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A LONG-WAVE ABSORBING FORM OF CHLOROPHYLL *a* RESPONSIBLE FOR THE "RED DROP" IN FLUORESCENCE AT 298 °K AND THE F723 BAND AT 77 °K

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SUMMARY

When Chlorella cells are ruptured at pH 4.6 by sonication in air, its absorption spectrum can be best explained if one assumes that a long-wave chlorophyll *a* form (Chl *a* 693) is preferentially destroyed. Using these preparations, and comparing them with the algal suspension and the sonicates prepared at pH 7.8 under argon, we make the following conclusions: (a) The red drop beginning at about 675–680 nm in the action spectrum* of fluorescence at 298 °K must be due to the presence of a non- (or weakly) fluorescent form of chlorophyll *a*. We suggest that this form is Chl *a* 693. The red drop is absent in the aerobic sonicates. (b) The red drop in fluorescence in whole algal cells is not due to any errors in absorption measurements; this drop is clearly present in the anaerobic sonicates. (c) The emission band at 723 nm, discovered by S. S. BRODY in whole Chlorella cells at 77 °K, may be due to increased fluorescence efficiency of Chl *a* 693 at low temperature; the F723 band is absent in aerobic sonicates.

INTRODUCTION

Since the discovery of two pigment systems[1,2] in photosynthesis, several attemps[3–10] have been made to separate the pigment complexes which are postulated to perform light reactions I and II.

In the present work, we ruptured Chlorella cells by sonication with the hope of preferentially destroying pigment system I or II. We report here the fluorescence properties of the cell-free sonicated material, prepared in presence of air at pH 4.6, at room and liquid-nitrogen temperatures. It shows a loss of Chl *a* 693 (pigment system I?) accompanied by the absence of the red drop in the action spectrum of fluorescence (at 298 °K) and of the emission band at 723 nm (at 77 °K). These spectroscopic properties of the sonicate prepared under anaerobic conditions (pH 7.8), however, remain unchanged when compared with those of the algal suspension.

METHODS

Chlorella pyrenoidosa was grown in inorganic medium for several days as described in ref. 11. Algal cells were suspended in the culture medium, the pH of which

* Φ(fluorescence yield) = f(λ).

was adjusted to 4.6. A 50-ml suspension was sonicated for 30 min in a Raytheon sonic oscillator, model D.F. 101 in the presence of air. The pH remained unchanged after sonication. These samples are referred to as aerobic. In other samples, the pH of the culture medium was adjusted to pH 7.8 and the suspension was sonicated under an atmosphere of O_2-free argon (99.995 %). The pH remained unchanged after sonication. These samples are labelled anaerobic. In both cases, the sonicator was operated at 10 kcycles and 200 W. During sonication the temperature was maintained at 290 °K. A 20-ml sample of the sonicated suspension was centrifuged at 3600 rev. per min for 5 min and the supernatant liquid was called sonicated material. This material was cell-free. The residue, containing unbroken cells and cell debris, was discarded.

The absorption spectra of the original algal suspension and the sonicated material were measured on a Bausch and Lomb recording spectrophotometer (Spectronic 505) equipped with an integrating sphere. The optical cell had a path length of 1 cm. The absorbance values for the path length (d) of the excitation light in the sample were obtained by multiplying the absorbance values at 1-cm path length by d. These absorbance values were converted to percent absorption values.

The emission and the excitation spectra of fluorescence were measured by a spectrofluorometer[10–14] with a bandwidth of 6.6 nm. A Corning C.S. 2-73 filter was used in front of the analyzing monochromator when excitation spectra were measured at 77 °K. When emission spectra of 77 °K were measured, Corning C.S. 4-72 filter was used before the exciting light (480 nm) hit the sample. In other cases, no filter was used with the exciting monochromator. The emission spectra were corrected for the spectral sensitivity of the photomultiplier and the transmission efficiency of the analyzing monochromator. The excitation spectra were corrected for the variations in the incident quanta at different wavelengths.

The quantum yield of chlorophyll *a* fluorescence as a function of wavelength of exciting light was obtained by dividing the excitation spectra of fluorescence by the percent absorption spectra. We collect only a small fraction of total quanta emitted, and thus the quantum yields of fluorescence are presented in relative units.

