THE LONG WAVELENGTH FORMS OF CHLOROPHYLL *a*

JEANETTE S. BROWN *and* C. STACY FRENCH

From the Department of Plant Biology, Carnegie Institution of Washington, Stanford, California

ABSTRACT When *Euglena gracilis* is cultured with light of low intensity (*ca.* 250 ft-c), an absorption band at 695 mμ is formed in an amount equal to about 20 per cent of the total chlorophyll absorption in this red region. An equally large proportion of C_a695 is observed in *Ochromonas danica,* irrespective of light intensity. Other algae tested appear to contain approximately 3 to 5 per cent of their chlorophyll as C_a695; this proportion does not increase as strikingly with lowering of the light intensity as it does in *Euglena.* C_a695 bleaches more readily than the other chlorophyll forms both reversibly, in whole cells, and irreversibly, in homogenates. Cells containing a large proportion of C_a695 have a fluorescence maximum at 708 mμ, as contrasted to the 687 mμ maximum in other algae. Occasionally, old cultures of *Euglena* contain cells with an absorption band at approximately 710 mμ. This absorption band is quite stable in aqueous extracts; when the pigment is transferred to ether an equivalent amount of pheophytin *a* is found to be present. Conditions leading to the formation of the 710 mμ absorption band are not yet known.

INTRODUCTION

The study of chlorophyll *a* in living plants and aqueous green extracts has revealed the presence of two forms of this pigment (1). These forms, with absorption peaks at about 673 and 683 mμ, occur in various proportions. They may be seen clearly in the derivative absorption spectra of typical green algae (2). In *Chlorella* the proportions of these two forms may be changed by rupturing the cells and partially bleaching the aqueous extract (3). A third form of chlorophyll *a,* having an absorption maximum at approximately 695 mμ, is evident in *Euglena gracilis* (4), *Ochromonas danica* (5), and in some other algae, including *Scenedesmus,* when the derivative spectra are measured at liquid air temperature (6). Chlorophyll *a* 695 is of particular interest in photosynthesis because the light it absorbs appears to be used only in conjunction with light absorbed by accessory pigments (7).

In this paper we will discuss the culture conditions which favor the formation of C_a695 in *Euglena* and some of its characteristics, in particular its photostability.

Another absorption band, at about 710 mμ, occasionally observed in *Ochromonas* and a *Chlorella* mutant (8) is here reported for *Euglena*. The conditions leading to its formation are not known, but its presence coincides with a decrease in absorption at 695 mμ and an increase in the amount of pheophytin *a*. Some observations on this pigment will also be presented.

CULTURE CONDITIONS

Euglena gracilis "T," (Chick, Emerson, Starr Collection No. 752) was grown in light, in the synthetic medium of Cramer and Myers (9) and in the dark, in the organic medium recommended by Brawerman and Chargaff (10).

Several arrangements for varying the light intensity during the growth of the cultures gave similar results. The following arrangement due to Myers (11) was found most convenient. 50-ml test tubes were suspended in a 26°C water bath from a rack. 5 per cent carbon dioxide in air bubbling through the medium kept the culture stirred. Maximum illumination was provided by a bank of three Westinghouse, cool-white, 20 W fluorescent bulbs on each long side of the rectangular water bath. The illumination at the position of the tubes was approximately 700 ft-c from one side and 500 ft-c from the other. By means of wire screens, the illumination on individual tubes could be reduced. The average intensity of light incident on the individual *Euglena* cell decreases during the growth of the culture due to mutual shading. Therefore the cells were harvested before the culture density became so great as to influence markedly the amount of light received by each cell.

RESULTS AND DISCUSSIONS

Properties of C_a695 in Whole Cells

The Effect of Culture Conditions on C_a695 Formation. The derivative absorption spectra of *Euglena gracilis* cells, grown at high (*ca.* 1200 ft-c) and at low (*ca.* 250 ft-c) light intensities, are shown in Fig. 1. The presence of a pigment with an absorption maximum of 695 mμ is indicated by a minimum in the derivative spectrum at about 700 mμ. The accumulation of C_a695 has been observed in several varieties of *E. gracilis,* but not in *E. mutabilis*. The positions of the other peaks and shoulders on these curves indicate the presence of the same chlorophyll *a* forms as described previously for *Chlorella* (3). The cells grown with light of low intensity have a much greater proportion of the 695 mμ form than have those grown with light of high intensity.

