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DOES THE RATE OF COOLING AFFECT FLUORESCENCE PROPERTIES OF CHLOROPLASTS AT −196 °C?

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SUMMARY

The question addressed in the title was examined by measuring fluorescence emission spectra and light-induced fluorescence-yield changes of chloroplasts which had been frozen to −196 °C rapidly, as very thin samples adsorbed into substrates which were plunged directly into liquid nitrogen, or slowly by the cooling action of liquid nitrogen through the wall of the cuvette. Contrary to previous reports, we found that the rate of cooling had no influence on the shape of the emission spectrum, the extent of the variable fluorescence or the fraction of the absorbed quanta which are delivered initially to Photosystem I.

INTRODUCTION

It was reported recently [1, 2] that the fluorescence properties of chloroplasts and *Chlorella* cells at −196 °C were altered by artifacts due to membrane damage if the rate of cooling was less than 10^4 degrees/s. According to those studies the fluorescence emission spectrum, the ratio of variable to non-variable fluorescence and the value of α (i.e. the fraction of the absorbed quanta delivered initially to Photosystem I) as calculated by Butler and Kitajima [3], were all subject to the freezing artifacts. Surprisingly large effects were reported. With the rapidly frozen samples the ratio of variable to non-variable fluorescence, F_V/F_o, at 687 nm was only one-fourth the ratio obtained with more slowly frozen samples; also the values of α were about one-third those of the slowly frozen samples. It was assumed that the very low values of F_V and of α obtained from the rapidly frozen samples were characteristic of the undamaged system while the generally accepted larger values found with the more usual freezing techniques were the result of membrane damage. It is clear that if the data of Harnischfeger [1, 2] are correct that most of the previous work based on quantitative measurements of fluorescence from photosynthetic systems at −196 °C must be thrown out or, at least, reexamined.

Harnischfeger varied the rate of cooling in his studies by changing the sample thickness. The most rapidly frozen samples were obtained by adsorbing chloroplasts or *Chlorella* cells on discs of cheesecloth which was immersed directly into liquid nitro-

gen. The samples frozen slowest were in a 2 mm cuvette which was immersed into liquid nitrogen. An intermediate rate of freezing was obtained with a 1 mm cuvette. It seemed possible to us that different rates of cooling might result in different scatter and absorption properties of the samples and that the thicker samples might be subject to more self-absorption for the same amount of chlorophyll. Self-absorption could alter the shape of the emission spectrum but self-absorption should not alter the ratio F_V/F_o at 690 nm or the value of α calculated by Butler and Kitajima [3]. (In that calculation of α, the measurements of fluorescence at 690 and 730 nm are always used in ratios of two different measurements at the same wavelength so that any effects of self-absorption should cancel out.) Since we were skeptical of the results reported by Harnischfeger, especially the large variations in the ratio of F_V/F_o and α, we have repeated these measurements on chloroplasts frozen to −196 °C with different rates of cooling. Measurements were compared between very thin samples (adsorbed onto cheesecloth or tissue paper) frozen rapidly by direct contact with liquid nitrogen or relatively slowly through the wall of the cuvette. Comparisons were also made between the rapidly frozen thin samples and slowly frozen thick (2 mm) samples. The concentration of chloroplasts was examined over a 40-fold range to determine whether chlorophyll concentration or self-absorption had any effect. We find that the value of α and the extent of the variable fluorescence (relative to F_o or F_M) at 694 and 730 nm are constant within small limits of experimental error regardless of the rate of cooling, the thickness of the sample, or the amount of chlorophyll present.

