. The duration of pre-illumination was made sufficiently long so that the number of quanta absorbed in the pre-illumination time would be comparable to the number of chlorophyll *a* molecules in the chloroplasts. This pre-treatment did not significantly affect the induction of F684 and F695 or that of F-l, on subsequent illumination with 475-mμ light. This fact indicates that the induction of F-l, as well as of F684 and F695, is mainly caused by the change in rate of photoreaction II during the induction period.

Heat treatment of the chloroplasts eliminated the induction as shown in Fig. 3. It affected the time courses in a similar manner in F684, F695 and F-l; the treatment at 45° for 5 min was found to be critical for all three emissions. This treatment has also been shown to be critical in destroying photoreaction II in spinach chloroplasts[9]. The present experimental result also confirmed the above-mentioned involvement of photoreaction II in the induction of F-l in spinach chloroplasts. The fluorescence spectrum was not altered by this heat treatment of the chloroplasts.

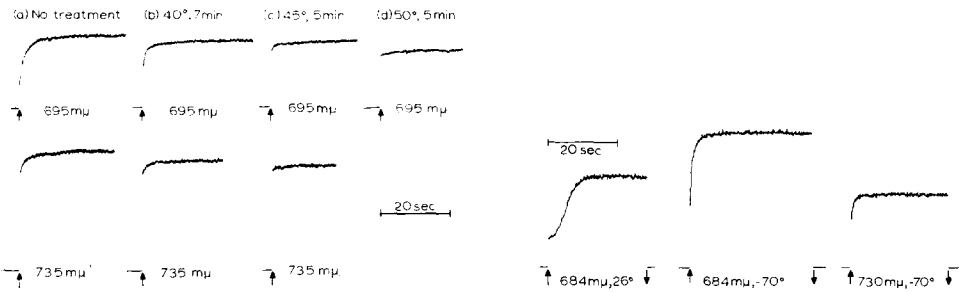


Fig. 3. Effect of heat treatment of spinach chloroplasts on time courses of fluorescence at —196°. Fluorescence was measured at 695 mμ and 735 mμ with a 10-mμ band width. Excitation light, 475 mμ, 0.23 nEinstein/cm^2·sec. Treatments: (a) no treatment, (b) 40°, 7 min, (c) 45°, 5 min, (d) 50°, 5 min.

Fig. 4. Time courses of fluorescence in spinach chloroplasts at 26° and —70°. Excitation light, 475 mμ, 0.37 nEinstein/cm^2·sec. Fluorescence was measured with a 15-mμ band width.

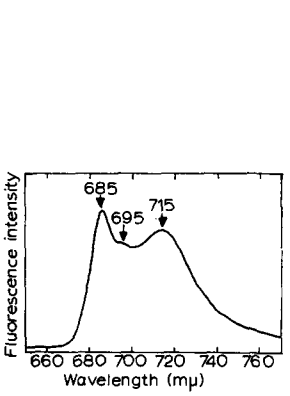
Dithionite at a concentration of 10 mM eliminated the induction of the three emissions at —196°. The work integral and f_t decreased markedly. The level of the steady state, however, was not significantly affected. The same result was obtained at room temperature. These findings suggest that the intensities of the emissions at —196° are controlled by the oxidation–reduction state of the primary electron acceptor of photoreaction II.

The effects of photosynthetic inhibitors, 3-(4′-chlorophenyl)-1,1-dimethylurea (CMU) and 5-bromo-3-isopropyl-6-methyluracil (BIMU), on the induction of fluorescence at —196° were also investigated. At the concentration of 10^{-5} M, these inhibitors lowered the steady level of fluorescence, but had little effect on the induction pattern of fluorescence at —196° nor did they change the value of the work

integral. These findings are in clear contrast to their behavior at room temperature, at which the work integral is decreased to about one-tenth by their addition.

The induction of fluorescence was also observed in spinach chloroplasts at —70° (Fig. 4). At this temperature, F695 was not observed[3]. While F-l was observed, it was not so prominent as at —196° (see Fig. 2 in ref. 3). As shown in Fig. 4, fluorescence measured at 684 mμ and 730 mμ showed induction at this temperature. f_t of F684 was little changed by cooling to —70° (see Fig. 2). On the other hand, the work integral for F684 was reduced to one-quarter.

