SHORT COMMUNICATIONS

BBA 43182

Chlorophyll fluorescence near 720 mμ in Euglena extracts

Fluorescence emission spectra and the induction kinetics of fluorescence in algae and chloroplast fragments can be used as an index of the activity and physical-chemical state of chlorophyll *in vivo*[1]. The emission maximum of chlorophyll fluorescence in most plants is near 680 mμ and comes from a form of chlorophyll absorbing near 670 mμ (C*a*670). The chlorophyll form absorbing near 680 mμ (C*a*680) is largely nonfluorescent at room temperature, and may or may not be the source of emission near 690 mμ at very low temperature (—196°)[2]. The long wavelength chlorophyll form, C*a*695, fluoresces near 720 mμ in intact cells of Ochromonas[3], Euglena[4] and Phaeodactylum[5].

GOEDHEER[6] demonstrated the artificial enhancement of the long wavelength emission from the vibrational level of chlorophyll *a* dissolved in methanol when measured at —196° in a concentrated preparation due to reabsorption of the emission at shorter wavelengths. This raises the question whether the large emission at long wavelengths and low temperature observed in most plant cells is due to C*a*695 or to reabsorption of shorter wavelengths.

In this paper, we compare the emission at 720 mμ from C*a*695 with a similar emission from a concentrated extract not containing C*a*695.

Of the three species of algae mentioned above which have been shown to contain C*a*695, it has been possible only in Euglena to break the cells and still retain a significant proportion of C*a*695[7] and long wavelength fluorescence[8].

The Euglena extract was prepared as previously described[7] except that the cells were broken in a buffer containing NaCl, 0.35 M; EDTA, 0.01 M; Tris–HCl, 0.02 M (pH 7.4). The supernatant from the centrifugation at 10000 × *g* for 30 min, used for this experiment, contained 170 μg total chlorophyll per ml.

An aliquot of the green extract was treated with the proteolytic enzyme, protease (from *Streptomyces griseus*, Type VI, Sigma Chemical Co.), at a concentration of 1 μg protease per 1 μg chlorophyll at 33°. After 30-min incubation under nitrogen the sample was cooled to 4° and measured as soon as possible.

Both the absorption and fluorescence emission spectra were measured in cuvettes with a metal finger projecting into liquid nitrogen to cool the samples to approx. —195°. The spectrophotometer and special cuvettes described previously[9] were designed to minimize light scattering errors in the recorded spectrum.

The spectrofluorimeter[4] automatically corrects the recorded spectra for the changes in sensitivity of the apparatus with wavelength. For excitation the 436-mμ mercury line was isolated with Balzer filter No. 435 and Corning filter No. 9782.

For the fluorescence spectra the extract, before and after enzyme treatment, was diluted with the same buffer as used for the extraction and mixed with $BaSO_4$ powder. This white paste was placed in a 0.2-mm deep groove in an aluminum bar

and covered with a microscope glass coverslip. We routinely add $BaSO_4$ to samples to minimize the changes in fluorescence yield upon freezing due to increased light scattering. Since only frozen samples were compared in this particular experiment, the precaution was not relevant.

Fig. 1 illustrates the absorption spectra measured at —195° of the Euglena extract before and after enzymatic digestion. Clearly the enzyme caused a preferential destruction of *Ca*695. There was also an increase in absorption at 670 mμ.

Fluorescence emission spectra at —190° of the extract before treatment are shown in Fig. 2 and after enzymatic digestion in Fig. 3. A 10-fold dilution of the untreated extract which contained *Ca*695 had little effect on the shape of the emission spectrum. But after the destruction of *Ca*695, dilution to a similar chlorophyll concentration (2 μg/ml) caused a marked reduction in the long wavelength fluorescence relative to the 683-mμ maximum. Obviously measurement of a too concentrated sample can cause an apparent emission maximum nearly identical to the real emission band of *Ca*695.

A comparison of the total emission between the more dilute samples of Figs. 2 and 3 was made by measuring the area under the curves and calculating the relative

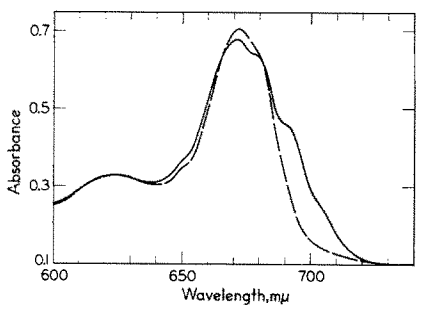

Fig. 1. Absorption spectra of aqueous Euglena extract measured at —195° before (———) and after (— — —) enzymatic digestion.

