

BBA 46318

RESTORATIVE EFFECT OF WARMING ON THE FLUORESCENCE INTENSITY AND FLUORESCENCE INDUCTION OF PHOTOSYNTHETIC MATERIAL AT 77 °K

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(Received December 23rd, 1971)

SUMMARY

Irradiation of spinach chloroplasts or *Chlorella* at 77 °K causes a lowering of the fluorescence intensity¹⁻³. In the present work we find that warming the irradiated material above 77 °K (but without melting) gives rise to a restoration of the fluorescence intensity as well as a recovery of the fluorescence induction at 77 °K. The degree of recovery and restoration are related to the temperature to which the sample is warmed. An internal fluorescence standard was employed to adjust for the variables introduced by freezing. An Arrhenius plot of the recovered fluorescence induction does not give a linear relationship. On the other hand, an Arrhenius plot of the restored fluorescence intensity gives linear relationships for each of the three fluorescence bands. For chloroplasts the activation energies for F735, F695 and F685 are -150, -300 and -170 cal, respectively. The activation energies for the restoration of the fluorescence bands indicate that different reactions are involved for each of the fluorescence centers. It is clear that the process operating for recovery of induction is different from that for restoration of fluorescence intensity.

INTRODUCTION

Spinach chloroplasts at 77 °K have three fluorescence bands with maxima at 735, 695 and 685 nm. Irradiation of frozen spinach chloroplasts with strong actinic light (at 77 °K) results in a lowering of the fluorescence intensity^{1,2}. This is also observed with *Chlorella pyrenoidosa*³. It has been shown that lowering of fluorescence intensity for each of the three bands proceeds at different rates^{1,3}. If the irradiated chloroplasts are maintained at 77 °K and in the dark, the fluorescence intensities remain constant. Fluorescence induction at low temperature was reported by several workers and its relationships to the primary events in photosynthesis has been discussed^{4,5}.

In the present work, we find that warming the irradiated chloroplasts above 77 °K gives rise to recovery of the fluorescence induction and also restores the fluorescence intensity of the bands referred to as I725, I695 and I685. The degree of recovery of fluorescence induction and restoration of fluorescence intensity is related to the temperature to which the chloroplasts are warmed.

MATERIALS AND METHODS

Spinach chloroplasts were prepared according to the method of Witt and Stiehl¹⁶ and stored in high concentration at 77 °C. Whole *Chlorella pyrenoidosa* cells were cultivated according to the method of Pirson and Ruppel⁷. These cells were used without storage.

When used, the chloroplasts were suspended in 0.05 M Tris buffer at pH 7.2 along with a fluorescence standard. The mixture of chloroplasts and standard was adjusted to give both a chlorophyll concentration of about $4 \cdot 10^{-7}$ M and an approximately equal fluorescence intensity from both the standard and the chloroplasts when excited with blue light (436 nm). The *Chlorella* suspension was also made up to about $4 \cdot 10^{-7}$ M chlorophyll.

An internal fluorescence standard is necessary for low temperature fluorescence spectroscopy because of the many uncontrollable variables introduced by freezing aqueous suspensions. The standard must be inert in regards to the material under study. It is also necessary that the fluorescence spectrum of the standard not overlap the spectrum from the biological material.

With these considerations, Day Glo Signal Green type A-18 fluorescent water-color with a fluorescence maximum at 545 nm (a solid solution of fluorescent dye in a melamine-sulfonamide-formaldehyde resin; particle size about 4 μ m in diameter)⁸ was chosen for this investigation.

Fig. 1 shows fluorescence spectra of spinach chloroplasts and Signal Green fluorescence standard at low temperature, before and after 15 min of irradiation. Changes in fluorescence intensity from the chloroplasts are referenced to the intensity of the fluorescence standard. The mixture of standard and chloroplast or *Chlorella* was placed in a sample vessel (a 5-ml beaker) and kept in the dark for 10 min. The mixture was then frozen by immersion in liquid nitrogen. Samples were placed in a special sample holder in an optical dewar. In Fig. 1 note that the chloroplast spectrum has changed in intensity, whereas the intensity of the standard has not.