If *Euglena* is grown with low light intensity until the maximum proportion of C_a695 has appeared (about 1 week) and then placed in strong light for a week, C_a695 gradually disappears and the cells become yellowish, oval in shape, and less motile. However, a culture grown in high light intensity for the same total length of time is normal in appearance and motility.

Euglena grown in the dark is colorless. If these colorless cells are placed in dim light in the same growth medium, the chlorophyll formed shows a spectrum like

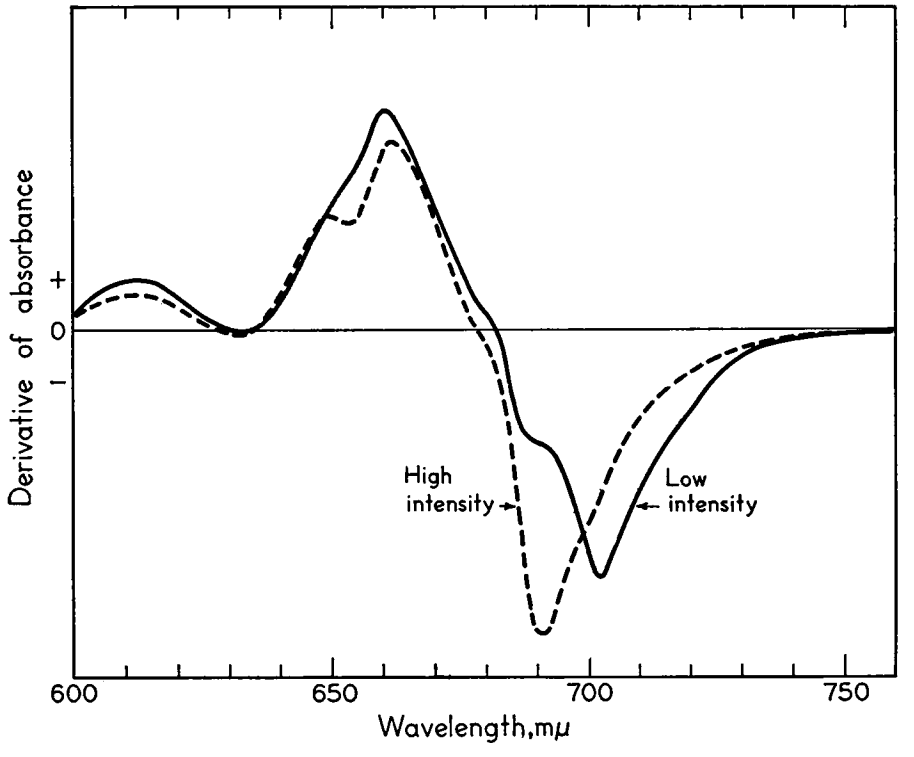

FIGURE 1 Derivative absorption spectra of *Euglena* cells grown with high and low light intensities.

that of cells grown in low light. Furthermore, colorless cells placed in high light intensities develop a chlorophyll spectrum typical of cells grown at high light intensities. However, if these dark-grown cells are resuspended in a nitrogen-free medium prior to illumination, no C_a695 is found, regardless of the intensity. In other words, C_a695 does not form when growth or nitrogen assimilation cannot occur.

C_a695 Formation in Relation to Chlorophyll Concentration. A study was made of the relationship between the total amount of chlorophyll per cell and the formation of C_a695. For this purpose chlorophylls *a* and *b* were measured together according to the method of Comar and Zscheile as described by Smith and Benitez (12). The cell number was determined by hemocytometer counts in aliquots of *Euglena* cultures in the logarithmic growth phase. These measurements and the corresponding derivative absorption spectra were determined for cells grown with a series of light intensities. The cultures obtained at the lowest intensity (*ca.* 50 ft-c) had the maximum amount of C_a695 and contained nearly three times as much chlorophyll *a* per cell as those grown at 1200 ft-c, which had no measurable C_a695. Thus it appears that when *Euglena* grows slowly with a low

light intensity, the cells accumulate more chlorophyll and produce an increased proportion of C_a695. This proportion may amount to approximately 20 per cent of the total chlorophyll content as determined by analysis of the derivative spectra (3).

In contrast with our findings in *Euglena,* Myers (13) reported that the amount of chlorophyll per cell does not vary when *Chlorella* is grown with different light intensities. It is therefore possible that the correlation between total chlorophyll and C_a695 accumulation found in *Euglena* is not typical of all algae.

