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FLUORESCENCE SPECTRA OF CHLORELLA IN THE 295–77°K RANGE*

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SUMMARY

A relation of fluorescence spectra of Chlorella (in the 295–77°K range) to the changes in the phase of the ice is suggested in this paper.

When Chlorella cells are first cooled to 77°K and then warmed slowly, the change of the fluorescence intensity as a function of temperature is different for different bands as earlier reported in spinach chloroplasts. In the 80–150°K range, the F698 decreases rapidly, F687 remains almost unchanged, and f680 increases; above 150°K (the temperature at which ice changes from vitreous to cubic phase), F687 decreases more rapidly than any other band. The F725, that decreases smoothly in the 77–295°K range, is composed of several bands (F717 and F725).

If Chlorella cells are first cooled to 77°K, and then warmed to 260°K, and recooled to 77°K before melting, f685, f698 and f730—but not f717—are lowered; this fluorescence decrease is not due to changes in the reabsorption of fluorescence or to major changes in the cellular structure due to freezing.

The F698 band is greatly influenced by the aqueous environment; the phase of the ice and the addition of polar solvents (10 % dimethylsulfoxide) greatly influence it. These data are consistent with the hypothesis that F698 is from an energy trap (at low temperatures) of System II.

INTRODUCTION

A number of investigators have studied the structure of ice crystals at low temperatures by X-ray diffraction[1], by electron diffraction[2], and by thermal analysis[3]. Three phases of ice are known at low temperatures: 'vitreous' (or amorphous with random molecular arrangement), 'cubic' (having oxygen atoms in a diamond cubic lattice), and 'hexagonal' (built up of layers of oxygen and hydrogen atoms, where oxygen atoms form a network of hexagonal rings). Upon rapid cooling to temperatures below 113°K, vitreous phase occurs. With a warming rate of 5°K/min, the conversion of vitreous to

Abbreviation: The prefix F will be used to denote a band, and f to denote the fluorescence intensity at a certain wavelength. For ease in presentation, the main band will be designated as F687, F698 and F725 even though their actual locations may vary.

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cubic phase occurs[1] at about 150°K, and of cubic to hexagonal phase occurs irreversibly at about 210°K (also see refs. 4 and 5). Fluorescence spectra of Porphyridium[6] and of chloroplasts suspensions from spinach[7] as a function of temperature (77–298°K) have provided information concerning the nature of different fluorescence bands at these low temperatures. In view of the available physical data on the phase change of ice, we have compared the fluorescence data of Chlorella *in vivo* in the 295–77°K range, obtained by warming a 77°K sample at an approximate rate of 5–10°K/min, with the known changes in the ice phase.

MATERIALS AND METHODS

Chlorella pyrenoidosa was grown as described earlier[8]. Absorption spectra, excitation spectra of fluorescence, and the fluorescence spectra were measured as described in other publications[9–11]. Additional details will be mentioned under RESULTS AND DISCUSSION. The half-band widths of all the slits were 6.6 nm; the excitation spectra were corrected for the number of incident quanta, and the fluorescence spectra were corrected for the transmission efficiency of the monochromator (Bausch and Lomb), and the spectral efficiency of the photomultiplier (EMI 9558B).

RESULTS AND DISCUSSION

Fluorescence spectra of C. pyrenoidosa from 77 to 295°K

It is difficult to obtain consistent results on the fluorescence spectra *in vivo* at low temperatures due to difficulties in obtaining identical path lengths; there are multiple reflections due to the presence of ice crystals. Differences in the reabsorption of fluorescence in different samples cause changes in the shape of the spectra. The peak positions may vary between different experiments by $\pm$ 0.5 nm and the relative heights among F687, F698 and F725 may change many fold due to variations in the rate of cooling and the concentration of the sample that affect the reabsorption of fluorescence (Fig. 1). In this experiment, the dilute and the concentrated samples had absorbance values of 0.1 and 0.8, respectively, at 675 nm for 1 cm path length. Quick cooling was obtained by dipping the coverslip spread with Chlorella cells into liquid N_2. 77°K is reached within seconds. Slow cooling was obtained by blowing cold air (evaporating liquid N_2) onto the sample; freezing was obtained in 1–2 min. Our data show that the most reliable spectra (*i.e.* with least reabsorption of fluorescence) are obtained when dilute samples are quickly cooled. The fluorescence spectra at different temperatures were measured by first quickly cooling the cells to 77°K and then warming the cells. The rate of warming varied between 5 to 10°K/min depending upon the temperature range. At 100°K, the rate was approx. 8°K/min. The changes caused by the temperature variations during the short time of measurement of a spectrum were then corrected by assuming that the fluorescence change as a function of temperature is a continuous function.

