BBA 4190

THE SEPARATION OF THE FORMS OF CHLOROPHYLL *a* AND THE ABSORPTION CHANGES IN EUGLENA DURING AGING

JEANETTE S. BROWN

Carnegie Institution of Washington, Stanford, Calif. (U.S.A.)

(Received April 29th, 1963)

SUMMARY

The spectral changes which occur as green Euglena are allowed to age in the dark have been described. Previous studies have been concerned with the striking differences in the chlorophyll *a* absorption bands between Euglena grown with strong and weak illumination. Here we have shown that the absorption changes during aging of these two cell types also follow a different course. In cells grown with intense light, the chlorophyll absorption maximum shifts towards shorter wavelengths during aging whereas in cells grown with weak light, it shifts in the opposite direction. Both types gradually form a pigment absorbing at 710 mμ and also pheophytin *a*.

There appears to be a relationship between the decrease in absorption at 670 mμ and the increase at 710 mμ. At intermediary stages in the aging process two particulate fractions may be obtained—one with absorption maxima at 670 and 710 mμ and the other with a major band just above 680 mμ. This is presented in support of the hypothesis that the two major forms of chlorophyll *a*, C_a670 and C_a685, break down by different processes and are attached to different lipoprotein subunits.

INTRODUCTION

There are two major holochromatic forms of chlorophyll *a* which occur generally in green plants[1]. A third complex is evident in Euglena at 695 mμ when the culture is grown with relatively weak light. The appearance of a fourth absorption band at 710 mμ in Euglena after 1 week or more of aging in the dark has also been reported[2-4]. Study of this new band has partially revealed the nature of the combined forms of chlorophyll *a* which occur *in vivo*.

Nondestructive fractionation of the different chlorophyll *a* complexes has been impossible previously although preferential separation has been accomplished by destruction of the longer wavelength forms by intense light, heat, certain detergents and organic solvents. After the formation of the 710-mμ absorption band (P_a710), two centrifugal fractions can be obtained which have substantially different proportions of the 670- and 685-mμ (C_a670 and C_a685) absorption bands. This result indicates that these forms of chlorophyll may be different lipoprotein subunits.

Recently ALLEN *et al.*[5] have been able to extract very small photoreactive particles from Chlorella which have an absorption maximum at 672 mμ.

MATERIALS AND METHODS

Euglena gracilis (Klebs, Cambridge "T" strain)[6] was grown in 2-l batches of a neutral medium devised by CRAMER AND MYERS[7]. A 3-l Fernbach flask was placed in a bath of running tap water over a 60-W incandescent lamp. After 3 weeks of growth, the dense cell suspension was removed to the dark, cold room. These aging cells were used for study of the 710-mμ absorption band which slowly developed.

The procedure used to make fine suspensions of pigment-protein particles has been described[3]. This procedure was devised to preserve the full relative absorption at 695 mμ. However, the absorption at 710 mμ is not nearly so labile, and its preservation may not require all the precautions of this procedure.

The cells were centrifuged from their growth medium and resuspended in a smaller amount of a buffer containing 0.01 M glycine, 0.05 M KOH, 0.02 M cysteine–HCl, 20% glycerine at pH 9.5. They were then forced through the needle-valve pressure cell[8] at a rate and pressure designed to break most of the cells, but not the chloroplasts. The chloroplasts were washed, resuspended in more of the same buffer and again forced through the needle-valve at a higher pressure, 12 500 lb/in^2. The broken chloroplasts were centrifuged for 20 min at 12 000 $\times$ g to remove the larger fragments, and the relatively clear supernatant was used for the study of the chlorophyll forms. Spectrophotometric checks were always made to see whether this supernatant contained the same relative proportions of the same forms as the original whole algae. Comparison of spectra assured us that the extraction procedure itself did not alter the chlorophyll–protein complexes.

Further centrifugal studies were carried out on the Spinco Model-L centrifuge.

RESULTS AND DISCUSSION

Separation of pigment particles

Fig. 1 illustrates the absorption spectra of aqueous extracts from Euglena with chlorophylls *a*-670, *a*-685 and *a*-695 and from similar cells after a 2-week period in darkness when the absorption band at 710 mμ (P_a710) has become apparent.