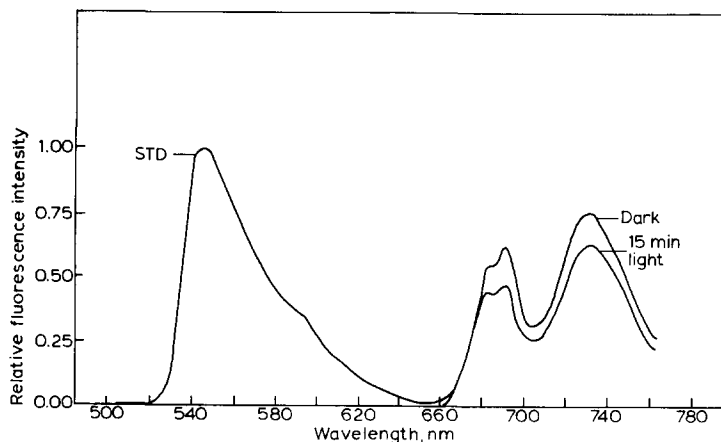


Fig. 1. Fluorescence emission spectra at 77 °K from a suspension containing spinach chloroplasts (chlorophyll concentration $4 \cdot 10^{-7}$ M) and fluorescence standard (maximum at 545 nm; labelled STD). Fluorescence spectrum before irradiation is labelled "Dark". The spectrum after 15 min irradiation with blue light (10^5 erg/cm² per s) is labelled "light".

The sample holder was constructed of solid brass and had eight 2-W resistors located in the brass. The resistors were insulated electrically and cemented in place with a heat conducting epoxy. After freezing and exposing a sample to actinic light, the sample was warmed by applying a voltage to the resistors. Samples could be warmed to any desired temperature in 5–60 s, depending upon the change in the temperature required. The sample was recooled by forcing liquid nitrogen droplets into the optical dewar by pressurizing a liquid nitrogen reservoir.

The recording spectrofluorimeter differs from one described previously⁹ in that a 1600-W high-pressure xenon arc lamp was used for fluorescence excitation. An EMI 9684C photomultiplier tube refrigerated with dry ice and a phase sensitive amplifier (Type HR-8, Princeton Applied Research) were used. The output of this amplifier was used to drive a dual pen Hewlett-Packard recorder (Model 17500A). A copper-constantan thermocouple referenced to ice-water and attached to the above recorder was employed for temperature measurements of the sample. The thermocouple was calibrated using liquid nitrogen, ice water, room temperature and solid CO₂-solvent mixtures.

Actinic light was provided by a 1000-W, water cooled, high pressure mercury arc lamp (GE, Type AH-6). Blue light was isolated from this source using a Corning 5-59 filter and focused to form an image of the arc on the sample. The intensity of light on the sample was 10⁵ erg/cm² per s.

The monochromator used to sensitize fluorescence was set to give a half bandwidth of 10 nm. This yielded a light intensity on the sample of 10³ erg/cm² per s (at 436 nm). The monochromator used to analyze the fluorescence was set to give a half bandwidth of 1.6 nm. All light intensities incident on the sample were measured with an Eppley thermopile calibrated by the National Bureau of Standards.

The intensities of the three fluorescence bands at L735, L695 and L685 were resolved into individual components by correcting for spectral overlap. The contributions of the individual components F735, F695 and F685 was calculated using the method of determinants. The normalized curves required in this analysis were obtained from spectra previously published for the bands at 735¹⁰, 695¹¹ and 685¹².

RESULTS AND DISCUSSION

Fig. 2 shows a series of fluorescence induction curves for a sample at 77 °K. Curve A shows the fluorescence induction for the initial irradiation of the sample (with 10³ erg/cm² per s). No further induction or recovery of induction occurs after a dark period, provided the sample is maintained at 77 °K (see curve B). Curve C shows the extent of induction recovery after the sample has been warmed to 296 °K (no melting occurred) in the dark and then recooled to 77 °K. In order to quantitatively determine the fraction of fluorescence induction recovered, the area integral was measured. The area integral is shown in Fig. 2 by the shaded portion for Curve A and C. The ratio of Area C to Area A is the fractional recovery.