Figs. 2 and 3 show the fluorescence spectra of Chlorella from 77 to 295°K. At 77°K bands at 686 (referred to as F687), 697.5 (F698), 717.5 (F717) and 725 nm (F725) are found. The band around 720 nm was discovered by BRODY[12] in Chlorella and around 698 nm by LITVIN *et al.*[13] in leaves. In spinach chloroplasts these bands are at 685, 696 and 738 nm (ref. 7); it is believed that F725 in Chlorella corresponds to the 738-nm band in chloroplasts.

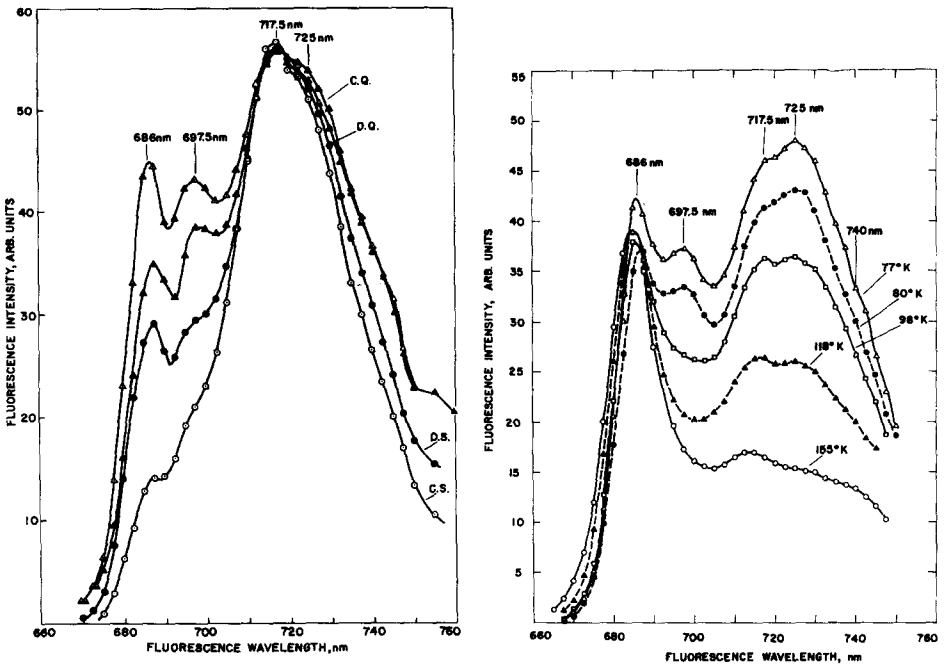


Fig. 1. Fluorescence spectra of *C. pyrenoidosa* at 77°K. D.Q. = dilute sample (absorbance, 0.1 at 675 nm) quickly (*i.e.* within seconds) cooled (open triangles with dots); D.S. = dilute sample slowly (within minutes) cooled (solid dots); C.Q. = concentrated (absorbance 0.8) sample quickly cooled (solid triangles); C.S. = concentrated sample slowly cooled (open circles with dots).

Fig. 2. Fluorescence spectra of *C. pyrenoidosa* in the 77°–155°K range. Wavelength of excitation = 485 nm.