At 4° the absorption band at 710 mμ is evident after 1 week in the dark, and it continues to increase for 3–4 weeks. At room temperature in the dark P_a710 forms more rapidly.

Bubbling the aging cultures with N_2 did not seem to increase the rate of formation of the P_a710.

During the aging the number of motile, green cells decreases and the proportion of non-motile, brownish cells increases. There is also a change in the shape of the cells from long cylindrical cells, with one end more pointed than the other, to ellipsoidal cells with both ends symetrically rounded. When the change has occurred in about half of the population, it is relatively easy to separate the two types of cells by allowing a mixed culture to sit in room light for about 0.5 h. The green, motile cells settle to the bottom, and the brownish cells remain suspended during this short period. P_a710 is found only in the non-motile, brownish cells.

The older the culture at the beginning of the dark, aging period, the faster P_a710 becomes apparent.

In cells aged for 3 weeks in the dark P_a710 was mostly within the chloroplasts.

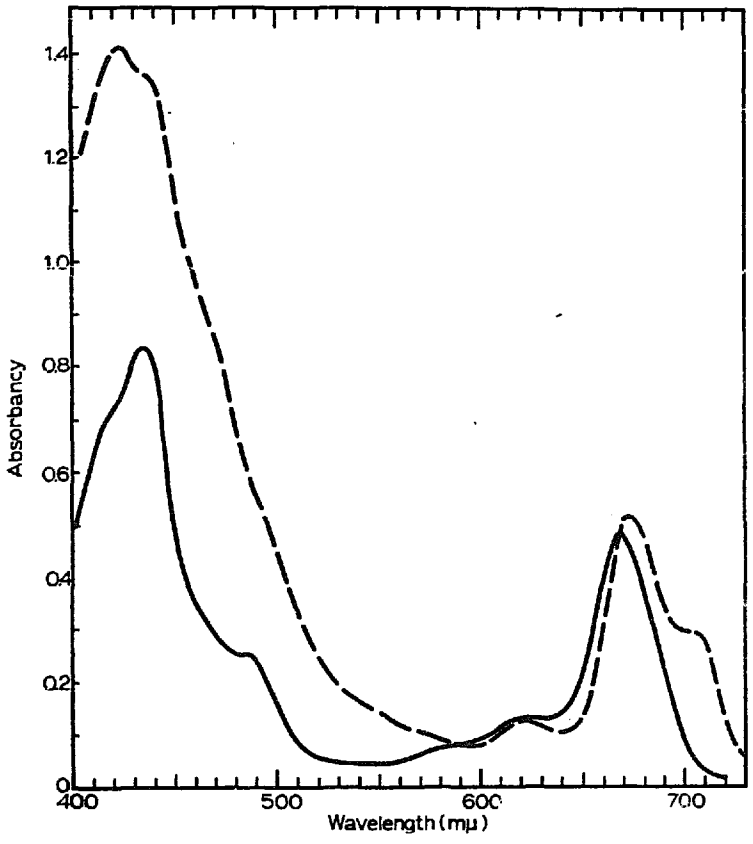

Fig. 1. Comparison of absorption spectra of aqueous extracts from Euglena cells grown with weak light before (———) and after (– – – –) being aged in the dark for 2 weeks.

When these chloroplasts were broken into small particles by the needle-valve, dialysed to remove the glycerin and then centrifuged at 144 700 × *g* for 1 h most of the P_a710-containing particles sedimented and particles with a main absorption peak at about 683 mμ remained suspended in the supernatant. Absorption spectra of the two particulate fractions are illustrated in Fig. 2.