A number of green algae, including *Chlorella, Chlamydomonas,* and *Dunaliella;* the yellow-greens *Botrydiopsis* and *Tribonema;* the diatom, *Navicula;* the blue-green, *Anacystis nidulans;* and the red alga, *Porphyridium cruentum* were compared after growth with high and low intensity. The derivative spectra of *Chlorella* and *Dunaliella* do show a small increase in the proportion of C_a695 at the lower light intensity, but this increase is not evident in the other algae studied.

The Fluorescence Spectrum of C_a695. The presence of C_a695 can be seen in the fluorescence spectra of intact cells. The fluorescence spectra of young, intermediate, and old *Euglena* cultures were compared previously (14). In that work the curves were not corrected for the spectral sensitivity of the photomultiplier. Therefore, the fluorescence spectra of *Euglena* grown in low and high light intensities, as measured with the correction device in operation, are now presented in Fig. 2. The exciting wavelength was 436 mμ. The fluorescence maximum of the cells grown with low light intensity is about 710 mμ. This contrasts with the maximum at 687 mμ characteristic of *Euglena* grown with strong light, as well as of common green algae and leaves. Both spectra appear to be made up of the same components, but in very different proportions. Since the cells high in C_a695 also contained more total chlorophyll *a,* the possibility exists that the 687 mμ peak in these cells is masked by reabsorption within the individual cells. The fluorescence spectra of dense cultures show an increase in relative intensity in the 730 mμ region, due to reabsorption of shorter wavelengths (15). This effect is, however, strikingly different from the appearance of the 710 peak seen here.

The fluorescence spectra of three different strains of *Amphora* are also shown in Fig. 2. One of these (Starr No. 689) shows very little of the 708 mμ fluorescence peak from C_a695 while No. 688 shows a trace of it. In No. 685 the 708 mμ peak is predominant. These spectra are sharper than those of *Euglena* probably because of the high chlorophyll content per cell in *Euglena.* The cell concentration in all the measuring vessels was low enough to avoid distortion of the spectra by reabsorption in other cells.

The different proportions of fluorescence from the various chlorophyll *a* components in these three curves may result from differing degrees of energy transfer from one component to another, as well as from differing proportions of the various forms that are present. Derivative absorption spectra of the *Amphora* cultures 688