Fig. 5 shows the fluorescence spectrum of *C. reinhardi* at —196°. Three emission bands were observed at 685 mμ, 695 mμ and 715 mμ. In this alga, F695 was relatively insignificant compared with the other two emissions. Therefore, the time course of F684 could be measured without significant overlapping of F695. The time courses of fluorescence measured at 684 mμ and 715 mμ showed induction. f_t was 0.45 and 0.10 at 684 mμ and 715 mμ, respectively. The work integral for F684 was approx. 0.15 equiv/chlorophyll *a* at —196°, assuming $K = 0.65$. It could not be determined whether the less significant induction observed at 715 mμ was due to that of F684; the satellite band of F684 would overlap at this wavelength. At least, it is clear that the induction of F-l, if present, is much less marked than that of F684.



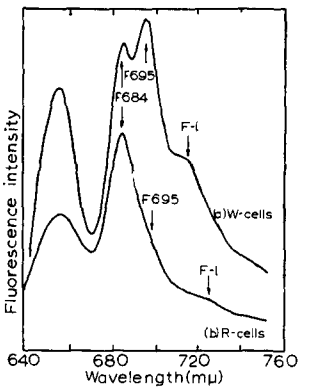


Fig. 5. Emission spectrum of fluorescence in *C. reinhardi* at —196°. Excitation, blue light with 470-mμ peak. Fluorescence was measured with a 5-mμ band width.

Fig. 6. Emission spectra at —196° in R-cells and W-cells of *A. nidulans*. Excitation light, 600 mμ. Fluorescence was measured with a 5-mμ band width. (a) W-cells, (b) R-cells.

In another group of algae containing phycobilins, the induction of fluorescence was also investigated at —196°. Fig. 6 shows the emission spectra in W-cells and R-cells of *A. nidulans* at —196° obtained on excitation of phycocyanin. F684, F695 and F-l were clearly seen in W-cells, while F695 and F-l were much weaker than F684 in R-cells. In W-cells, on excitation of chlorophyll *a*, F-l was much stronger than the other emissions (see Fig. 3 in ref. 3).

The time courses of fluorescence in W-cells of *A. nidulans* at —196° on excitation of phycocyanin were measured at 655 mμ, 682–684 mμ, 695 mμ and 715–720 mμ, corresponding to the fluorescence of allophycocyanin, F684, F695 and F-l, respectively. F684, F695 and F-l showed induction, while the emission from allophycocyanin did not. To compare the time courses of transient fluorescence, F_t, of the three

emissions, the time required for fluorescence to attain the half-level of the total transient fluorescence (half-rise time) was measured for the three emissions on excitation of phycocyanin. As seen in Table III, the half-rise time was the same in F684 and F695 within the limits of experimental error. The half-rise time of F-1, however, is shorter than those of F684 and F695. f_t was largest in F695; f_t was 0.26 for F695, 0.14 for F684 and 0.20 for F-1 (Table III). The work integral was 0.05 equiv/chlorophyll *a* for F695, assuming $K = 0.85$, according to the efficiency of excitation transfer in *A. nidulans* measured by TOMITA AND RABINOWITCH[18].

TABLE III

HALF-RISE TIME OF F_t ($t_{1/2}$) AND RELATIVE MAGNITUDE OF F_t TO THE TOTAL (f_t) AT −196° IN *A. nidulans*

Fluorescence was measured with a 5-mμ band width. *Expt. 1*: W-cells; excitation light, 620 mμ, 0.31 nEinstein/cm²·sec. *Expt. 2*: W-cells; excitation light, 600 mμ, 0.47 nEinstein/cm²·sec. *Expt. 3*: W-cells; excitation light, 435 mμ, 0.12 nEinstein/cm²·sec. *Expt. 4*: R-cells; excitation light, 580 mμ, 0.61 nEinstein/cm²·sec.

	λ (mμ)				$t_{1/2}$ (sec)				f_t			
Expt. No:	1	2	3	4	1	2	3	4	1	2	3	4
F684	684	682	—	684	2.1	1.8	—	—	0.16	0.12	—	0.16
F695	695	695	—	—	2.0	1.7	—	—	0.25	0.26	—	—
F-1	715	720	715	—	1.3	1.1	0.8	—	0.21	0.20	0.28	—

The induction of F-1 was also observed on excitation of chlorophyll *a*. f_t of F- was 0.28, and the work integral was 0.015 equiv/chlorophyll *a*, tentatively assuming $K = 1$. This induction must be caused by the change of rate of photoreaction I, because the light absorbed by chlorophyll *a* is mainly transferred to pigment system I in this alga. The half-rise time was 0.8 sec. Comparing the intensities of excitation light in the three experiments, the induction of F-1 caused by photoreaction I is expected to have a much shorter half-rise time than the induction caused by photoreaction II, if the same intensity of excitation light is used.