MATERIALS AND METHODS

Chloroplasts were prepared by methods previously described [4]. The samples were frozen in our vertical cuvette and Dewar system [5] and measured using the triple-arm, fiber-optic light pipe assembly described previously [6]. The glass rod, which serves as a mixing chamber for the three fiber-optic arms, was placed in the cylindrical cuvette directly on top of the frozen sample which was immersed in liquid nitrogen to insure temperature equilibration. Most of the fluorescence measurements were made from the top surface of the frozen sample with 633 nm excitation from a Ne-He laser (plus a 633 nm interference filter) being transmitted to the top surface by one of the light-pipe arms. Fluorescence emission spectra were measured with a Bausch and Lomb Double Grating Monochromator (2 nm passband) and a Ga-As phototube with input from one of the other light-pipe arms. Spectral measurements were made on line with a small computer which could plot the spectra as well as calculate and plot the ratio between two spectra. X-Y plots of fluorescence at 730 and 694 nm were made using two of the light-pipe arms (one terminated at a Bausch and Lomb High Intensity Monochromator set at 694 nm and a phototube, the other terminated with a 730 nm interference filter and a phototube) to measure fluorescence with the third arm used to transmit the 633 nm excitation to the sample. F_{730} was measured as a function of F_{694} as the 633 nm excitation beam transformed the fluorescence yield of the sample from the minimum, F_o, level to the maximum, F_M, level. A light-pipe assembly was also placed at the bottom surface of the Dewar so that measurements could be made on the fluorescence emanating from the back surface of the frozen sample. The X-Y plots were made with a recorder (Hewlett Packard, Model 7047A) with a 0.1 s time response. At the onset of irradiation the pen makes a rapid

excursion from the origin to the F_0 level of fluorescence at 730 and 694 nm. The X-Y plots presented are photographs of the raw data, including the meaningless trace from the origin to the F_0 level on the plot.

Thin samples consisted of a 10 μl aliquot of chloroplasts adsorbed onto 1.3-cm discs of cheesecloth or of Kimwipe tissue paper. The results were the same with either type of substrate but the tissue paper matrix was preferred because the samples were thinner and more reproducible. Thick samples consisted of 2-mm thick suspensions of chloroplasts in the cylindrical cuvette. Thin samples were frozen rapidly either by immersing the disc directly into liquid nitrogen and then transferring it to a prechilled cuvette or by placing the disc in the cuvette initially and pouring liquid nitrogen directly into the cuvette as well as into the Dewar around the cuvette. The results were the same with either type of rapid freezing procedure but the second procedure was generally used because it was more convenient. The thin samples were also frozen slowly by pouring the liquid nitrogen around the outside of the cuvette so that the sample froze slowly from the periphery in toward the center requiring about 90 s for complete equilibration. The same slow freezing technique was used with the 2-mm thick samples. After the temperature of the sample was down to −196 °C it was covered with liquid nitrogen and the prechilled light-pipe assembly was placed on top of the sample in the cuvette.

RESULTS AND DISCUSSION

Fluorescence emission spectra were measured at −196 °C on dilute suspensions of chloroplasts adsorbed onto tissue paper (1 μg chlorophyll/cm^2). The samples were frozen rapidly or slowly, as described in Materials and Methods, and the measurements were made with the fluorescence yield at the F_M level. It is apparent in Fig. 1 that the emission spectra of both samples have essentially the same shape. The ratio of the two spectra shown in the upper part of Fig. 1 reveals no significant variations with

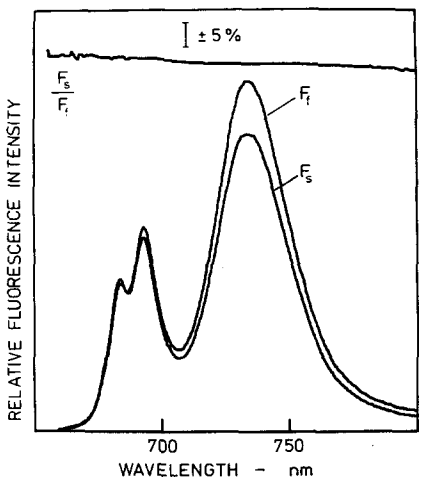

Fig. 1. Fluorescence emission spectra of chloroplasts (1 μg chlorophyll/cm^2) adsorbed onto tissue paper frozen to −196 °C rapidly (F_f) and slowly (F_s). The wavelength dependence of the ratio of the two spectra is also presented as F_s/F_f. The sensitivity scale at the top indicates a 5 % change in the ratio.