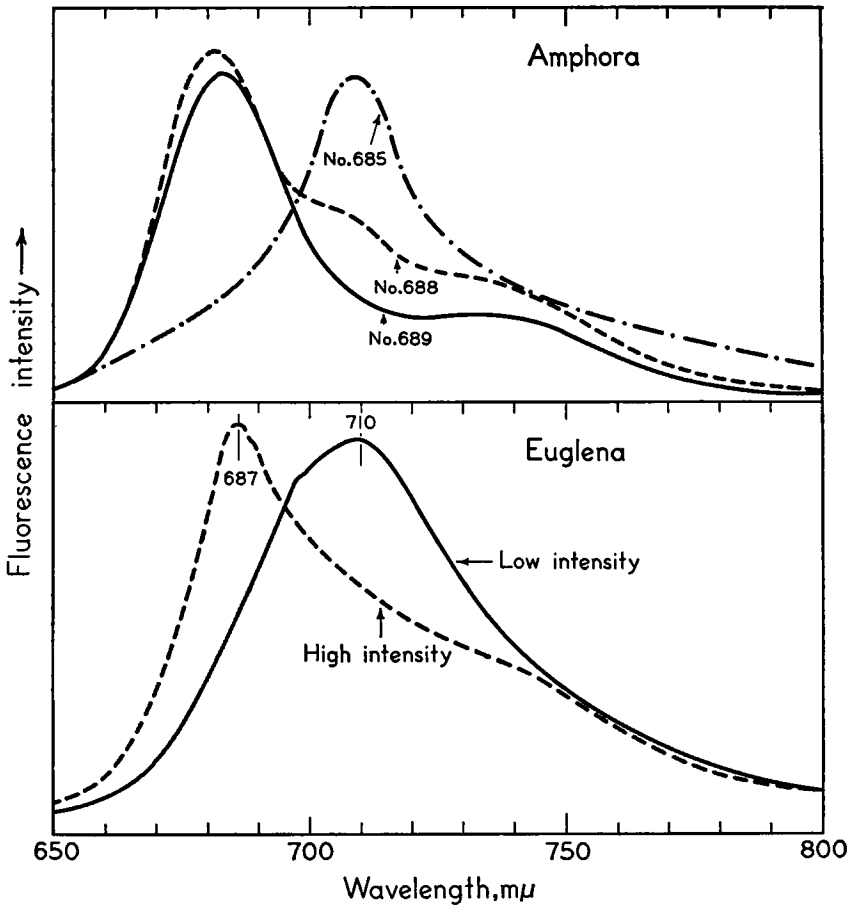

FIGURE 2 Fluorescence spectra of plants grown with different light intensities and containing various amounts of C_a695. The fluorescence peak of C_a695 lies at 705 to 708 mμ. The *Amphora* cultures were from the Starr collection and are identified by Starr's number.

and 689 were identical, suggesting that the stronger C_a695 fluorescence in No. 688 may have been due to a greater efficiency of energy transfer in this species. Strain No. 685 had a slightly greater absorption in the 695 mμ region than the other two strains.

Recently Butler (16) has observed a fluorescence band at 720 mμ in bean leaves when the measurements were carried out at −196°C. He attributes this band to the excitation of a chlorophyll form absorbing at 705 mμ. Krasnovskii (17) has observed a 710 mμ fluorescence band at low temperature in etiolated leaf homogenates which disappears after a brief illumination. Whether these bands are analogous to the C_a695 fluorescence at 710 mμ in algae and diatoms is not yet clear.

Brody and Brody (18) attribute the broadening of the red absorption band of chlorophyll *a* in concentrated alcohol to the presence of a dimer having a longer wavelength peak. They report action spectra for fluorescence emission in live algae to be in qualitative agreement with the absorption spectrum of the dimer in solution. Their conclusion that the short and the long wavelength-absorbing forms of chlorophyll *a* form two different intermediate products that then react with each other is in agreement with recent work of this laboratory (19). This conclusion

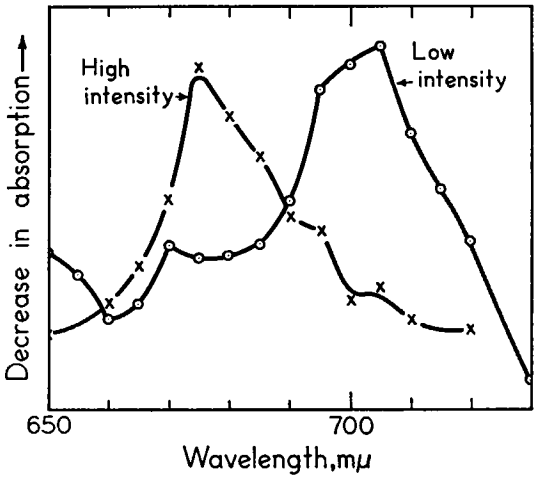

FIGURE 3 The absorption change in reversibly bleached *Euglena* grown with high and low light intensity.

is independent of the value of the monomer-dimer hypothesis as contrasted with the hypothesis that different chlorophyll protein complexes are the active substances in live cells.

Reversible Bleaching by Light. The photostability of C_a695 in relation to the other chlorophylls has been investigated in several ways. As noted earlier, C_a695 disappears from *Euglena* cultures placed in bright light for several days.

It was thought that cells high in C_a695 might show a greater reversible photobleaching in this spectral region than would the cells grown with strong light. This effect was observed and is illustrated in Fig. 3. However, another far more striking difference between the reversible bleaching spectra of the two types of cells was noted. The cells containing C_a695 gave a large reversible decrease in absorption around 705 mμ. The decrease at 695 mμ showed only as a shoulder on the 705 mμ band. The change at 695 to 705 mμ was twice as large as in the 670 to 680 mμ region for these cells. The reverse situation was observed in the cells grown with intense light.

These experiments were done through the courtesy of Dr. Bessel Kok with his

apparatus (20) designed to measure rapid, reversible absorption changes induced by light. The actinic light was a broad band at approximately 650 mμ. Only a fraction of 1 per cent of the total chlorophyll was bleached reversibly. Since the individual cells containing C_a695 also contained more total chlorophyll, the absorption maximum of the reversibly bleached pigment may be slightly skewed toward longer wavelengths.