Also in this alga the addition of CMU (10^{-5} M) had little effect on the induction at this temperature.

There remains a possibility that F684 did not exhibit induction and that the induction of fluorescence observed at 682–684 mμ was due to overlapping of F695 at this wavelength. A study of the induction of F684 in R-cells of *A. nidulans*, in which F695 is much weaker than F684, would supply information concerning this point. F684 exhibited induction also in this alga at −196°; f_t was 0.16 (Table III). The value coincided with that of F684 in the W-cells measured at 682–684 mμ. Therefore, it can be concluded that, in *A. nidulans*, F684 and F695 both show induction with the same half-rise time of F_t, and that the induction of F695 is more marked than that of F684, as shown by the larger value of f_t of F695.

The time course of fluorescence at −196° was also measured in *P. cruentum* on excitation of phycoerythrin by 570-mμ light. The emission spectrum of this alga at −196° is shown in Fig. 4 of ref. 3. The time course of F684 could not be measured because this emission was much weaker than F695 and F-1. As previously observed

at room temperature[13], the fluorescence of allophycocyanin exhibited no induction, while F695 and F-l measured at 695 mμ and 720 mμ showed induction. f_t was 0.55 for both F695 and F-l. The values for the work integral were approx. 0.11 and 0.10 equiv/chlorophyll *a* for F695 and F-l, respectively, assuming $K = 0.75$, according to the results obtained by TOMITA AND RABINOWITCH[18]. On excitation of chlorophyll *a* (440-mμ light), F-l showed a small induction. Pre-illumination experiments were designed to elucidate the cause of the induction. Fig. 7 shows the results of these experiments. The time courses of F695 and F-l were measured at 690 mμ and 720 mμ on excitation of phycoerythrin (570-mμ light) after pre-illumination of chlorophyll *a* or phycoerythrin with 440-mμ or 570-mμ light. The intensities of pre-illumination light were selected so that the quantity of the quanta absorbed during the pre-illumination time should be almost the same. As clearly seen in Fig. 7, the different effects of the two kinds of light were clear; while the pre-illumination of 570-mμ light eliminated the induction of both F695 and F-l, 440-mμ light only decreased the induction of both. It can be concluded that also in this alga, the induction of F-l and F695 on excitation of pigment system II is caused mainly by photoreaction II.

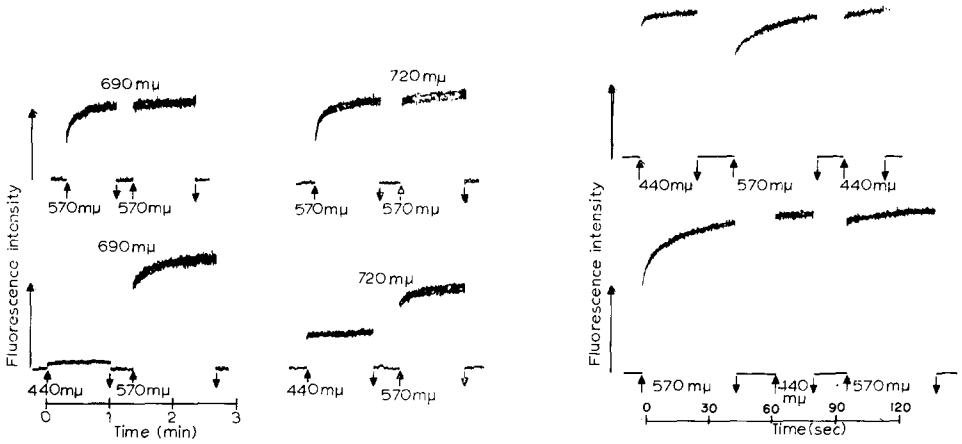


Fig. 7. Time courses of F695 and F-l in *P. cruentum* at −196°. Excitation light, 570 mμ, 0.19 nEinstein/cm²·sec and 440 mμ, 0.15 nEinstein/cm²·sec. Fluorescence was measured at 690 mμ and 720 mμ with 5-mμ band width.

Fig. 8. Time courses of F-l in *P. yezoensis* at −196°. Excitation light, 570 mμ, 0.28 nEinstein/cm²·sec and 440 mμ, 0.20 nEinstein/cm²·sec. Fluorescence was measured at 730 mμ with a 10-mμ band width.

