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DELAYED FLUORESCENCE FROM *CHLORELLA*: I. LOW TEMPERATURE OBSERVATIONS

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SUMMARY

Delayed fluorescence has been observed from *Chlorella* whole cells at 0.5 ms following flash excitation and at temperatures from 293 °K to 120 °K. Cells which are cooled while pre-illuminated before flashes produce less observed delayed fluorescence than cells cooled without pre-illumination. There exists a small component of delayed fluorescence whose magnitude is independent of pre-illumination effects. The effect of pre-illumination upon delayed fluorescence emission is eliminated by prior freezing of the algae.

INTRODUCTION

A major motivation for many investigations on delayed fluorescence in photosynthetic organisms is the possibility that observations of delayed fluorescence may serve as a probe into the microscopic physical interactions involved in the primary quantum conversion process. Evidence relating delayed fluorescence observations to Photosystem II activity has been well reviewed by Lavorel [1]. Motivated by a desire to separate the more physical from the more chemical aspects of delayed fluorescence emission from photosynthetic materials, a series of experiments has been initiated to examine this phenomenon at low temperatures. An apparatus is under development which incorporates a pulsed Laser for photo-excitation and liquid helium cryogenics. This system will be capable of observing the emission of delayed fluorescence from photosynthetic materials over the whole temperature range from 4 °K to 293 °K with 1 μs time resolution. The observations described here were performed with a simpler system in order to anticipate design problems encountered with low-temperature experiments and to repeat the observations of Tollin et al. [2] who made the original low-temperature delayed fluorescence observations. Emphasis was placed on observations at temperatures below 273 °K and at time less than 1 ms after the exciting flash.

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MATERIALS AND METHODS

All experiments were performed with whole cells of *Chlorella ovalis* Butcher. Cultures of this organism were kindly supplied by the Norwegian Institute for Water Research, where the material is cultivated for 24 h at 20 °C on a shaking table in a salt water medium under a continuous fluorescent light intensity specified as 6000 lux. The behaviour of this material with respect to the emission of delayed fluorescence was the same as previously observed with *Chlorella pyrenoidosa* [3].

The optical arrangement used in these experiments is much the same as previously described [3], with the exception that actinic pre-illumination was supplied by a slide projector and filter combination in place of a quartz–iodine lamp and monochromator combination.

For cooling experiments, the sample was contained in a 0.1 cm × 2.2 cm × 4 cm volume machined in the end of an aluminum bar and covered with 0.1 cm thick plastic windows. The other end of the bar was immersed in liquid nitrogen. The holder was surrounded by a nitrogen gas atmosphere to prevent water condensation in the optical path. Delayed fluorescence was observed at 90° to the excitation beam and the plane of the thin sample volume was oriented at 45° to the excitation beam.

The sample was suspended in growth medium at a final concentration of $2 \cdot 10^7$ cells/ml as counted in a hemocytometer. The observations at 293 °K prior to lowering the temperature should represent delayed fluorescence emitted while the sample was in reasonable physiological condition. The absorbance of the sample in this holder was approx. 0.02 at a wavelength of 685 nm. This was measured in a Shimadzu MPS 5000 spectrophotometer which has an optical geometry with little scattering artifact. The chlorophyll concentration in a sample was about 1 μg/ml as measured by methanol extraction using the extinction coefficients of Mackinney [4].

The measured incident flash intensity on the sample at the peak of the flash amplitude was approx. $6 \cdot 10^{18}$ photons/s per cm^2 in the spectral region from 400 nm to 550 nm. The width of the flash in time was 2 μs at half-maximum amplitude. This corresponds to roughly 1 photon absorbed per 1000 chlorophyll molecules during each flash. This is a value below saturation at room temperature [3]. The incident pre-illumination intensity was about $2 \cdot 10^{14}$ photons/s per cm^2 in the same spectral region. This corresponds to approx. 10 photons/s absorbed per 1000 chlorophyll molecules.

Delayed fluorescence was observed in the spectral region from 650 nm to 750 nm, with the limits determined by a glass cut-off filter and the photomultiplier sensitivity. The time resolution in this system was limited by the appearance at short times of a background emission from the Corning 2-62 cut-off filter with a roughly comparable amount from the plastic windows of the sample holder. The observation of fluorescence and luminescence from glass cut-off filters has been reported by Turner [5]. The observation time was arbitrarily chosen at 0.5 ms as the time at which the background artifact had decayed to a value which was about 1/10th the delayed fluorescence signal normally observed at 293 °K. The magnitude of the background was observed as a function of temperature and the data was corrected for this background.