Irreversible Photobleaching of C_a695 in Cell Homogenates

We are now able to prepare a clear aqueous extract from *Euglena* cells that has a derivative absorption spectrum identical with that of the whole cells. However, at the time the following experiments were done, a portion of C_a695 was lost during the rupturing of the cells. A dense suspension of *Euglena* cells was forced through a needle valve (21) under about 9000 psi to break the cells and the chloroplasts. After centrifuging this homogenate at 10,000 *g* for 15 minutes, the supernatant is an opalescent, green suspension of very fine chloroplast particles having a derivative absorption spectrum identical with that of the total homogenate. This green supernatant is clear enough to be measured in the Beckman DK-2 recording spectrophotometer using half-band widths of about 3 mμ.

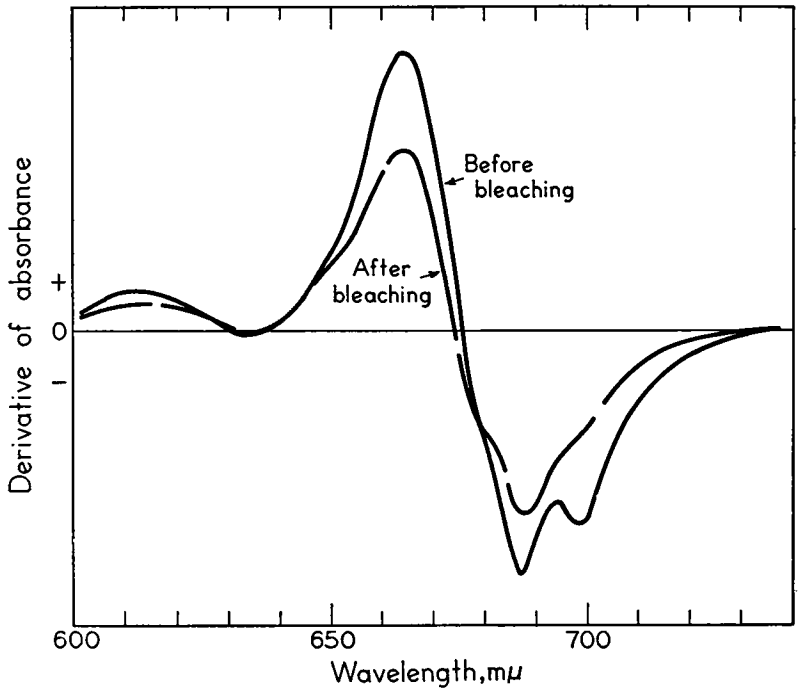

FIGURE 4 The derivative absorption spectra of an aqueous extract from *Euglena* before and after irradiation with intense red light for 20 minutes.

Since in our previous work with *Chlorella* the three forms of chlorophyll *a* had been found to differ in their photostability, a similar study was carried out on the supernatant of a *Euglena* homogenate. The green supernatant was partially bleached with red light isolated from a tungsten lamp, by means of Corning filter No. 2408 and a cooled water bath. The illumination at the position of the sample without the filter was 50,000 ft-c in one set of experiments and 100,000 ft-c in another. Within the intensity range of these experiments, the rate of photobleaching was directly proportional to the intensity.

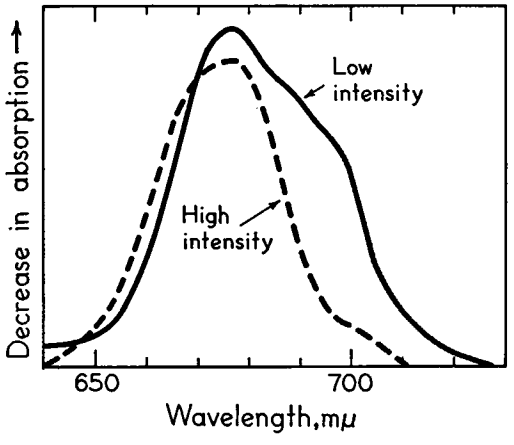

FIGURE 5 Changes in absorption spectrum caused by partial bleaching with red light for *Euglena* grown with both high and low light intensity.

The green extract was placed in a cuvette and both its absorbance and the first derivative of the absorbance were measured. Bleaching was then carried out in the same cuvette, keeping the temperature below 30°C. After illumination for several time periods, the same two absorption measurements were repeated. Derivative absorption spectra of a preparation from *Euglena* grown with dim light before and after partial bleaching are shown in Fig. 4.