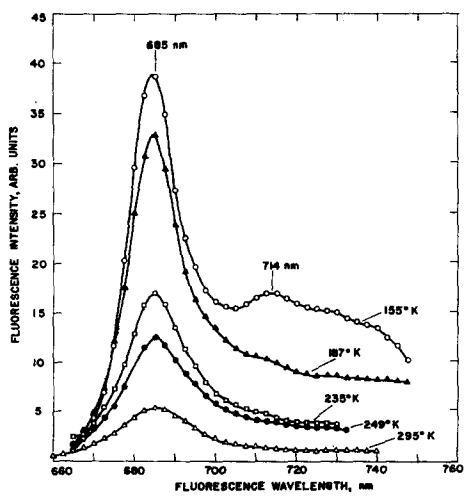


Fig. 3. Fluorescence spectra of *C. pyrenoidosa* in the 155–295°K range. Wavelength of excitation = 485 nm.

At about 150–155°K, where there is a change of ice phase, sharp changes in the fluorescence spectra and intensities occur (Figs. 2–4): (1) the f680 increases upon warming (from 80 to 150°K); (2) the intensity of F698 decreases rather rapidly upon warming (from 80 to 150°K); (3) the intensity of F687 — the intensity of which shows only a slight change in the 80–150°K range — decreases rapidly upon warming from 150 to 240°K; and (4) the F725 decreases faster than F717 as the temperature rises but on the whole f720 continuously decreases upon warming (from 80 to 240°K) and does not seem to be affected significantly by the change in the ice phase.

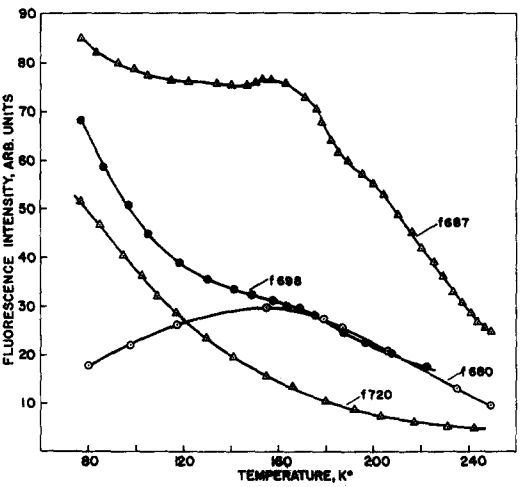


Fig. 4. Fluorescence intensities (f680, f687, f698 and f720) of *C. pyrenoidosa* as a function of temperature in the 80–240°K range. Wavelength of excitation = 485 nm.

At 155°K, the main fluorescence bands are at 684.5, 714, 725 and 740 nm. The F698 is hardly observable at this temperature. Spectra measured at 187, 235, 249 and 295°K show similar relative band heights (*i.e.* similar normalized spectrum) except that f714 is slightly decreased; the total fluorescence intensity decreases by 6–7-fold as the temperature increases from 187 to 295°K. Some bands appear as clear peaks only within a certain temperature range, but are shoulders or are hardly resolvable at other temperatures (Figs. 2–4). This is due to the different rate of change of fluorescence intensity of the various bands (Fig. 4). The long wavelength broad fluorescence band (F725) shows peaks at 714–717 and 740 nm at various temperatures confirming that the band discovered by BRODY[12] at about 720 nm, is composed of several sub bands.

The repeatability of the fluorescence spectrum at low temperatures

We examined whether the fluorescence spectra at 4 and 77°K can be repeated after warming up to 260°K, at which temperature most of the phase change has been completed. The purpose was to check if there is any relationship between the phase transitions of the ice crystals and the changes in the fluorescence spectra. The phase transitions from vitreous to cubic and then from cubic to hexagonal are irreversible. If these fluorescence changes are related to the changes in the phase of the ice one would expect the change of the latter to be irreversible also. Experimental results presented below confirmed this relation. Furthermore, from 4 to 130°K where there is no