Sample temperature was measured by a calibrated copper–constantan thermo-

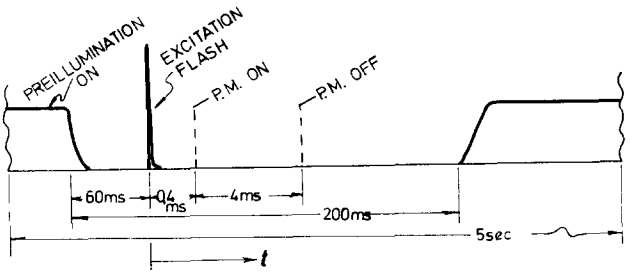

Fig. 1. Sequence of events in experiment. One cycle of a repetitive series is shown.

couple inserted into the sample. A measurement of the temperature difference between points in the sample judged to be the warmest and coldest when the sample is in equilibrium at a low temperature showed a difference of 60 °K. During the cooling, at which time data was taken, temperature differences may be larger than this amount. This experimental arrangement was considered sufficient for the qualitative observations being made. Data were plotted in terms of an estimated mean temperature of the sample, $\bar{T}_{est}$.

The main feature of this apparatus is that the delayed fluorescence observed is the result of a single flash of light applied at a variable sample temperature where actinic pre-illumination may be applied to the sample prior to each flash. The sequence of events in the observations is shown in Fig. 1.

RESULTS

Delayed fluorescence was observed at all temperatures below 273 °K. Fig. 2 shows examples of the data, oscilloscope pictures, as observed under several conditions. It should be noted that in this region of time the decay kinetics of the delayed

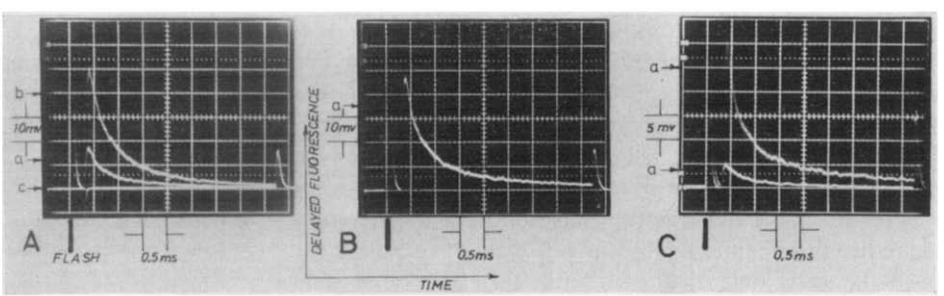

Fig. 2. Typical data observed in the low-temperature studies of delayed fluorescence. Flash and pre-illumination fluxes are stated in the text. The traces in each picture are labelled by an arrow to the value at 0.5 ms after the flash as: a, delayed fluorescence produced by flash without pre-illumination; b, delayed fluorescence produced by flash with pre-illumination; and c. delayed fluorescence produced by pre-illumination only. The individual pictures are: (A) Sample at thermal equilibrium at $T = 293$ °K (room temperature). (B) Sample at thermal equilibrium at $T = 160$ °K, having been cooled without pre-illumination prior to each flash but with one flash every 5 s. (C) Result of single flash after dark adaptation. The lower curve is at $T = 293$ °K and the upper curve is at $T = 120$ °K, where the sample was dark-adapted and then cooled in the dark.

fluorescence emission seem independent of temperature or treatment. This was born out by comparing plotted curves. The effect of pre-illumination has been described in a previous publication [3]. Briefly, pre-illumination produces a Photosystem II product whose presence can greatly affect the observed intensity of delayed fluorescence resulting from a single flash. This phenomenon was largely eliminated in samples which had been cooled and then subsequently observed at 293 °K. This was studied directly by rapidly pre-freezing samples in liquid nitrogen prior to observation at room temperature. Heat treatment was also used in comparison. Fig. 3 shows these observations.

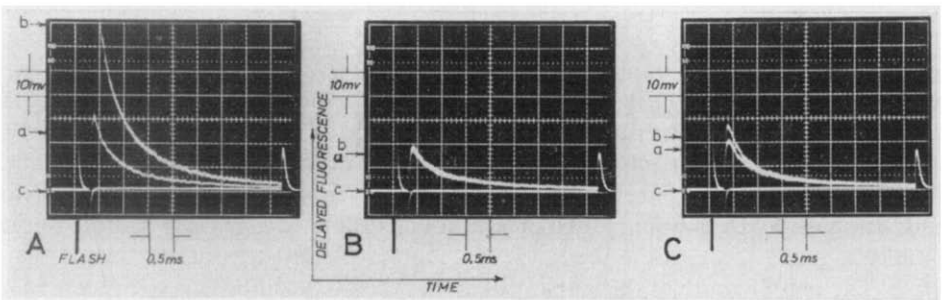

Fig. 3. Effects of treatment upon the flux of delayed fluorescence 0.5 ms after each flash with pre-illumination. The temperature is 293 °K for each picture. (A) Control. (B) Rapidly prefrozen in liquid nitrogen. (C) Pre-heated at 50 °C for 10 min in a water-bath.