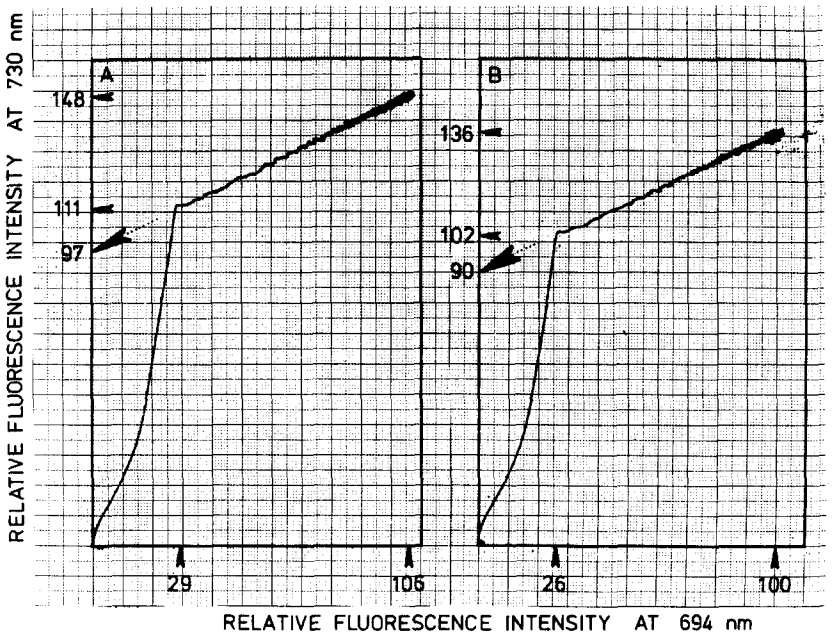

Fig. 2. X-Y plots of the fluorescence at 730 nm (F_{730}) as a function of the fluorescence at 694 nm (F_{694}) measured simultaneously as the 633 nm excitation light transformed the fluorescence yield of the sample from the F_0 to the F_M level. (A) For a sample of chloroplasts (1 μg chlorophyll/cm^2) adsorbed onto tissue paper frozen rapidly. (B) For a similar sample frozen slowly. Relative values of the fluorescence at the F_0 and F_M levels at 694 and 730 nm are indicated along the two axes. The relative value of $F_{I(\alpha)}$ is also indicated by the extrapolation of the X-Y plot back to the F_{730} axis.

wavelength. However, the intensity of emission is consistently greater with the rapidly frozen samples presumably because of a greater scatter intensification of absorption.

Samples were frozen to −196 °C either rapidly or slowly in the dark-adapted state to determine whether the value of α depended on the method of freezing. Kinetic measurements of fluorescence were made simultaneously at 694 and 730 nm from the same sample and plotted on an X-Y recorder as the fluorescence excitation beam converted the fluorescence yield from the F_0 to the F_M level (see Fig. 2). Such X-Y recordings of fluorescence at −196 °C always give straight line plots which can be extrapolated back to the F_{730} axis. The extrapolated value, $F_{I(\alpha)}$, indicates the intensity of the 730 nm fluorescence which is due solely to excitation of Photosystem I, i.e. to the fraction of absorbed quanta represented by α. If we normalize the value of $F_{I(\alpha)}$ on the maximum level of Photosystem I fluorescence, $F_{I(M)}$, we can define a normalized value, α_N, which can also be expressed in terms of photochemical parameters from the equations of our model [3, 7].

$$\alpha_N = \frac{F_{I(\alpha)}}{F_{I(M)}} = \frac{\alpha}{\alpha + \beta\varphi_{T(II\to I)(M)}} \tag{1}$$

where $\beta = 1-\alpha$ and $\varphi_{T(II\to I)(M)}$ is the yield of energy transfer from Photosystem II to Photosystem I when the Photosystem II reaction centers are closed.