phase transition of ice crystals, there is no sign of irreversibility of the fluorescence spectrum. Thus, for our data [9–11] in the 4 to 77 °K range, we are not concerned with the changes in the phase of the ice crystal. Most of the irreversibility occurs in the temperature range above 130 °K. One may, however, ask if the irreversibility of the fluorescence spectrum is caused by major changes in the cellular structure (*i.e.* by indirect mechanical stress) during phase transition. (Direct mechanical stress due to a volume change is not likely because hexagonal and cubic ice have equal density.) We compared the spectra at 77 °K after the cells had gone through the following cooling processes: (i) 295→77 °K, (ii) 295→77→260→77 °K, and (iii) 295→77→295→77 °K. Fig. 5 shows the results. If the decrease of F687 and F698 is directly caused by the phase transition, one should expect (ii) to be very different from (i), because ice cannot change from hexagonal back to vitreous or cubic phase by cooling (before melting). This is shown to be the case in Fig. 5. If the phase transitions of ice or the process of freezing the cells to low temperature did not cause major changes in the structure of the cells, one would expect the spectrum of (iii) to be very similar to that of (i). This is again found to be true. These results confirm that the change of phase of ice has a direct effect on the fluorescence spectrum. (However, repeated freezing and warming more than two times generally causes a shift of the bands, and the spectrum of (i) can no longer be repeated.)

One obvious interpretation for the reduction of F687 and F698 bands when the ice is in hexagonal phase is that there is an increased internal reflection. Consequently, F687 and F698 should be reabsorbed more due to their band overlap with chlorophyll *a* 678 absorption band. However, if there is a large increase in internal reflection, one would also expect an increase in the total absorption of the exciting light, which will in turn cause an increase in the long-wavelength fluorescence where no reabsorption takes place. (For complications due to secondary emission, see SZALAY *et al.*[14].) However, we see a decrease of f730 (Fig. 5). Fluorescence excitation spectra of F725 also show (Fig. 6) steady decrease of long-wavelength excitation when cells are warmed to

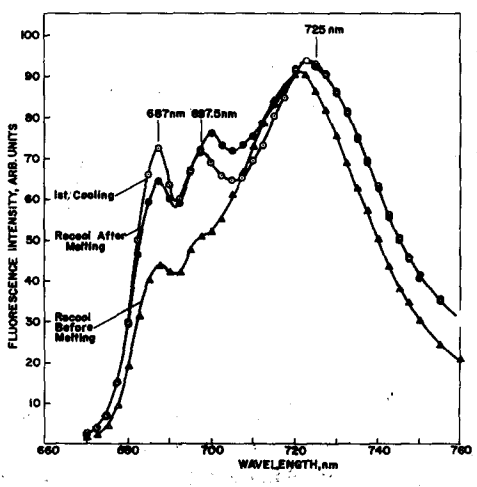
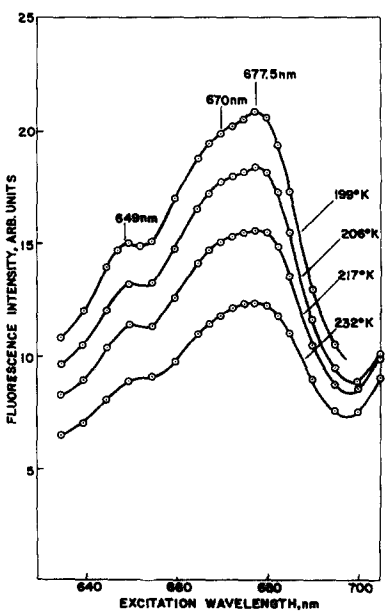


Fig. 5. Fluorescence spectra of *C. pyrenoidosa* at 77 °K. 1st cooling = sample cooled from 295 to 77 °K; recool before melting: sample first cooled to 77 °K, warmed up to 260 °K (*i.e.* not allowed to melt) and then recooled to 77 °K; recool after melting: sample first cooled to 77 °K, warmed up to 295 °K (*i.e.*, allowed to melt), and then recooled to 77 °K.

230°K suggesting no increase of absorption in the region when ice is in hexagonal phase. (For Chlorella, the excitation spectra at 77°K were first reported by BRODY AND BRODY[15] but no data were available in the red region; GOEDHEER[16] reported these spectra in the red region with bands at 672 and 682 nm.) Absorption at 680 nm is 2.2 times stronger than that at 687 nm. If decrease of F687 is caused by increasing internal reflection and reabsorption, one would expect the f680 to be suppressed by a much larger ratio (*i.e.* about 2.2 times more decrease than that of F687), but the ratio of suppression of f680 was less than that of F687. All these results confirm that there is a decrease of fluorescence intensities at 687, 698, and 720 nm (but not at 717 nm), as ice changes to hexagonal phase, and this fluorescence decrease is not caused by changes in the scattering properties or by the increase of reabsorption of fluorescence.