Fig. 3 is an Arrhenius plot of the fractional recovery of fluorescence induction. The abscissa is the reciprocal of the temperature to which the sample was warmed after irradiation at low temperature; the ordinate is the fractional recovery.

Fluorescence induction has been often described as representing the reduction of a quencher "Q" by transfer of electrons from the reaction center of System II^{4,5,13}.

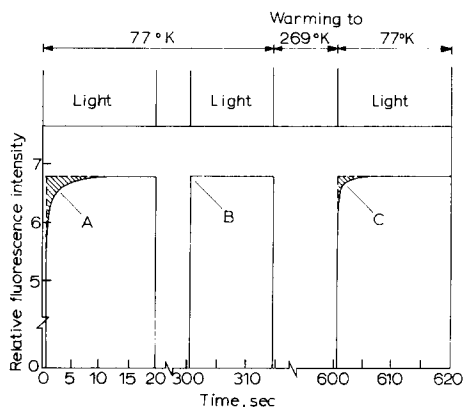


Fig. 2. Low temperature (77 °K) fluorescence induction of a spinach chloroplast sample observed at 695 nm. To sensitize fluorescence induction, blue light of 436 nm (10^3 erg/cm² per s) was used. The area integral for the first fluorescence induction is shown by the shaded portion for Curve A. The shaded area is a measure of the induction. Curve B shows the rise of fluorescence after a dark period of 280 s, following the initial excitation. The shaded portion for Curve C shows the area integral of fluorescence induction at 77 °K after warming the sample to 269 °K, in the dark.

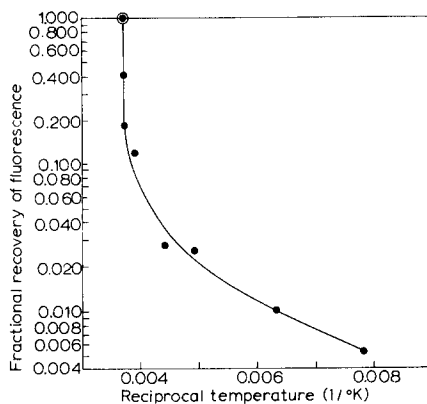


Fig. 3. Arrhenius plot of the fractional recovery (see text) of fluorescence induction. The circle is an estimated value for 273 °K.

After fluorescence induction at low temperature the electrons are trapped in Q provided the temperature remains at 77 °K.

That recovery of fluorescence induction can occur in the frozen state shows that processes resulting in a recovery of Q can still proceed. Furthermore, since the Arrhenius plot (Fig. 3) is not linear it can be assumed that the processes responsible for the recovery are heterogeneous in nature. This heterogeneity may represent variation in the physical arrangements between Q and the pool. Alternatively one may say there are different trap depths^{14,15} for the reduced Q.

Upon warming a sample above 77 °K after induction, electrons are released from Q to the pool. The degree of recovery of induction is related to the temperature to which the sample is warmed. In Fig. 3 it can be seen that a temperature above 200 °K is required to get significant recovery. This may be a measure of the highest traps.

In the case of restoration of fluorescence by warming, the samples were cooled to 77 °K in the dark. The fluorescence intensity after the induction period of the three bands was then measured using the spectrophotometer; these initial fluorescence intensities are referred to as L_{0735} , L_{0695} and L_{0685} . The sample was then exposed to actinic light for several minutes. The fluorescence intensities after irradiation for a time, t , is referred to as L_{t735} , L_{t695} and L_{t685} . Next, the sample was warmed in total darkness to some temperature, T . In no case was the temperature allowed to exceed 269 °K (*i.e.* the samples were never melted). The sample was then recooled to 77 °K and the fluorescence intensities remeasured; these intensities are referred to as L_{r735} , L_{r695} and L_{r685} .