It is apparent from Eqn. 1 that α_N, which can be determined readily from the X-Y plots, is not directly related to α. That relationship also involves $\varphi_{T(II\to I)(M)}$. Information as to whether $\varphi_{T(II\to I)(M)}$ changes with method of freezing can be obtained from the slope of the X-Y plots since the equations of our model show that that slope is proportional to the photochemical rate constant for energy transfer, $k_{T(II\to I)}$. If $k_{T(II\to I)}$ changes, $\varphi_{T(II\to I)(M)}$must change in the same direction but if $k_{T(II\to I)}$ remains constant, $\varphi_{T(II\to I)(M)}$ is also constant. Thus, we will collect data on the slope of the X-Y plots as well as the values of α_N.

X-Y plots of F_{730} vs. F_{694} are shown in Figs. 2A and 2B for thin tissue paper samples of chloroplasts (1 μg chlorophyll/cm^2) frozen rapidly and slowly, respectively. The numbers on the two axes represent the relative values of fluorescence at the F_0 and F_M levels at 730 and 694 nm as well as the extrapolated value, $F_{I(\alpha)}$. The slopes of the two plots are virtually the same; 0.48 for the rapidly frozen sample vs. 0.46 for the slowly frozen sample and the values of α_N are the same, 0.66, for both samples. Since the slopes are the same we conclude that $\varphi_{T(II\to I)(M)}$ is the same for both samples. It is apparent from Eqn. 1 that if α_N and $\varphi_{T(II\to I)(M)}$ are constant, α must also remain constant.

TABLE I

α_N AND FLUORESCENCE RATIOS TAKEN FROM X-Y PLOTS SIMILAR TO THOSE SHOWN IN FIG. 2 FOR THE SAMPLES INDICATED

Sample	Fast frozen				Slow frozen			
	F_V/F_M		$F_{V(730)}$	α_N	F_V/F_M		$F_{V(730)}$	α_N
	694	730	$F_{V(694)}$		694	730	$F_{V(694)}$	
1 μg chlorophyll/cm^2, thin	0.73	0.25	0.49	0.66	0.73	0.25	0.48	0.66
1 μg chlorophyll/cm^2, thin	0.72	0.25	0.48	0.65	0.74	0.24	0.50	0.68
1 μg chlorophyll/cm^2, thin	0.73	0.24	0.50	0.67	0.73	0.25	0.48	0.66
1 μg chlorophyll/cm^2, 2 mm	–	–	–	–	0.72	0.24	0.47	0.67
20 μg chlorophyll/cm^2, thin	0.73	0.25	0.48	0.66	0.72	0.25	0.46	0.65
20 μg chlorophyll/cm^2, thin	0.74	0.26	0.49	0.65	0.74	0.25	0.48	0.66
20 μg chlorophyll/cm^2, thin	0.74	0.25	0.49	0.66	0.74	0.26	0.43	0.65
20 μg chlorophyll/cm^2, 2 mm	–	–	–	–	0.72	0.24	0.49	0.67

Data from a number of experiments (X-Y plots) comparing comparable samples frozen rapidly or slowly are presented in Table I. The samples include the thin tissue paper samples with 1 or 20 μg chlorophyll/cm^2 as well as thick samples which were only frozen slowly. Ratios of F_V/F_M at 694 and 730 nm were taken from the X-Y plots and α_N was calculated as:

$$\alpha_N = 1 - \frac{(F_V/F_M)_{730}}{(F_V/F_M)_{694}} \tag{2}$$

which is equivalent to the extrapolation and normalization of $F_{I(\alpha)}$. The slopes of the X-Y plots are presented as the ratio of $F_{V(730)}/F_{V(694)}$. We conclude from these data showing that α_N and the slope values are the same for all of the different samples that α is constant among these various samples.