Fluorescence spectrum of chlorella treated with 10% dimethylsulfoxide

The experimental procedure was as follows. In order to prevent the exothermic (heating) effect of dimethylsulfoxide on the cells, it was first diluted 1 to 4 with water. This 20% dimethylsulfoxide solution was then added to an equal volume of Chlorella suspension. After 30 min of equilibration, the cells were cooled down, at the rate of 9°K/min, to 250°K, followed by quick cooling to 77°K. The fluorescence spectrum of these cells (Fig. 7) shows F687, F698 and F725 bands. We note that the F698 band is much better resolved in the dimethylsulfoxide-treated cells than in the normal cells. Since dimethylsulfoxide is believed to have strong affinity for water[17,18], the above result suggests that F698 is sensitive to the aqueous environment.



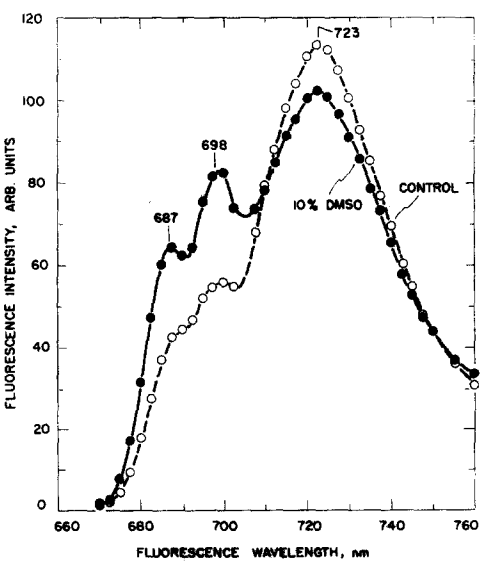


Fig. 6. Excitation of F725 of *C. pyrenoidosa* (in the 640–700 nm range) in the 199–232°K range. The last point around 705 nm is mainly due to the leakage of light.

Fig. 7. Fluorescence spectra of *C. pyrenoidosa*, treated with 10% dimethylsulfoxide (DMSO) at 77°K. Control (= untreated sample) of the same concentration (*i.e.* absorbance) is shown for comparison.

It has been suggested[19,20] that F698 is from the trap of System II. At low temperatures, conditions are favorable for detecting fluorescence from "traps": internal conversion is greatly decreased, steady-state photosynthesis is completely stopped and the trap depth is much greater than kT (where k = Boltzman constant). We have shown here that F698 is sensitive to the change of the phase of ice and the addition of polar molecules. Our results are, thus, consistent with the view that F698 is linked to a trap that is highly sensitive to the water environment. These properties, our suggestion[9] that F698 originates in a molecular species (present in very low concentrations) absorbing at about 688 nm, and the recent experiments of MURATA[21] and of DONZE AND DUYSENS[22], are in agreement with the assignment that F698 is from a trap species. DÖRING and co-workers[23–25] have recently discovered an absorbance change due to the active chlorophyll *a* in System II with peaks at 682 nm (in System II particles) and at 690 nm (in chloroplasts). We consider it likely that DÖRING's active chlorophyll *a* II (present in small quantities, λ is about 690 nm), F698 (at low temperatures) and F693 (ref. 26) (at room temperature and high intensities in Porphyridium) are related components.

Viability of frozen cells

The viability of frozen cells was tested by inoculating cells and observing growth. We failed to get any viability of Chlorella cells after they were frozen (without dimethylsulfoxide) regardless of the rate of cooling. However, when cooled in the presence of dimethylsulfoxide, cells were viable after they were warmed. The protective effect of dimethylsulfoxide was found to be better than that of glycerol, probably, because the former can penetrate the cells more readily[18]. The mechanism of the protection by dimethylsulfoxide is not clear; however, it is believed to be caused by its affinity for water[17]. The viability of these cells after thawing suggests that the internal structure of the cells was not severely distorted or damaged during freezing to 77°K.

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