The same relationship between the induction of F-l and photoreaction II was also observed in *P. yezoensis*. In this alga on excitation of phycoerythrin, F-l is much more intense than the other two emissions (see Fig. 5 of ref. 3). The induction of F-l was much more marked on excitation with 570-mμ light than on excitation with 440-mμ light (Fig. 8). The work integral of F-l was calculated to be about 0.13 equiv/chlorophyll *a* for excitation with 570-mμ light, assuming $K = 0.75$, and about 0.006 equiv/chlorophyll *a* for 440-mμ excitation light, assuming $K = 1$. Fig. 8 shows the time course of F-l measured at 730 mμ on successive excitation of pigment system I with 440-mμ light and pigment system II with 570-mμ light. The intensities of 440-mμ light and 570-mμ light were controlled to give almost the same intensity of F-l

at the steady level. The pre-illumination of 440-mμ light did not eliminate the induction with excitation of 570-mμ light. On the contrary, the pre-illumination of 570-mμ light eliminated the induction with excitation of 440-mμ light. This fact suggests that, also in this alga, the induction of F-l at —196° is caused mainly by the change in rate of photoreaction II.

DISCUSSION

In previous studies on fluorescence of chlorophyll in various plants and in subchloroplasts particles, it has been concluded that $C_{f_{684}}$ and $C_{f_{695}}$ are contained in pigment system II (refs. 3–8). The induction of F684 at room temperature has been fully explained by the assumption that $C_{f_{684}}$ is connected with photoreaction II (refs. 9–13). In addition, GOVINDJEE AND YANG[4] and KREY AND GOVINDJEE[5] have suggested that $C_{f_{695}}$ might be the energy trap of pigment system II. In the present study, we investigated the kinetics of induction of fluorescence at —196° to elucidate the mechanism by which $C_{f_{684}}$ and $C_{f_{695}}$ are connected with photoreaction II.

The most pertinent points in the kinetic behavior of F684 and F695 described above are as follows: (1) the time courses of the transient components of F684 and F695 are the same; (2) f_t of F684 is smaller than f_t of F695.

In the following analysis, two models connecting photoreaction II and excitation transfer in pigment system II are compared (Fig. 9).

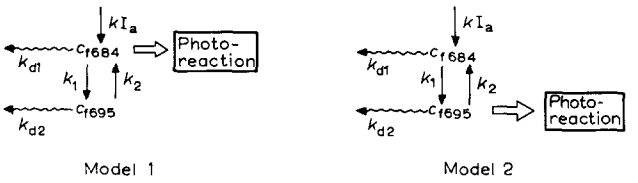


Fig. 9. Models for excitation transfer in pigment system II.

The following symbols and notations are used: k_1: rate constant of excitation transfer from excited $C_{f_{684}}$ to $C_{f_{695}}$; k_2: rate constant of excitation transfer from excited $C_{f_{695}}$ to $C_{f_{684}}$; k_{d_1} and k_{d_2}: rate constants of radiative and radiationless degradation of excited molecules of $C_{f_{684}}$ and $C_{f_{695}}$, respectively; r: fraction of the active reactant to the total, *i.e.*, (fraction of oxidized form to the total primary electron acceptor) $\times$ (fraction of reduced form to the total primary electron donor); k_r: rate constant of the photoreaction II. The rate of photoreaction II can be presented as $k_r f(r)$; $F_1(r)$ and $F_2(r)$: probabilities with which the absorbed quanta are emitted as fluorescence from $C_{f_{684}}$ and $C_{f_{695}}$, respectively; $V(r)$: probability with which the absorbed quanta are used for photoreaction II; c_1 and c_2: ratios of rate constants of radiative *versus* total degradation for $C_{f_{684}}$ and $C_{f_{695}}$, respectively.

In Model I, $C_{f_{684}}$ is directly connected with photoreaction II, whereas in Model II, $C_{f_{695}}$ is directly connected with photoreaction II. In both models it is postulated that $C_{f_{695}}$ is excited only through excitation transfer from $C_{f_{684}}$, and that there occurs a return transfer of excitation from $C_{f_{695}}$ to $C_{f_{684}}$. The return transfer is essential in Model II for the presence of induction of F684; *i.e.*, if it were omitted from Model II, there would be no induction in F684, as is actually the case in the fluorescence of phycobilins[13].

The fundamental concept common to both models in correlating the fluorescence intensity with the photochemical process is that one exciton is consumed in carrying one electron through the photochemical process. The primary electron acceptor and donor participating in this photochemical process, therefore, act to quench the fluorescence. The general equation for the rate of photoreaction will be a function of their concentrations.