RESULTS AND DISCUSSION

Shift of the red absorption maxima; loss of Chl a 693 in the aerobic sonicates

Fig. 1 shows the absorption spectra of Chlorella suspension and the aerobic sonicated material. Although the position of the peak at 437 nm remains unchanged, a shift of 5 nm towards the shorter waves is noticed in the red absorption peak in the sonicated material. Preferential disruption of a long-wave pigment complex may be responsible for the shift in absorption peak mentioned above. Anaerobic sonicated material did not show any significant shift in the location of the red band (Fig. 2, I). Difference absorption spectrum (Fig. 2, III) between the anaerobic and aerobic sonicated material shows a peak at 693 nm. BROWN AND FRENCH[15] earlier suggested the existence of a chlorophyll *a* band in this wavelength range. Our results can be best explained in terms of the preferential loss of this long-wave form of chlorophyll *a* and some carotenoids with peaks at 440 and 493 nm in the aerobic low pH sonicate.

There is a lower value (1.55) of the ratio of absorbance at 678 nm (chlorophyll *a*) to 650 nm (chlorophyll *b*) in the aerobic sonicated material than in the suspension

(1.79). This indicates lesser relative amount of chlorophyll *a* in this sonicated material. This is not due to the decreased sieve effect (see ref. 16, p. 1863–1866, Vol. II, Part II) which would cause a sharpening and an increase in the intensity of the main band, resulting in a higher value of the ratio of absorbance at 678 to 650 nm in the sonicated material than in the suspension[17].

Absence of pheophytins in the sonicates

One question may arise here, whether there is some formation of pheophytin due to loss of magnesium in the low pH aerobic sonicate. To check this, we have measured the absorption spectra of the methanol extracts of the aerobic and anaerobic

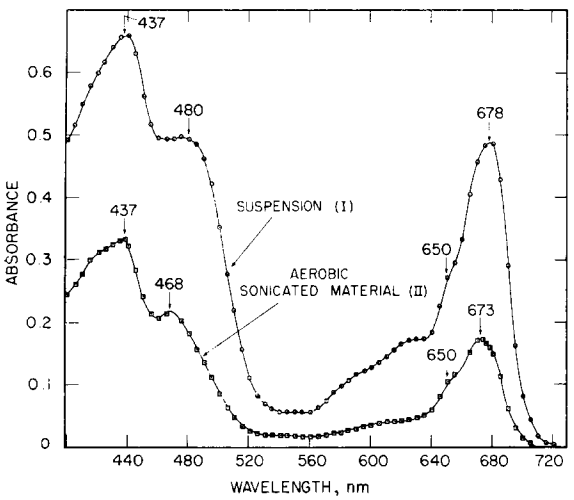


Fig. 1. Absorption spectra of cell suspension of *Ch. pyrenoidosa* and of the aerobic sonicated material (pH 4.6).

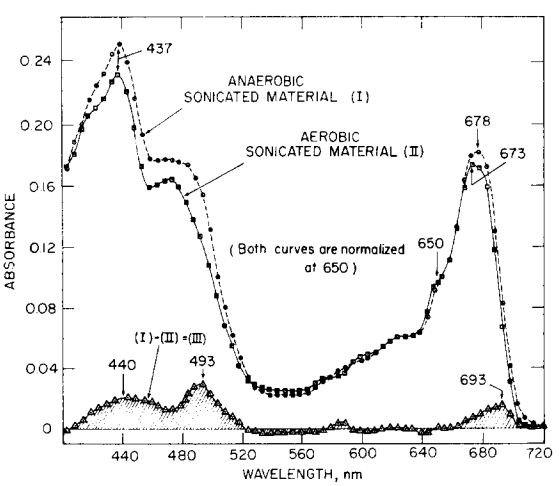
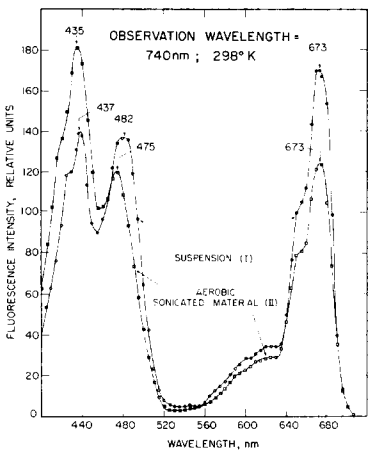


Fig. 2. Absorption spectra of aerobic (pH 4.6) and anaerobic (pH 7.8) sonicated material and the difference spectrum between the two*.

* *Note added in proof:* The absorbance value at 703 nm of the aerobic sonicated material is 0.147; the value of 0.046 is in error. (Received October 9th, 1967).

sonicated material. GOEDHEER[18] has shown that the formation of pheophytin from chlorophyll in ether solution is accompanied by (i) a shift of the red absorption maximum towards the longer wavelength, (ii) a shift of the blue maximum toward the shorter wavelength and (iii) the formation of new peaks in the 500–600 nm region. A comparison of the solution spectra of the aerobic and anaerobic sonicated material did not show these changes. We can thus assume that the pheophytin is absent in the low pH sonicated material. Although the formation of pheophytin in traces cannot be disproved, most of the pigments remaining in the sonicated material are chlorophyll *a*, *b*, and carotenoids.