A similar separation of the two kinds of particles occurred when the dialysed extract was centrifuged through a sucrose gradient. A narrow, brownish, particulate

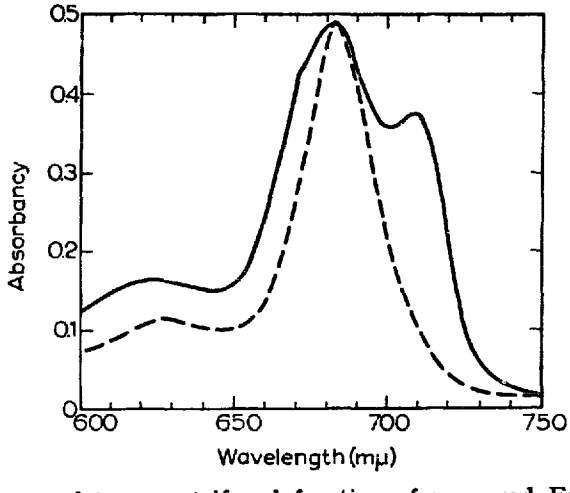

Fig. 2. Absorption spectra of two centrifugal fractions from aged Euglena. ———, sediment; – – – –, supernatant.

layer formed about 1 cm from the bottom of the tube. These particles contained P_a710, corresponding to the sediment from the direct centrifugation. Above this a wider, clear-brown layer corresponded to the supernatant. Each centrifugal fraction contained some contaminating absorption from the other. We were not able to eliminate this by further centrifugation either with or without sucrose.

Although the formation of pheophytin in dark-adapted Euglena has been studied to some extent by GREENBLATT AND SCHIFF[9], the formation of the absorption maximum at 710 mμ *in vivo* was not noted. Apparently considerable time in the dark is necessary before this band becomes evident. It may be also that growing and aging the cells in a neutral medium is favorable to the formation of P_a710 since acidifying the green cells or an aqueous extract from them forms pheophytin readily but not P_a710. In Ochromonas[3], however, P_a710 forms when the cells are stored overnight in the dark coldroom, and in a Chlorella mutant[10] it forms while the culture is still in the light.

In our earlier work we aged Euglena which had been grown with low light intensity, and C_a695 disappeared as P_a710 formed. This suggested a conversion of one to the other.

More recently we have observed that in the dark, under N_2, P_a710 is formed in high-light grown cells which contain no detectable C_a695. We know from previous experiments that C_a695 is much more photosensitive than the other chlorophyll forms. Thus it may be that when Euglena is grown with intense light, C_a695 is bleached as rapidly as it is formed, but when these cells are removed from the light, C_a695 is metabolized to P_a710. On the other hand it may be that P_a710 is formed directly from C_a670 and the presence or breakdown of C_a695 is coincidental.

We wish to emphasize in this study that P_a710 is mostly associated with the 670-mμ-absorbing form of chlorophyll *a*. This shows clearly in Fig. 2 where most of the absorption at 670 mμ and 710 mμ is in one fraction and the absorption at 685 mμ in another. With longer aging and greater breakdown of the chlorophyll (as illustrated below in the two fractions of Fig. 5) most of the chlorophyll absorption from the P_a710-containing particles disappears.

We cannot distinguish between the hypotheses: (1) that C_a670 is converted to P_a710 enzymically or by autocatalysis or, (2) that C_a670 is degraded to a non-absorbing substance and P_a710 is formed from some unknown porphyrin-type precursor.

Nevertheless it does appear that the two major forms of chlorophyll *a*, C_a670 and C_a685, are degraded to different products. This is partial support for the hypothesis that these two chlorophyll *a* forms are distinct lipoprotein pigment particles.

Absorption changes in aging cells

The changes in the chlorophyll absorption spectrum of aging Euglena, which had been grown with high and low illumination, are illustrated in Figs. 3 and 4. Figs. 3A and 4A are derivative absorption spectra of whole cells suspended in their culture medium. The shoulder at about 645 mμ is due to chlorophyll *b* absorption; the shoulder at about 680 mμ is caused by the combined absorption of C_a670 and C_a685. These three absorbing entities are present in all Euglena. In addition the cells grown with weak light have an absorption band at 695 mμ which may be seen as a shoulder on the derivative curve at about 690 mμ.

The chlorophyll absorption maximum due to the combined, overlapping absorp-

tion of chlorophyll *b* and the forms of chlorophyll *a* is represented by the point at which the derivative spectrum crosses the zero line going from left to right.