This cycle of irradiating, warming and cooling was repeated, with the same sample, for a series of T values between 77 and 269 °K. Following each fluorescence intensity measurement, the intensity of the fluorescence standard was measured. The

latter was used to standardize the values obtained for the fluorescence from the chloroplasts.

A typical series of measurements is shown in Fig. 4. The temperature of the sample is given on the vertical axis on the right; the relative fluorescence intensity from the sample is given on the vertical axis on the left. The temperature of the sample is indicated by the dashed curve. The intensities of the 735, 695 and 685 bands are indicated with the appropriate notation (*i.e.* L_0 735, L_r 735, *etc.*). The intensity of the standard during the various portions of the light-warming cycles is indicated by L_0 545, L_t 545 and L_r 545. The time course of the experiment is also indicated in the figure. The lowering of fluorescence in light was monitored for the 735 band and is indicated by L_{735} in Fig. 4.

If the sample was kept at 77 °K in the dark after irradiation, no change in fluorescence intensity either up or down was noted. This implies that whatever products are formed are quite stable and that there is insufficient activation energy at 77 °K to permit further reaction.

As soon as the sample is warmed there is some restoration of fluorescence intensity. The intensity of the restored fluorescence is reached rapidly for that temperature, within the time it takes to warm the sample. Within the experimental accuracy of this study the time that a sample remains at any temperature between 77 °K and 273 °K, does not appear to affect the value of L_r for any of the fluorescence bands. The invariance of L_r with time was shown by warming the sample to a given temperature and maintaining it there for periods ranging from 15 min to 5 s, before recooling to 77 °K.

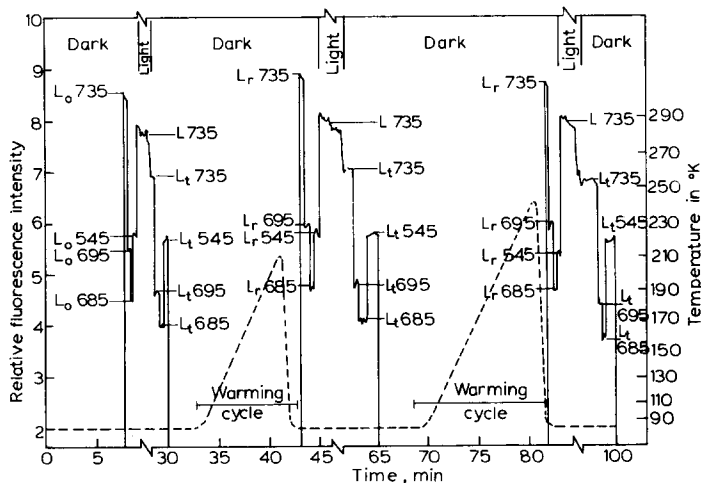


Fig. 4. Effect of warming (in darkness) on the intensities of the fluorescence bands from spinach chloroplasts at 735, 695 and 685 nm, at 77 °K. For all cases fluorescence was sensitized with low intensity (10^3 erg/cm² per s) blue light (436 nm). The initial fluorescence intensities of these bands are indicated by L_0 735, L_0 695 and L_0 685, respectively. The fluorescence intensity of the standard is shown by L_0 540. Fluorescence intensities after irradiation with high intensity blue light (10^5 erg/cm² per s) for 10–20 min are shown by L_t 735, L_t 695, L_t 685 and L_t 545. The restored fluorescence intensity after a warming cycle is indicated by L_r 735, L_r 695, L_r 685 and L_r 545. L_{735} was the fluorescence intensity monitored during the irradiation period (2 different chart speeds were used). Temperature of the sample is shown by the dashed line. The term "Light" indicates the irradiation period.

To determine the energy of activation for the restored fluorescence, ΔE , the data were resolved to determine the contributions of the F values of the individual components F735, F695 and F685 as described in Materials and Methods. These values of F were graphed using an Arrhenius equation in the form $(F_r - F_t)/(F_o - F_t) = e^{-E/KT}$. ΔE was determined by fitting the best straight line to the data in Fig. 5. The subscripts have the same meaning as described previously for the I values.