Similar green supernatants from *Euglena* grown with high and low light intensities were prepared as described above, and bleached to approximately the same extent. The absorption spectra before and after bleaching were measured for each of the two types of cells with the Beckman DK-2. Plots of the difference between the initial and final curves are shown in Fig. 5. These difference spectra represent the absorption of the bleached pigment. C_a695 is clearly evident in the absorbance of the bleached pigment from the cells grown under low illumination.

An Absorption Peak at 710 mμ in Euglena

Under conditions which are not as yet clearly defined, old cultures of *Euglena* may have an absorption peak at 707 to 710 mμ. The substance giving this band is

believed to be a complexed form of pheophytin *a* and is therefore tentatively called P_a710. Cells containing P_a710 in large amounts are oval in shape, have brownish green, dense chloroplasts, and are non-motile. This latter characteristic makes it possible to separate them partially from active, green cells in the same culture. The derivative absorption spectrum of cells containing P_a710 is shown in Fig. 6. An aqueous extract of the cells can be prepared by extrusion through a needle valve without apparent loss of the 710 mμ pigment.

When cells containing P_a710 were extracted first with 80 per cent acetone and

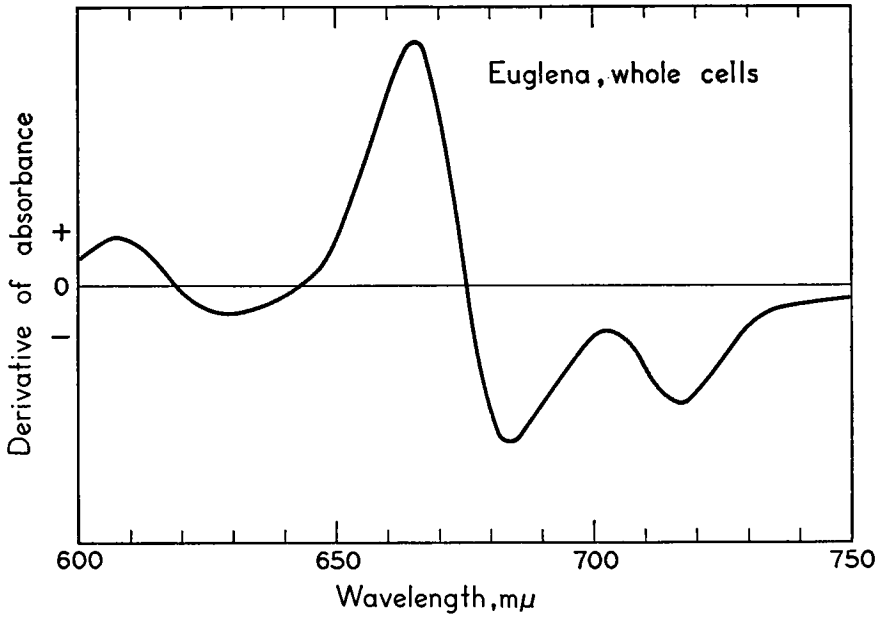

FIGURE 6 Derivative absorption spectrum of *Euglena* cells possessing an absorption band at 705 to 710 mμ.

then with ether, according to Smith and Benitez (12), the spectrum of the ether solution showed a large amount of pheophytin *a*. The extract was chromatographed on paper, and the gray and green bands were redissolved in ether. These bands were identified as belonging to pheophytin *a* and chlorophyll *a* respectively, and occurred with approximately the same relative intensity as that with which the 710 mμ band and the chlorophyll absorption band have appeared in the whole cells. Extracts from normal green *Euglena* have at most a trace of pheophytin.

Because P_a710 appears to be a complex of pheophytin *a* formed from C_a695 *in vivo* we tried to produce it by acid treatment of cells and extracts containing C_a695. It has not been possible to make P_a710 by this means. Once formed in the cells by some natural process it is stable, in cells or in homogenates, to pH 1.6

overnight in the cold. Similar acidification of C_a695 containing cells or homogenates removes the absorption band at 695 mμ without forming a new band at 710 mμ. Wolken *et al.* (22) reported the formation of pheophytin in *Euglena,* but did not observe an absorption band at 710 mμ.

The appearance of an absorption band high enough to show as a very distinct shoulder in the absorption spectrum of cell suspensions is of considerable interest (8). Since this band appears to be due to a pheophytin complex and is found in old cultures rather than in young ones, it may well be a degradation product rather than a functional part of the photosynthetic system.