In addition, there have been two alternative concepts concerning the excitation transfer between the "units" in pigment system II. MALKIN AND KOK[10], in their analysis of the time course of fluorescence, used a model in which there is no excitation transfer between the units and, therefore, the rate of photoreaction is proportional to the active fraction of the reactant. In another model proposed by JOLIOT[19], there is an excitation transfer between the units, and the rate of photoreaction is a rather complicated function of the active fraction of the reactant. In JOLIOT's formulation, the rate of the photochemical process is expressed as some function of the active fraction of the reactant, r. However, for our present discussion, consideration of the following two circumstances suffices.

At the onset of illumination, r and $f(r)$ are equal to unity and the rate of the photochemical process, $k_r f(r)$, will be maximum and equal to k_r. After a sufficient time of illumination, when a steady level of fluorescence has been reached, r and $f(r)$ are equal to zero and the rate of the photochemical process, $k_r f(r)$, will be zero. During the induction period, r and $f(r)$ decrease with time from unity to zero, and $k_r f(r)$, from k_r to zero.

Model I

In Model I, $F_1(r)$ and $F_2(r)$ will be obtained as functions of r as follows:

$$F_1(r) = c_1 K I_a \frac{k_{d1}}{k_1 + k_{d1} + k_r f(r)} \left\{ 1 + \frac{k_1}{k_1 + k_{d1} + k_r f(r)} \cdot \frac{k_2}{k_2 + k_{d2}} + \ldots + \left(\frac{k_1}{k_1 + k_{d1} + k_r f(r)} \cdot \frac{k_2}{k_2 + k_{d2}} \right)^n + \ldots \right\}$$

$$F_1(r) = c_1 K I_a \frac{k_{d1}}{k_1 + k_{d1} + k_r f(r)} \cdot \frac{1}{1 - \dfrac{k_1}{k_1 + k_{d1} + k_r f(r)} \cdot \dfrac{k_2}{k_2 + k_{d2}}} \tag{1}$$

$$F_2(r) = c_2 K I_a \frac{k_{d2}}{k_2 + k_{d2}} \cdot \frac{k_1}{k_1 + k_{d1} + k_r f(r)} \cdot \frac{1}{1 - \dfrac{k_1}{k_1 + k_{d1} + k_r f(r)} \cdot \dfrac{k_2}{k_2 + k_{d2}}} \tag{2}$$

From Eqns. 1 and 2, the following equation is derived:

$$\frac{F_1(r)/F_1(r = 0)}{F_2(r)/F_2(r = 0)} = 1 \tag{3}$$

In Model I, therefore, f_t of $F_1(r)$ and $F_2(r)$ should be the same. This does not fit the relationship observed between the time courses of F684 and F695 in chloroplasts and in *A. nidulans*, in which f_t of F684 was always smaller than f_t of F695.

Model II

In Model II, $F_1(r)$, $F_2(r)$ and $V(r)$ will be obtained as follows:

$$F_1(r) = c_1 K I_a \frac{k_{d1}}{k_1 + k_{d1}} \cdot \frac{1}{1 - \frac{k_1}{k_1 + k_{d1}} \cdot \frac{k_2}{k_2 + k_{d2} + k_r f(r)}} \tag{4}$$

$$F_2(r) = c_2 K I_a \frac{k_1}{k_1 + k_{d1}} \frac{k_{d2}}{k_2 + k_{d2} + k_r f(r)} \cdot \frac{1}{1 - \frac{k_1}{k_1 + k_{d1}} \cdot \frac{k_2}{k_2 + k_{d2} + k_r f(r)}} \tag{5}$$

$$V(r) = K I_a \frac{k_1}{k_1 + k_{d1}} \cdot \frac{k_r f(r)}{k_2 + k_{d2} + k_r f(r)} \cdot \frac{1}{1 - \frac{k_1}{k_1 + k_{d1}} \cdot \frac{k_2}{k_2 + k_{d2} + k_r f(r)}} \tag{6}$$

In Eqn. 4, if the return transfer were neglected, $F_1(r)$ should be independent of the active fraction of reactant, r. This condition does not fit the observed characteristics of F684. This indicates that the return transfer is essential for Model II.