As the Euglena cells grown with intense light age in the dark, the absorption maximum shifts towards shorter wavelengths as if C_a685 disappeared. This is illustrated in Fig. 3A. As the C_a695-containing cells age, the maximum shifts slightly towards longer wavelengths and P_a710 becomes apparent as a negative derivative peak at about 715 mμ. This is illustrated in Fig. 4A. The reasons for these differences when the two types of cells are aged are not known.

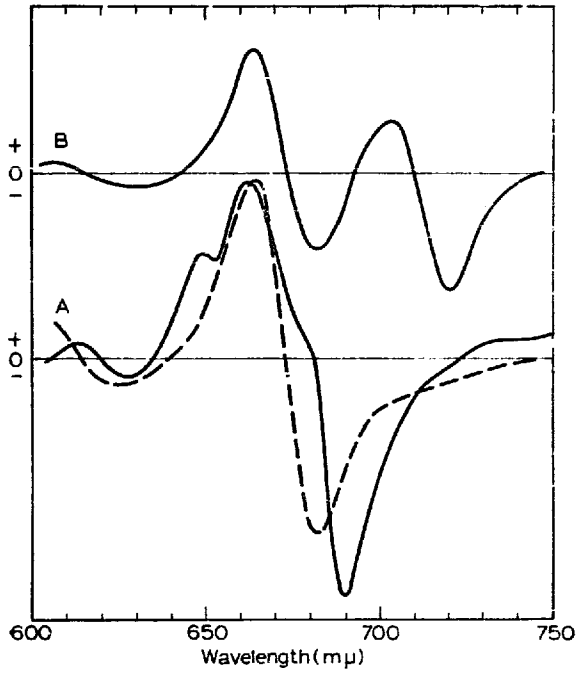

Fig. 3. A, derivative absorption spectra of Euglena cells grown with intense light before (———) and after (– – – –) 27 days in the dark with N_2 gas bubbling through the culture. B, spectrum of an aqueous extract of the 27-day cells.

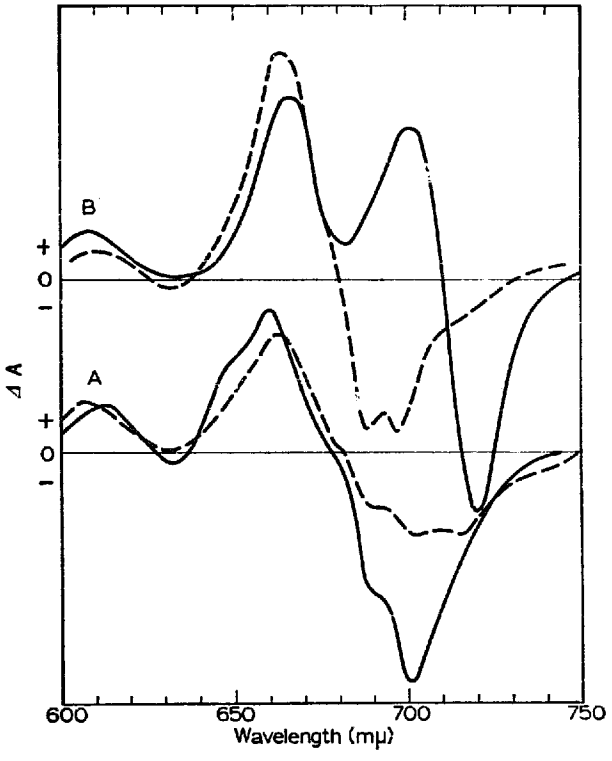

Fig. 4. A, derivative absorption spectra of Euglena cells grown with weak light before (———) and after (– – – –) 34 days in the dark with N_2 gas bubbling through the culture. B, spectra of aqueous extracts of the chloroplasts (– – –) and cytoplasm (———) of the 34-day cells.

Although the apparent absorption coefficient of all the pigment-lipoprotein material increases when the chloroplasts are broken into small particles, the increase in absorption at 710 mμ is relatively much greater than at the chlorophyll maximum. This effect can be seen by comparing the spectrum in part B of Fig. 3 and the fractions in Fig. 4B with the corresponding spectrum of the aged, whole cells. Extraction of the whole cells and of an equal proportion of the broken cells by acetone demonstrates that the amount of pigment is not changed by the breaking process. The reasons for the different apparent changes in the absorption coefficients are not understood, but are probably related to the different types of pigment-binding within the cell.