In order to determine the absolute value of α, $\varphi_{T(II \rightarrow I)(M)}$ was measured on rapidly frozen and slowly frozen thin samples (1 μg chlorophyll/cm^2) by the method described previously [8] and was found to be approx. 0.27 for both types of sample (data not shown). Using that value and a value of 0.67 for α_N, α is calculated from Eqn. 1 to be 0.35. These experiments were all carried out with 633 nm excitation but, with mature chloroplasts, the value of α or of α_N are largely independent of the excitation wavelength between 400 and 675 nm [8, 9].

In order to examine the effects of self absorption, the X-Y plots of F_{730} vs. F_{694} were measured from the front and back surfaces of a 2 mm thick sample with 40 μg chlorophyll/cm^2 frozen slowly. The data (not shown) gave a value of α_N of 0.68 from the front surface and a value of 0.67 from the back surface, both being consistent with the values presented in Table I.

An attempt was made to increase any freezing damage that might occur by going through a slow thaw and slow refreeze cycle. A thin sample (2 μg chlorophyll/cm^2) was frozen rapidly and the X-Y plot was made. The sample was then allowed to warm to room temperature slowly in the Dewar, to remain in the dark at room temperature for approx. 30 min and was then refrozen by the slow freeze technique. The results taken after the slow refreeze are compared with the initial results in Table II. Any membrane damage that may have occurred was not reflected in the low temperature fluorescence measurements. The values of α_N for both measurements were the same as those observed with the other samples.

TABLE II

EFFECT OF THAWING AND REFREEZING ON α_N

	Fast frozen				Thaw →	Slowly refrozen			
2 μg chlorophyll/cm^2, thin	694	730	$\frac{F_{V(730)}}{F_{V(694)}}$	α_N		694	730	$\frac{F_{V(720)}}{F_{V(694)}}$	α_N
F_o	42	140	–	–		35	118	–	–
F_M	148	185	–	–		122	153	–	–
F_V/F_M	0.72	0.24	0.42	0.67		0.71	0.23	0.40	0.68

Although Harnischfeger [1] states that his values of α were calculated by the method of Butler and Kitajima [3], that is not the case. Harnischfeger used what he considered to be an equivalent, simplified formula [2] in which:

$$\alpha = \frac{F_{I(\alpha)}}{F_{I(M)} + F_{II(M)}}$$

It should be noted that this is presented as a formula for α, not α_N. The theoretical basis of that formulation is not apparent, but it is not equivalent to the method of Butler and Kitajima [3]. In the study by Butler and Kitajima [3] on the effects of Mg^{2+} on energy distribution in chloroplasts, relative values of α in the absence and presence of Mg^{2+} were obtained from the measurements of $F_{I(\alpha)}$. Calculation of the absolute values of α, however, required the solution of six simultaneous equations which

provided the value of $\varphi_{T(II \to I)}$ needed to calculate the value of α [7]. That information is not available in the formula of Harnischfeger. Furthermore, it is apparent that values of α calculated by that formula would be sensitive to self absorption, since $F_{I(M)}$ and $F_{II(M)}$ appear separately. α is a property of the chloroplast and, as such, should be independent of any sample preparation artifacts such as self absorption. Note that in Eqn. 2 intensities of fluorescence appear only as the ratio of two measurements made at the same wavelength so that the effects of any sample preparation artifacts will cancel out.

In conclusion we find no merit in the suggestion by Harnischfeger [1, 2] that the fluorescence properties of chloroplasts at −196 °C are affected by the rate at which the sample cooled nor in the doubt he casts on the previous determinations of α. On the contrary, when α is calculated properly (from properly made measurements) it is remarkably independent of the freezing techniques employed.

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