From Eqns. 4, 5 and 6, $F_1(r)$ and $F_2(r)$ can be derived as a function of $V(r)$ as follows:

$$\frac{F_1(r = 0) - F_1(r)}{F_1(r = 0)} = \frac{k_2}{k_2 + k_{d2}} \frac{V(r)}{K I_a} \tag{7}$$

$$\frac{F_2(r = 0) - F_2(r)}{F_2(r = 0)} = \frac{V(r)}{\frac{k_1}{k_1 + k_{d1}} K I_a} \tag{8}$$

Therefore,

$$\frac{F_1(r = 0) - F_1(r)}{F_1(r = 0)} = \frac{k_1}{k_1 + k_{d1}} \frac{k_2}{k_2 + k_{d2}} \cdot \frac{F_2(r = 0) - F_2(r)}{F_2(r = 0)} \tag{9}$$

This relationship between $F_1(r)$ and $F_2(r)$ in Eqn. 9 fits the experimentally obtained characteristics of the induction of F684 and F695, thus indicating that C_{f695} may be connected with photoreaction II according to Model II. Considering the existence of the return transfer of excitation from C_{f695} to C_{f684}, it is preferable to call C_{f695} the "energy sink" rather than the "energy trap". C_{f684} constitutes the "bulk chlorophyll *a*" in pigment system II.

In spinach chloroplasts, f_t was 0.65 for F684 and 0.85 for F695. From these values, the following relationship can be derived according to Eqn. 9:

$$\frac{k_1}{k_1 + k_{d1}} \cdot \frac{k_2}{k_2 + k_{d2}} \cong \frac{0.65}{0.85} \tag{10}$$

It will be reasonable to assume that the energy transfer from the bulk chlorophyll *a* to the energy sink must be a highly efficient one. In other words, k_{d1} must be much smaller than k_1. It follows, then, that,

$$k_2 \cong 3 k_{d2} \tag{11}$$

Unfortunately, the life time of F695 has not been measured. If k_{d_2} is assumed to be $3 \cdot 10^8$ sec^{-1} (the value for the reciprocal of the life time of F-1 in bean leaf at —196°

measured by BUTLER AND NORRIS[20]), k_2, the rate of return transfer, is estimated to be approx. $1 \cdot 10^9$ sec^{-1} at —196° in spinach chloroplasts. However, the excitation energy, in general, can be transferred with much lower efficiency from a molecule with lower excitation energy to a molecule with higher excitation energy. Therefore, the rate constant of return transfer, k_2, must be much smaller than the rate constant of forward transfer, k_1, at both liquid-N_2 and room temperatures.

The energy difference between the excitation levels corresponding to wavelengths 684 mμ and 695 mμ (0.029 eV) is comparable to the thermal energy at 25° ($\boldsymbol{k}T$ = 0.025 eV), but not at —196° ($\boldsymbol{k}T$ = 0.0066 eV). In the process of return transfer, the energy between the two levels is supplied by the thermal energy. At —196°, the amount of available thermal energy is much smaller than the energy difference. Therefore, the rate constant of return transfer at —196° should be much smaller than that at room temperature.

The ratio of $F_2(r=0)$ and $F_1(r=0)$ is obtained from Eqns. 5 and 6;

$$\frac{F_2(r=0)}{F_1(r=0)} = \frac{c_2}{c_1} \cdot \frac{k_1}{k_{d1}} \cdot \frac{k_{d2}}{k_2 + k_{d2}}$$

As seen in Eqn. 11, k_2 is approx. 3 times as large as k_{d_2} at —196°. Therefore;

$$\frac{k_{d2}}{k_2 + k_{d2}} \cong \frac{k_{d2}}{k_2}$$

and,

$$\frac{F_2(r=0)}{F_1(r=0)} \cong \frac{(c_2 k_{d2})}{(c_1 k_{d1})} \cdot \frac{k_1}{k_2} \tag{12}$$

where $(c_1 k_{d_1})$ and $(c_2 k_{d_2})$ are the rate constants of the radiative degradation process in C_{f684} and C_{f695}, respectively. For the reasons mentioned above, on cooling the chloroplasts to —196°, k_2 decreases much more significantly that the other factors, *i.e.*, $(c_2 k_{d_2})/(c_1 k_{d_1})$ and k_1. This decrease in rate constant of return transfer, k_2, is the main reason why F695 is clearly demonstrated only at low temperatures.