Very few chloroplasts were visible in the aged high-light grown Euglena, so no attempt was made to separate a chloroplast fraction from the rest of the cell components. However, this can be done easily with the low-light cells containing C_a695

as described above. Fig. 4B shows derivative spectra of the cytoplasmic and broken chloroplast fractions.

Analogous spectra made with the Beckman DK-2 recording spectrophotometer presented in Fig. 5 allow comparison of the absorption maxima in the blue region of the spectrum also.

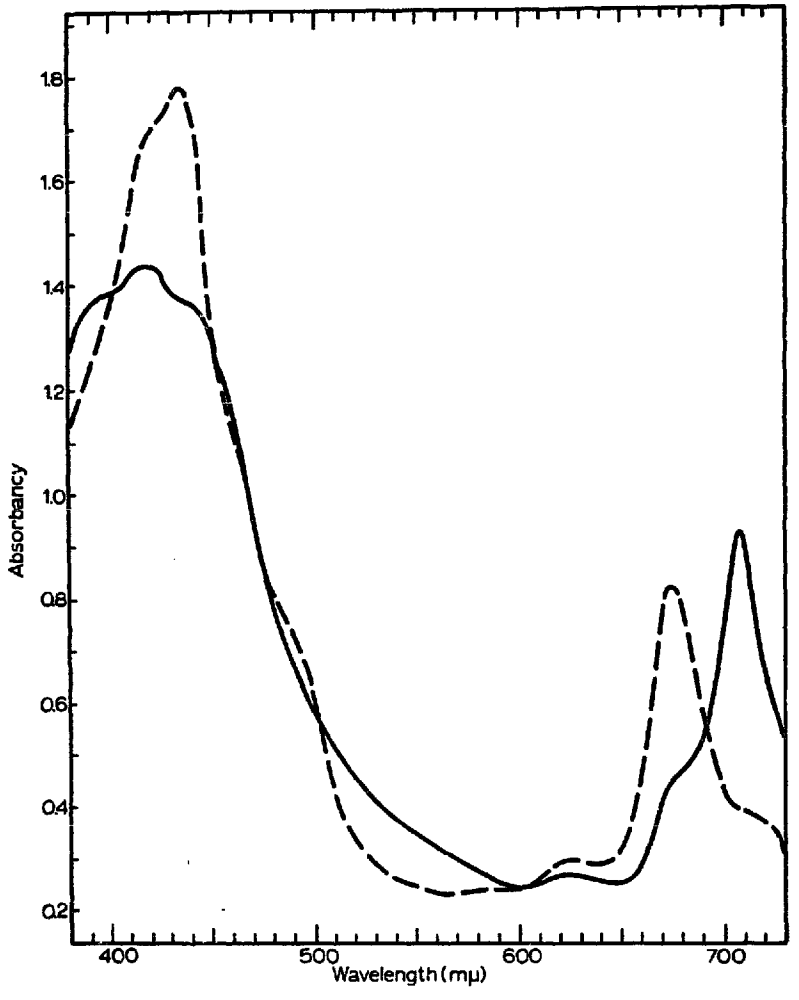

Fig. 5. Absorption spectra of aqueous extracts of the chloroplasts (– – –) and cytoplasm (———) from Euglena cells grown with weak light and aged for 34 days in the dark at 4°.

Nothing is known about possible absorption maxima of the natural forms of chlorophyll *a* in the blue region of the spectrum. This is partly because so much of our work has been done with the aid of the derivative spectrophotometer[11] which does not function below 430 mμ and partly because of the overlapping absorption of the carotenoids in this region.

It is well known that the combined absorption from the two major chlorophyll forms which peaks at about 675 mμ in Euglena has another maximum at about 435 mμ. Fig. 5 illustrates that the pigment absorbing at 710 mμ also has a maximum at about 420 mμ. We still have no information about absorption of the forms of chlorophyll *a* in other than the red region of the spectrum.