Fig. 4 shows the dependence of delayed fluorescence upon the temperature of the sample at the time of illumination under different experimental conditions. Clearly, the nature of the delayed fluorescence observed was very dependent upon the experimental conditions used. Although there was considerable variability between samples when these observations were repeated, the general features of the data selected for presentation were reproducible. We have put a good deal of data in one figure, at the risk of some possible confusion, because it facilitates comparison. There are several distinct qualitative features to be noted. While the application of pre-illumination at room temperature always increased the observed delayed fluorescence emission in viable cells, its application during cooling reduced the delayed fluorescence observed at the lower temperatures. Under a pre-illumination light intensity higher by roughly one order of magnitude than was normally used in our experiments, it was possible to produce a curve of delayed fluorescence versus temperature with pre-illumination which was almost a monotonically decreasing curve as the temperature was reduced. The final steady state delayed fluorescence signal at low temperature was much the same regardless of illumination history or sample treatment. This is suggested by the grouping of terminal points in the cooling experiments as indicated by the cross hatched area in Fig. 4. Samples which were pre-heated or rapidly pre-frozen exhibited delayed fluorescence which increased as the temperature was decreased.

The application of pre-illumination to a sample which had been brought to a steady-state temperature in the dark produced several distinct results depending upon the temperature. After the application of pre-illumination the delayed fluorescence was observed on subsequent flashes. This is compared with the delayed fluorescence ellicited by a single flash before the application of pre-illumination. When applied to

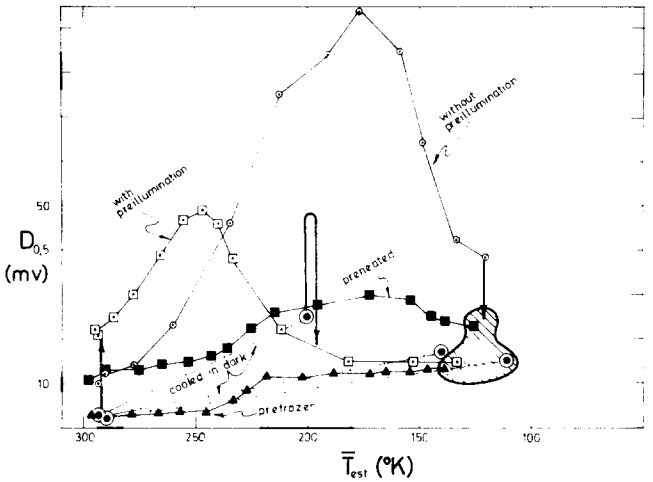

Fig. 4. Delayed fluorescence observed during cooling of *Chlorella* whole cells. $D_{0.5}$ is proportional to the flux of delayed fluorescence emitted at 0.5 ms after a flash of light. Three series of data show results obtained during cooling under different experimental conditions. ⊙, cooling with one measuring flash every 5 s. ⊡, cooling with pre-illumination between flashes. ◉, dark-adapted at room temperature and cooled in the dark. The lines, – · –, connect the observations on dark-adapted samples at room and low temperatures. The arrows show the effect when pre-illumination prior to a flash was added at the end of a cooling series. The arrow follows the trajectory of the delayed fluorescence magnitude in time after the pre-illumination was added. Flash and pre-illumination fluxes are stated in the text. Also shown, are two series of data where the sample was treated prior to cooling: ■, pre-heated; and ▲, rapidly pre-frozen. The darkened sections of the ordinate and abscissa indicate the estimated uncertainties in the plotted values at those points.

samples at 200 °K, pre-illumination first produced an increase in delayed fluorescence, but upon continued observation the delayed fluorescence decreased below its initial value. Thus the character of the effect of pre-illumination changes somewhere in the neighbourhood of 200–250 °K. Above this temperature interval the net effect of pre-illumination is a steady state increase in delayed fluorescence and below this temperature the net effect is a steady state decrease in delayed fluorescence. At temperatures below 150 °K pre-illumination produced no effect, when applied to samples cooled in the dark. However, when applied to samples which had been cooled to the lowest temperature in the low average level of illumination of slowly repeating measuring flashes, pre-illumination caused a decrease in the observed delayed fluorescence.

DISCUSSION

The first reports of the observation of delayed fluorescence at temperatures below 273 °K are those of Tollin and coworkers of which ref. 2 is the one dealing with ms times of observation. The design of Tollin's experiments is similar to the one used in our experiments. Subsequent to Tollin's report, there are statements in the literature that delayed fluorescence is not observed at low temperatures. There are several comments by Arnold that delayed fluorescence observed after cooling disappears based on observations with a phosphoroscope [6, 8]. This was confirmed by Lumpkin and Hillel [7] in whose study the delayed fluorescence disappeared upon freezing, again as observed with a phosphoroscope.