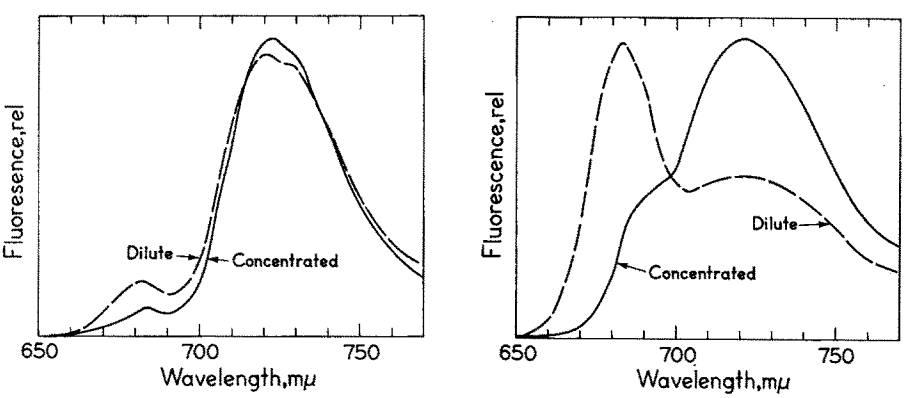

Fig. 2. Fluorescence emission spectra of an aqueous Euglena extract mixed with $BaSO_4$ measured at —190°. Excitation at 435 mμ. Concentrated (———) 17 μg, dilute (— — —) 1.7 μg chlorophyll per ml extract.

Fig. 3. Fluorescence emission spectra of an aqueous Euglena extract after digestion with protease, mixed with $BaSO_4$ measured at —190°. Excitation at 435 mμ. Concentrated (———) 56 μg, dilute (— — —) 2.0 μg chlorophyll per ml extract.

response of the fluorimeter. The total fluorescence on a chlorophyll basis of the original extract was nearly 5 times greater than that of the treated extract. This suggests: First, that the protease does not break up the pigment complex into a highly fluorescing form as do some detergents, and, second, C*a*695 at low temperature has a high fluorescence yield.

These results emphasize the practical difficulty in distinguishing between the real emission by C*a*695 and the artificially enhanced emission caused by reabsorption within chloroplasts. An approximate calculation of the chlorophyll concentration in Euglena chloroplasts from the data in ref. 10 gives a value of about 30 μg/ml. The chlorophyll concentration in the samples for the curves in Fig. 3 were 56 and 2 μg/ml, respectively. An accurate comparison between chloroplasts within a cell and our extract mixed with $BaSO_4$ is impossible, but at least the chlorophyll concentrations are in the same order of magnitude. Probably in most algae, the apparent enhancement of the long wavelength fluorescence relative to the main emission maximum observed when spectra measured at room temperature and low temperature are compared results from reabsorption of the shorter wavelengths by chlorophyll.

However, the existence of C*a*695 and its corresponding fluorescence has been demonstrated in three species of algae. The simplest test for C*a*695 is to compare the fluorescence near 720 mμ before and after some mild deleterious treatment such as freezing and thawing, heating to 40° for 10 min, or incubation for several hours in 10 % ethanol. These treatments should destroy C*a*695 and decrease F 720 but have no effect upon the artifact due to reabsorption.

These data illustrate why caution must be exercized when citing long wavelength fluorescence as an index of chlorophyll activity or form *in vivo*.

One of the authors (M. R. M.-W.) is a Fellow of Carnegie Institution of Washington, 1967.

*Department of Plant Biology**,
Carnegie Institution of Washington,
Stanford, Calif. (U.S.A.)

JEANETTE S. BROWN
M. R. MICHEL-WOLWERTZ**

1 W. L. BUTLER, in L. P. VERNON AND G. R. SEELY, *The Chlorophylls*, Academic Press, New York, 1966, p. 343.
2 G. PAPAGEORGIOU AND GOVINDJEE, *Biophys. J.*, 7 (1967) 375.
3 J. S. BROWN, *Carnegie Inst. Wash. Yearbook*, 66 (1967) in the press.
4 J. S. BROWN, *Carnegie Inst. Wash. Yearbook*, 65 (1966) 483.
5 J. S. BROWN, *Biochim. Biophys. Acta*, 143 (1967) 391.
6 J. C. GOEDHEER, *Biochim. Biophys. Acta*, 88 (1964) 304.
7 J. S. BROWN, *Carnegie Inst. Wash. Yearbook*, 60 (1961) 366.
8 J. S. BROWN, *Biochim. Biophys. Acta*, 120 (1966) 305.
9 C. S. FRENCH, *Carnegie Inst. Wash. Yearbook*, 66 (1967) in the press.
10 J. S. BROWN, *Carnegie Inst. Wash. Yearbook*, 59 (1960) 331.

Received September 22nd, 1967

* Publication No. 428.
** Permanent address: Laboratory of Plant Physiology, Research Station of Gorsem, Sint Truiden, Belgium.