In Eqn. 8, the intensity of F695 is related to the quantum yield of photoreaction II at a given value of r. The value of $V(r=1)/KI_a$ measured at the onset of illumination, when r is equal to unity and $V(r)$ is maximum, represents the maximum quantum yield of the photoreaction. As k_{d_1} can be assumed to be much smaller than k_1 (see above), $k_1/(k_1+k_{d_1}) \cong 1$. Therefore, f_t of F695 is equal to the maximum quantum yield of photoreaction II. f_t of F695 was 0.85 in spinach chloroplasts and 0.25 in *A. nidulans* at —196°, indicating that photoreaction II proceeds at —196° with a high efficiency, comparable to that at room temperature (Fig. 2 in this paper and Fig. 10 of ref. 13). Thus, photoreaction II is a temperature-independent reaction, as are photoreaction I in green plants[21,22] and the photoreaction in photosynthetic bacteria[23,24].

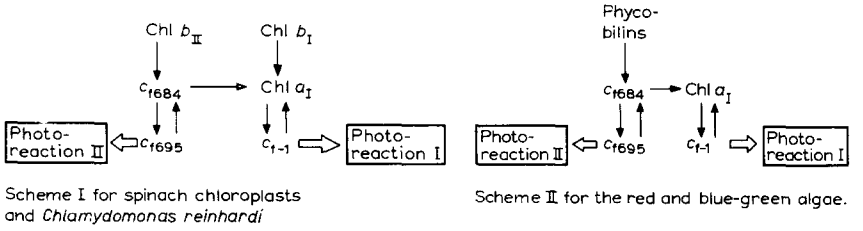
It is not quite clear whether C_{f-1} is contained in pigment system I. For the excitation of F-1, chlorophyll *a* showed a slightly higher efficiency than chlorophyll *b* in spinach chloroplasts[3–5] or phycobilins in *P. yezoensis*[3], *P. cruentum**, *Grateloupia turuturu** and *Anabaena variabilis**. However, in *A. nidulans*, F-1 is more strongly excited by the illumination of phycocyanin than by illumination of chlorophyll *a*[3,25]. In studies of detergent-treated subchloroplast particles, it has been concluded that

* N. MURATA AND K. SUGAWARA, unpublished data.

F-1 is emitted from pigment system I (refs. 6–8). In the present study it is assumed that F-1 is emitted rom pigment system I.

The dependence of F-1 on temperature will be explained in a similar way as in the case of F695, if C_{f-1} is the energy sink of pigment system I. In our previous study with spinach chloroplasts[3], F-1 was insignificant at room temperature, but it was clearly demonstrated at —70° and —196°. F695, however, was not observed at —70°. As seen in the case of pigment system II, the sensitivity of the energy sink fluorescence to temperature will depend on the amount of energy required for the return transfer of excitation from the energy sink to the bulk chlorophyll *a*. The energy difference between C_{f-1} and the bulk chlorophyll *a* in pigment system I will be much larger than that between C_{f695} and C_{f684}. The thermal energy available at —70° (0.017 eV) will be insufficient to fulfill the energy deficit. F-1 appears at 735–740 mμ in spinach chloroplasts, and it seems that the bulk chlorophyll *a* in pigment system I emits at a wavelength shorter than 700 mμ. If this is true, the amount of energy required for the return transfer will be larger than 0.09 eV. This may be the reason why F-1 appeared at —70° but F695 did not.

The present study confirms that the induction of F-1 at —196° is not only due to photoreaction I but also due to photoreaction II. This indicates the presence of an excitation transfer from pigment system II to C_{f-1}. The transfer occurs from C_{f684} to C_{f-1} through the bulk chlorophyll *a* in pigment system I. Schemes shown in Fig. 10 are proposed to represent the excitation transfer between the pigments. Chl a_I is the bulk chlorophyll *a* in pigment system I. The nature of its fluorescence is still unclear. In these schemes, C_{f-1} is assumed to be the energy sink of pignent system I, but this requires further elucidation.



Scheme I for spinach chloroplasts and *Chlamydomonas reinhardi*

Scheme II for the red and blue-green algae.

Fig. 10. Schemes for excitation transfer between pigments in photosynthetic systems.

The distinctive feature discriminating spinach chloroplasts from the red and blue-green algae is the difference in relative contents of chlorophyll *a* in the two pigment systems. In spinach chloroplasts, the amount of Chl a_I is considered to be comparable to that of C_{f684}, since F684 and F695 are excited with good efficiency by light absorbed by chlorophyll *a* as well as by light absorbed by chlorophyll *b* (refs. 3, 4). In the red and blue-green algae, the amount of Chl a_I is considered to be much larger than that of C_{f684}, since F684 and F695 are excited with much poorer efficiency by light absorbed by chlorophyll *a* (refs. 3, 5).