Action Spectra of Photosynthesis in Euglena Grown at Various Light Intensities

From the work of Emerson (23, 24), Blinks (25), and Rabinowitch (26) and from experiments in this laboratory (27-30) it appears that C_a695 functions in photosynthesis only when accessory pigments such as chlorophyll *b* or C_a673 are also illuminated. Because the amount of C_a695 can be made to vary in *Euglena,*

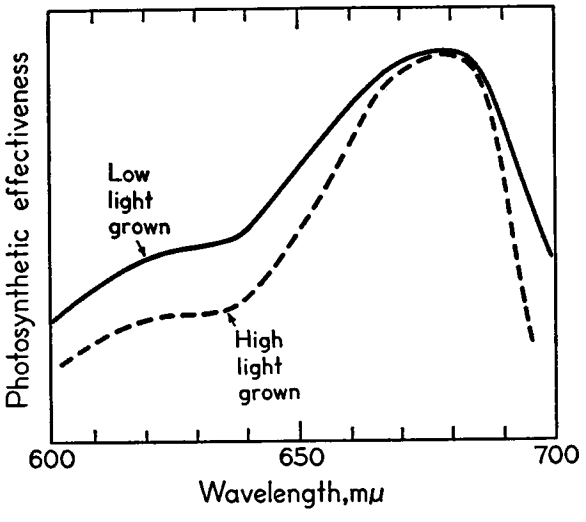

FIGURE 7 Action spectra of photosynthesis for *Euglena* grown with low and high light intensity.

action spectra of these organisms grown under different conditions were measured. However, the shapes of these two action spectra, shown in Fig. 7, appear to differ more because of the flattening effect of high chlorophyll content in the cells grown with low light intensity, than because of a difference in the proportion of C_a695.

The Emerson enhancement effect, usually found when green cells are illuminated with light absorbed by long wavelength chlorophyll *a* together with light absorbed

by an accessory pigment, was barely detectable in these cells. Lack of such enhancement in some cultures has also been reported by McLeod (31) and may be correlated with the exhaustion of nutrient. It remains to be seen whether it is possible to develop large enough amounts of C_a695 to show significant changes in the action spectra without the cells becoming too old to give any enhancement at all.

In *Euglena* the amount of chlorophyll *b* in relation to that of chlorophyll *a* is smaller than in typical green algae. This fact may also in part account for the lack of significant enhancement in these experiments.

We wish to thank Dr. Bessel Kok and the staff at the Research Institute for Advanced Study, Baltimore for making it possible to carry out the reversible bleaching experiments.

Received for publication, April 3, 1961.

REFERENCES

1. KRASNOVKSY, A. A., and KOSOBUTSKAYA, L. M., Different conditions of chlorophyl in plant leaves, *Doklady Akad. Nauk, SSSR*, 1953, **91**, 343.
2. FRENCH, C. S., and HUANG, H. S., The shape of the red absorption band of chlorophyll in live cells, *Carnegie Institution Washington Yearbook No. 56*, 1957, 266.
3. BROWN, J. S., and FRENCH, C. S., Absorption spectra and relative photostability of the different forms of chlorophyll in *Chlorella, Plant Physiol.*, 1959, **34**, 305.
4. FRENCH, C. S., The variability of chlorophyll in plants, Photobiology, (R. W. Newburgh, editor), Corvallis, Oregon State College Press, 1952, 52.
5. ALLEN, M. B., FRENCH, C. S., and BROWN, J. S., Native and extractable forms of chlorophyll in various algal groups, *in* Comparative Biochemistry of Photoreactive Pigments, (M. B. Allen, editor), New York, Academic Press, Inc., 1960, 33.
6. FREI, Y. F., The derivative absorption spectra of algae and leaves at low temperature, *Carnegie Institution of Washington Yearbook* 1960 *No. 59*, 333.
7. EMERSON, R., Dependence of yield of photosynthesis in longwave red on wavelength and intensity of supplementary light, *Science*, 1957, **125**, 746.
8. ALLEN, M. B., Evidence for pigments absorbing at 705 to 710 mμ in photosynthetic organisms, Life and Light, (W. D. McElroy and B. Glass, editors), Baltimore, The Johns Hopkins Press, 1961, 479.
9. CRAMER, M., and MYERS, J., Growth and photosynthetic characteristics of *Euglena gracilis, Arch. Mikrobiol.*, 1952, **17**, 384.
10. BRAWERMAN, G., and CHARGAFF, E., Changes in protein and ribonucleic acid during the formation of choroplasts in *Euglena gracilis, Biochim. et Biophysica Acta*, 1959, **31**, 164.
11. MYERS, J., The culture of algae for physiological research, *in* The Culturing of Algae, A symposium, (J. Brunel, G. W. Prescott and L. H. Tiffany, editors), Yellow Springs, Ohio, The Charles F. Kettering Foundation, 1950, 45.
12. SMITH, J. H. C., and BENITEZ, A., Chlorophylls: Analysis in plant materials, *in* Modern Methods of Plant Analysis, (K. Paech and M. V. Tracy, editors), Julius Springer, Berlin, 1955, **4**, 142.
13. MYERS, J., Culture conditions and the development of the photosynthetic mechanism, *J. Gen. Physiol.*, 1946, **29**, 419.
14. FRENCH, C. S., and ELLIOT, R. F., The absorption spectra of chlorophylls in various algae, *Carnegie Institution of Washington Yearbook No. 57*, 1958, 278.
15. FRENCH, C. S., Fluorescence spectrophotometry of photosynthetic pigments, *in* The Luminescence of Biological Systems, (F. H. Johnson, editor), American Association for the Advancement of Science, Washington, D. C., 1955, 51.