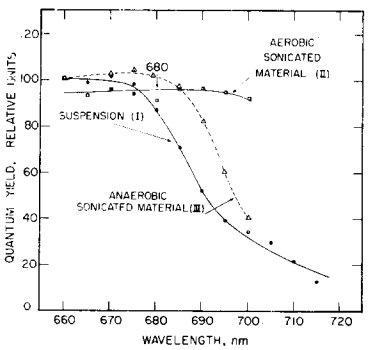


Fig. 3 (upper left). Excitation spectra of cell suspension of *Ch. pyrenoidosa* and of the aerobic sonicated material (pH 4.6) at room temperature (298 °K). λ observation = 740 nm.

Fig. 4 (upper right). Fluorescence yield (quanta emitted/quanta absorbed) of the suspension of *Ch. pyrenoidosa*, aerobic (pH 4.6) and anaerobic (pH 7.8) sonicated materials at room temperature. The scale for the quantum yield is in arbitrary units.

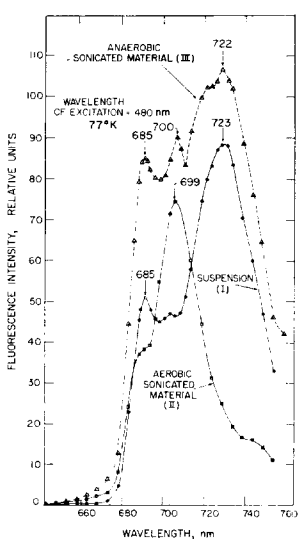


Fig. 5. Emission spectra of cell suspension of *Ch. pyrenoidosa*, aerobic (pH 4.6) and anaerobic (pH 7.8) sonicated materials at liquid nitrogen temperature (77 °K). λ excitation = 480 nm.

Variations in the transfer efficiencies from the accessory pigments to chlorophyll a

Fig. 3 shows the excitation spectra, measured at 740 nm, of the suspension and the aerobic sonicated material at room temperature (298 °K). The excitation spectrum of the suspension (Fig. 3, I) shows 3 peaks: at 673 nm (due to chlorophyll *a*), 482 nm (chlorophyll *b* + carotenoids), 437 nm (chlorophyll *a* + carotenoids) and a shoulder at 650 nm (chlorophyll *b*). In the aerobic sonicated material (Fig. 3, II), the same peaks are observed at 673, 475 and 435 nm.

A comparison of the excitation spectra of the fluorescence and absorption spectra suggests that on sonication, the efficiency of energy transfer from chlorophyll *b* to chlorophyll *a* did not change significantly. In suspension and in the sonicated material, the ratio of fluorescence yields (Φ) at 650 nm (chlorophyll *b*) and 675 nm (chlorophyll *a*) is approx. 1.0. However, it seems that the transfer efficiency from carotenoids to chlorophyll *a* has changed since Φ measured at 740 nm excited by 675 nm (chlorophyll *a*) to that excited at 435 nm (chlorophyll *a* + carotenoids) was 1.70 and 1.33 in the cell suspension and in the sonicated material, respectively.

Red drop in chlorophyll a fluorescence

DUYSENS[19] measured the quantum yield of fluorescence as a function of excitation wavelength in dense suspensions of Chlorella. He observed a decline in the quantum yield of fluorescence at wavelengths longer than 675 nm (the red drop). TEALE (cited by WEBER[20]) has shown that optically dilute suspensions of Chlorella in sucrose do not show red drop up to 690 nm. TEALE had no measurements beyond 690 nm. We have calculated the quantum yield of chlorophyll *a* fluorescence measured at 740 nm, as a function of excitation wavelengths of light (660 nm) in optically dilute (A for 1-mm path at 680 nm = 0.01 to 0.05) suspensions of Chlorella. Fig. 4 shows the plot of the quantum yield of chlorophyll *a* fluorescence, in relative units, adjusted to give the same value at 660 nm, as a function of excitation wavelength in the aerobic and anaerobic sonicated materials and in the Chlorella suspensions. The red drop in chlorophyll *a* fluorescence begins at 675 nm in the Chlorella suspensions (Fig. 4, I). The red drop in photosynthesis is known to begin at about 680 nm (ref. 21). The red drop in Chlorella fluorescence was recently found by SZALAY *et al.*[22], by an independent method which uses data on absorption and the emission intensities in the region of the overlap of the two spectra, also to begin at about 680 nm. The exact location of the red drop in Chlorella fluorescence as measured in several experiments is not exactly the same. It varies from 668 to 680 nm. Perhaps, the history of the algal culture and the intensity of the exciting light effect the location of the red drop in fluorescence.