Clearly, under appropriate conditions the experiment of Tollin et al. [2] can be repeated. That is, the delayed fluorescence emission increases and goes through a maximum as the temperature is lowered when elicited by single flashes at low repetition rates. However, when observed with constant illumination between flashes or with flashes at a high repetition rate, the signal may be observed as one which appears to decrease with a reduction in temperature. It is tempting to suggest that in phosphoroscope experiments, which are always essentially steady illumination experiments and are often run at saturating intensities, delayed fluorescence is observed to decrease with temperature mainly due to experimental conditions. The signal will be thought to disappear when it drops below the noise level of the apparatus, which may be larger than we have here. This can only be a tentative explanation of the disparity of published observations of delayed fluorescence at low temperature. The differences in experimental conditions, especially light intensities, sample preparation and concentration, times of observations and the difficulty in comparing apparatus sensitivity from published information preclude a more definite statement.

Arguments that delayed fluorescence from photosynthetic organisms is dependent upon a variety of factors such as the physical interactions at the reaction center, electron transport and phosphorylation activity at the thylakoid membrane are presented by Lavorel [1]. The question which is raised here is whether low-temperature observations of delayed fluorescence will allow us to separate the various processes which might contribute to the production of delayed fluorescence in order to better understand the mechanism by which delayed fluorescence is produced. There are two related, but possibly distinct, phenomena under discussion. One is the production of delayed fluorescence by a single flash of light. The other is a change in the observed delayed fluorescence ellicited by a flash as the result of pre-illumination of the photosynthetic system. The latter effect appears to us to be highly correlated with events surrounding phosphorylation at the thylakoid. Sufficiently concrete evidence to demonstrate this point has not been obtained yet, so we will take this as an assumption for purposes of this publication. The rational for our pre-illumination experimental design is to attempt to separate these two aspects of delayed fluorescence production. Thus, a non-saturating flash was used at a low frequency to examine delayed fluorescence, while the general state of the system was largely governed by an independent actinic light. That these two aspects of delayed fluorescence production are separable is partly demonstrated by the effect of freezing and thawing upon delayed fluorescence emission, where the effect of pre-illumination was largely eliminated while the delayed fluorescence from a single flash had much the same appearance as it did from normal cells in a dark steady state. Related observations have been made by others [7, 9], but these observations were made with phosphoroscopes and may not be comparable. The results of Heber and coworkers [10, 11] on the effect of a freeze–thaw treatment on chloroplasts are consistent with our assertion that the effect of pre-illumination is correlated with phosphorylation activity. In these studies phosphorylation activity is lost after such treatment, and the loss is interpreted as due to changes in the thylakoid membrane permeability which occur during freezing.

Whatever the mechanism for the pre-illumination effect may be, it is clear that this effect can occur at some low temperatures and that it reflects events associated with the more chemical aspects of the photosynthetic mechanism. Pre-illumination

has two opposing effects. It produces an intermediate whose presence affects the delayed fluorescence emission after a single flash. It may be that this intermediate is a precursor to one of several types of delayed fluorescence production. The decrease in delayed fluorescence ellicited by a flash observed after pre-illumination at lower temperatures suggests that pre-illumination also leads to processes which decrease the level of the intermediate. This would be expected if one of these processes were the production of delayed fluorescence (which would not be observed in this experiment). The effect of pre-illumination involves processes of considerable complexity, perhaps lying at the root of the problem which has been encountered in understanding the mechanisms of delayed fluoresecence production. While lowering the temperature down to 200 °K does not result in a greatly simplified situation, temperatures below 100 °K eliminate this effect and may well lead to a situation in which simple physical models are applicable.

This brings us to discussion of the small residual signal which seems to exist in dark-adapted samples, at steady state at low temperatures, and under several conditions of sample treatment. Certainly this signal has some interesting characteristics which bear further investigation. One is the apparant temperature dependance in which the magnitude of this signal increases with decreasing temperature. Another is the suggested independance of its kinetics upon temperature. However, the present apparatus lacks the temperature control or the time resolution to perform the desired quantitative measurements on the magnitude or kinetics of this signal.

The usual evidence which is put forth to argue that delayed fluorescence is a meaningful aspect of photosynthetic observations cannot be applied to this signal as it may well be smaller and perhaps different than the delayed fluorescence which has been observed and discussed to date. Thus, there exists the possibility that this emission is only indirectly dependant on the photosynthetic process. On the other hand, there is no evident reason to exclude the more optimistic possibility that subsequent investigations will find this small signal to be intrinsic to the physical mechanism operating in the photosynthetic process and will help to answer the question of the relationship between the observation of delayed fluorescence and the physical as well as the chemical interactions of primary quantum conversion.

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