Each of these aqueous pigment fractions was extracted with acetone and the

pigment transferred to ethyl ether. Spectra of these ether extracts are presented in Fig. 6.

Paper chromatograms of these two fractions showed the presence of chlorophyll *b*, chlorophyll *a*, pheophytin *a* and pheophorbide *a*. The ratio of pheophytin to chlorophyll is much higher in the cytoplasmic P_a710-containing fraction[12] as would also be

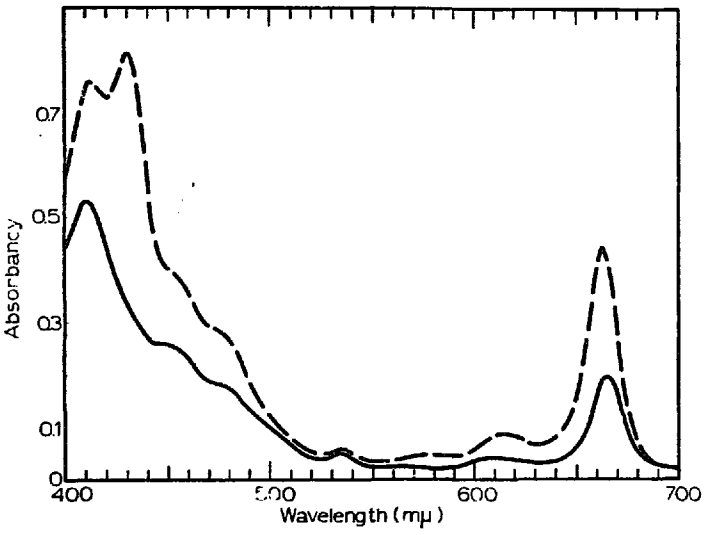

Fig. 6. Absorption spectra of acetone extracts transferred to ethyl ether of the same fractions as shown in Fig. 5. ----, chloroplast fraction; ———, cytoplasmic fraction.

predicted from Fig. 6. The ratio of pheophytin *a* to pheophorbide *a* in the cytoplasmic fraction varies with different aged Euglena preparations. Apparently the older the culture, the lower the ratio indicating that pheophorbide is a step further than pheophytin in the degradation process.

ACKNOWLEDGEMENTS

We are grateful to Mrs. H. FULK for technical assistance and to Drs. C. S. FRENCH and J. H. C. SMITH for criticism of the manuscript.

REFERENCES

1 J. S. BROWN, *Photochem. Photobiol.*, 2 (1963) 159.
2 J. S. BROWN, *Carnegie Inst. Washington Year-Book*, 59 (1960) 330.
3 J. S. BROWN, *Carnegie Inst. Washington Year-Book*, 60 (1961) 366.
4 J. S. BROWN AND C. S. FRENCH, *Biophys. J.*, 1 (1961) 539.
5 M. B. ALLEN, J. C. MURCHIO, S. W. JEFFREY AND S. A. BENDIX, *Jap. Soc. Plant Physiol., Microalgae and Photosynthetic Bacteria*, Univ. Tokyo Press, 1963, p. 407.
6 R. C. STARR, *Am. J. Botany*, 47 (1960) 82.
7 M. CRAMER AND J. MYERS, *Arch. Mikrobiol.*, 17 (1952) 384.
8 C. S. FRENCH AND H. W. MILNER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods of Enzymology*, Vol. I, Academic Press, New York, 1955, p. 64.
9 C. L. GREENBLATT AND J. A. SCHIFF, *J. Protozool.*, 6 (1959) 23.
10 M. B. ALLEN, S. A. BENDIX AND J. C. MURCHIO, *Arch. Mikrobiol.*, 42 (1962) 36.
11 C. S. FRENCH, *Instrumentation and Control Symposium*, sponsored by Northern California Secion Instrument Soc. of America, Berkeley, 1957, p. 83.
12 J. H. C. SMITH AND A. BENITEZ, in K. PAECH AND M. V. TRACEY, *Modern Methods of Plant Analysis*, Vol. IV, Springer Verlag, 1955, p. 142.