That the beginning of the decline in the quantum yield of fluorescence at 675–680 nm in algal suspensions is not due to any errors caused by scattering of light in the suspensions, is clearly shown by the presence of a decline at about 680 nm in the cell-free sonicated material prepared under anaerobic conditions (Fig. 4, III). In the aerobic sonicated material (Fig. 4, II), however, there is no red drop in fluorescence.

It is generally believed that the red drop in chlorophyll *a* fluorescence is due to the existence of a weakly fluorescent form of chlorophyll *a* (see ref. 19). We suggest that the disappearance of the red drop of fluorescence in the aerobic sonicated material is due to the preferential loss of Chl *a* 693 which is weakly fluorescent at room temperature.

The anaerobic sonicates (sonicating time, 30 min; pH 7.8) used in the present work show a red drop in fluorescence beginning at about the same location as in Chlorella suspensions. We have observed that sonication for longer times (90 min) extends the location of the red drop even in anaerobic preparations (pH 7.8) to about 690–695 nm. However, when the pH of the medium, during sonication is acidic (pH 4.6), the red drop is absent even in anaerobic preparations when sonicated for longer times (60–90 min). On the other hand, the red drop in fluorescence is present in aerobic sonicates if the pH of the medium, during sonication, is made alkaline (pH 7.8). These results suggest that during sonication both O_2 and hydrogen ions somehow affect the destruction of non- (or weakly) fluorescent form of chlorophyll *a*.

Absence of F723 in sonicates that lack Chl a 693

Fig. 5 shows the emission spectra of Chlorella suspension and the aerobic and anaerobic sonicated materials, at 77 °K. For review of earlier work at 77 °K see refs. 12 and 13. In these experiments, one drop of the sample, under a cover slip, was frozen on the bottom window of the Dewar flask and then covered with liquid nitrogen to a depth of 5 cm. In each experiment the total intensity of fluorescence was several fold greater than at room temperature. In case of the suspension, 2 main peaks at 685 nm (F685) and at 723 nm (F723), and a less prominent peak at 697 nm (F697) are observed (Fig. 5, I and Table I). In the aerobic sonicated material (Fig. 5, II), F723 is absent but a strong band at 699 nm (F699) and a shoulder at 682 nm are present.

The absence of F723 and the increase in the intensity of F697 in the aerobic sonicated material may be due to the lack of energy transfer from the short-wave absorbing species to that which fluoresces at 723 nm. This must be so if the latter species is preferentially destroyed by sonication. Absorption data (Fig. 2) have been explained in terms of the loss of Chl *a* 693 in aerobic sonicates. It has been suggested[13,23] that F723 belongs to pigment system I and F700 (also referred as F696) to pigment system II. Thus, the cell-free extract, prepared by sonication in air at pH 4.6, is poor in system I.

We raise the question whether the 685 nm fluorescence band is not significantly absorbed within single Chlorella cells, causing an artificial increase in the ratio of F723 to F685. One may suggest that in the aerobic sonicated material Chlorella cells are broken into extremely small fragments, and the problem of reabsorption of the

TABLE I

DATA FROM EMISSION SPECTRA AT 77 °K

Excitation wavelength, 480 nm. F refers to fluorescence intensity and the number following it to the wavelength of observation in nm.

Sample	*Peaks (P) and shoulders (S) (nm)*	*Ratio of fluorescence intensities*	
		$\frac{F723}{F685}$	$\frac{F699}{F685}$
Chlorella suspension	685 (P), 697 (S), 723 (P)	1.73	0.91
Anaerobic sonicate	685 (P), 700 (P), 716 (S), 722 (P)	1.25	1.04
Aerobic sonicate (Chl *a* 693 is absent)	685 (S), 699 (P)	0.62	1.94

685 nm fluorescence band within each particle is reduced and thus the ratio of F723 to F685 decreases tremendously. However, this is not the case since the anaerobic sonicated material gives results similar to those of the cell suspension (Fig. 5, III). However, the ratio of F723 to F685 is lower in the anaerobic sonicated material than in the suspension of Chlorella cells. This may mean that even in anaerobic sonicates either (a) reabsorption of 685 nm fluorescence distorts to some extent the emission spectrum of Chlorella cells (at 77 °K) or (b) there is some loss of Chl *a* 693.

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