Another difference between the organisms containing chlorophyll *b* and those containing phycobilins is the contribution of the accessory pigments to the two pigment systems. In spinach chloroplasts and *C. reinhardi* (Scheme I), chlorophyll *b* is contained in both pigment systems. The reasons are as follows: the induction of C_{f-1} was much less marked than that of F684 on excitation of chlorophyll *b*. This fact can

be explained if it is assumed that the excitation energy should be transferred from chlorophyll *b* to Chl a_I in two ways, *i.e.*, directly or through $C_{f_{684}}$, and that there is no influence of photoreaction I on the intensity of F-l. Consequently, in these organisms, some portion of chlorophyll *b* is contained in pigment system I as well as in pigment system II. On the other hand, in the organisms containing phycobilins as accessory pigments (Scheme II), the induction of F-l, which is mainly caused by photoreaction II, has almost the same values of f_t and work integral as those of F695 in *P. cruentum* and F684 in *A. nidulans*. This fact indicates that the excitation energy is transferred from phycobilins to Chl a_I through $C_{f_{684}}$. Therefore, in these organisms, the phycobilins are assumed to be only in pigment system II.

As shown in the blue-green and red algae, F-l exhibits induction due to photoreaction I on excitation of chlorophyll *a*. The value of work integral for photoreaction I is much smaller than that for photoreaction II. In the presence of the excitation transfer from $C_{f_{684}}$ to C_{f-1}, the intensity of F-l reduced by photoreaction II will be further diminished by photoreaction I during the induction period on excitation of phycobilins. This reasoning explains why f_t of F-l is larger than f_t of F684 on excitation of phycobilins and why the half-rise time of F_t is shorter in F-l than in F684 and F695.

The consideration of the excitation transfer may in some way modify the interpretation of k_{dI} in the above discussion. Then, k_{dI} must represent the sum of the rate constants of the radiationless and radiative degradation and also of the excitation transfer from $C_{f_{684}}$ to Chl a_I.

While the relative magnitude of transient fluorescence, f_t, of F684 did not significantly change on cooling to —70° and —196° in spinach chloroplasts, the work integral of F684 at —70° was much smaller than that of F684 at room temperature. This fact might be explained by the diminution of the size of electron pool at a step near photoreaction II. On further cooling to —196°, the work integral of F684 recovered to a value similar to that at room temperature, indicating that the size of electron pool associated with photoreaction II is larger at —196° than at —70°. It is not certain whether the shrinkage of the electron pool at —70° is recovered on further cooling. This curious change in the size of the electron pool should be clarified in future study.

At —196°, CMU and BIMU did not change the value of work integral. This means that, at this temperature, the effective size of the electron pool associated with photoreaction II is not changed by the addition of these inhibitors. DELOSME[12], investigating the induction of fluorescence at room temperature, arrived at a conclusion that 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) blocks the temperature-dependent reaction next to photoreaction II. This mode of action of the inhibitor proposed by DELOSME, explains why the same value of work integral is obtained in the presence and absence of the inhibitor. However, it does not explain why the value is not diminished, but remains unaltered, on lowering the temperature to —196°. In fact, in spinach chloroplasts the value of work integral of F684 at room temperature in the presence of the inhibitor has been estimated to be 0.01–0.005 equiv/chlorophyll *a* (refs. 9,13), whereas the values of the work integrals of F695 and F684 in the presence and absence of the inhibitors at —196° obtained in the present study were approx. 0.05 equiv/chlorophyll *a*, which was almost the same as that for F684 at room temperature in the absence of the inhibitors. The simplest inference to be deduced from these experimental facts will be that inhibitors of electron flow, such as

CMU and BIMU, which are effective at room temperature, for some reason loose their effectiveness at liquid-N_2 temperature. The undiminished value of the work integral at —196° tells that the flow of electrons from the primary electron acceptor (X_1; see ref. 9) to the subsequent members of the electron pool (X_2,X_3) would not be abolished even at this low temperature, although the rate of the electron flow becomes greatly suppressed, as reflected by the longer time required to attain the final steady level of fluorescence.

ACKNOWLEDGEMENTS

The author wishes to express his thanks to Professor A. TAKAMIYA for helpful advice and kind suggestions. Thanks are also due to Drs. T. OOHUSA and K. ARAKI of the Yamamoto Nori Research Laboratory for the supply of *P. yezoensis*. This work was supported by a grant from the Ministry of Education, Japan.

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