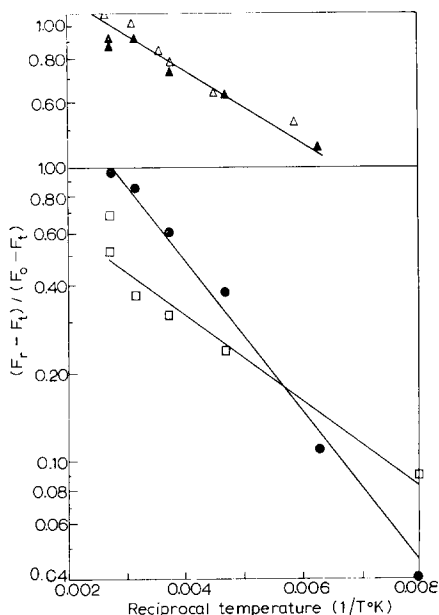


Fig. 5. Upper curve: Arrhenius plot of the relationship between temperature to which the sample is warmed and the restoration of fluorescence intensity. The fluorescence intensities at 735, 695 and 685 for spinach chloroplasts were corrected for spectral overlap to obtain the contributions of the individual components F735, F695 and F685, respectively. F735 from spinach chloroplasts at 77 °K is indicated by \blacktriangle . The fluorescence intensity at 720 nm from *Chlorella pyrenoidosa* is indicated by \triangle . The large open triangle is an estimated value at 273 °K. From the slope of the line the average activation energy is -150 cal. Lower curves: Arrhenius plot of the relationship between the temperature to which the sample was warmed and the restoration of fluorescence intensity. F695 and F685 from spinach chloroplasts is indicated by \blacksquare and \square , respectively. The activation energy for restoration of F695 is -300 cal and for 685 is -170 cal.

Chlorella pyrenoidosa also displays both a lowering of fluorescence upon irradiation at 77 °K and a restoration of the fluorescence on warming. The fluorescence bands of *Chlorella* have maxima at 720, 695 and 685 nm. This study was carried out in the same manner as described earlier for chloroplasts. The results for I720 are shown in Fig. 5 by open triangles.

For chloroplasts ΔE is -300 and -170 cal for F695, and F685, respectively. The average value of ΔE for *Chlorella* and chloroplasts is -150 cal for F720 and F735.

Assuming there is complete restoration of fluorescence if the samples are warmed to 273 °K, one would expect the graphs to go to unity at this temperature. Extending the line drawn through the data points to the horizontal axis shows that the above assumption is satisfactory for F735, F720 and F695, since restoration is almost complete at 273 °K. For F685 the extension does not go to unity, however, the data show

that the line curves upward as 273 °K is approached and that it does indeed go to unity. Therefore in the case of F685 there is a nonlinear region near the melting point and so diffusion is required for restoration at these temperatures.

The linearity of restoration of fluorescence intensity of the other bands (Fig. 5) indicates that a single process is involved. The components of the restoration process are probably in a highly ordered environment.

The mechanism of restoration for F735, F695 and F685 are probably different since they have different ΔE values associated with their reactions. That different reactions are involved in restoring the fluorescence intensity is understandable if one considers the origin of the various bands. Probably F735 is associated with System I, F695 is associated with System II and F685 with bulk chlorophyll^{12, 16-18}. As mentioned earlier, it has also been shown that lowering of fluorescence intensity for each of the three bands proceeds at different rates^{1, 3}.

It is quite clear that different mechanisms are involved in the low temperature restoration of fluorescence and recovery of fluorescence induction. In the former case a linear Arrhenius plot is obtained while in the latter a nonlinear relationship is obtained.

ACKNOWLEDGEMENTS

Parts of this paper are based upon a thesis to be submitted by R. J. in partial fulfillment of the requirements for the Doctor of Philosophy Degree.

This work was supported in part by National Science Foundation Grant (G.B. 18354) and also by a U.S. Public Health Career Development award (K3-GM17918).

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