16. BUTLER, W. L., A far red absorbing form of chlorophyll, *Biochem. and Biophysics Research Com.*, 1960, **3**, 685.
17. KRASNOVSKII, A. A., and BYSTROVA, M. I., Study of the formation of chlorophyll in homogenates of etiolated leaves using a fluorescence spectrophotometric method *Biokhimiya*, 1960, **25**, 168.
18. BRODY, S. S., and BRODY, M., Spectral characteristics of aggregated chlorophyll and its possible role in photosynthesis, *Nature*, 1961, **189**, 547.
19. FRENCH, C. S., and FORK, D. C., Two primary photochemical reactions in photosynthesis driven by different pigments, (manuscript submitted December, 1960 for Fifth International Biochemical Congress, Moscow, 1961).
20. KOK, B., and GOTT, W., Activation spectra of the 700 mμ absorption change in photosynthesis, *Plant Physiol.*, 1960, **35**, 802.
21. FRENCH, C. S., and MILNER, H. W., Disintegration of bacteria and small particles by high-pressure extrusion, *in Methods of Enzymology*, (S. Colowick, editor), New York, Academic Press, Inc., 1955, 64.
22. WOLKEN, J. J., MELLON, A. D., and GREENBLATT, C. L., Environmental factors affecting growth and chlorophyll synthesis in *Euglena*, *J. Protozool.*, 1955, **2**, 89.
23. EMERSON, R., Yield of photosynthesis from simultaneous illumination with pairs of wavelengths, *Science*, 1958, **127**, 1059.
24. EMERSON, R., and CHALMERS, R., Speculations concerning the function and phylogenetic significance of the accessory pigments of algae, *Phycol. Soc. Am. News Bull.*, 1958, **11**, 51.
25. BLINKS, L. R., Action spectra of chromatic transients and the Emerson effect in marine algae, *Proc. Nat. Acad. Sc.*, 1960, **46**, 327.
26. GOVINDJEE, and RABINOWITCH, E., Action spectrum of the "second Emerson effect," *Biophysic. J.*, 1960, **1**, 73.
27. MYERS, J., and FRENCH, C. S., Evidences from action spectra for a specific participation of chlorophyll *b* in photosynthesis, *J. Gen. Physiol.*, 1960, **43**, 723.
28. FRENCH, C. S., and MYERS, J., Recording action spectra of photosynthesis automatically, *Carnegie Institution of Washington Yearbook No. 58*, 1959, 323.
29. MCLEOD, G. C., and FRENCH, C. S., The function of various plant pigments in photosynthesis, *Carnegie Institution of Washington Yearbook No. 59*, 1960, 336.
30. FRENCH, C. S., MYERS, J., and MCLEOD, G. C., Automatic recording of photosynthesis action spectra used to measure the Emerson enhancement effect, Comparative Biochemistry of Photoreactive Pigments, (M. B. Allen, editor), New York, Academic Press, Inc., 1960, 361.
31. MCLEOD, G. C., Variation of enhancement of photosynthesis with conditions of algal growth, *Science*, 1961, **133**, 192.