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ABSORPTION SPECTRA AND RELATIVE PHOTOSTABILITY OF THE DIFFERENT FORMS OF CHLOROPHYLL IN CHLORELLA[1]

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Two or more forms of in vivo chlorophyll a with different absorption spectra appear to exist together in green plants (8, 9, 14, 16, 18, 20, 23). This paper describes the various components of the chlorophyll absorption band in the red region in Chlorella, as to their peak positions, half widths, and relative stability to light in cell homogenates.

The decline in photosynthetic efficiency at longer wave lengths within the red absorption band of chlorophyll a discovered by Emerson and Lewis (5, 6) might be attributed to coexisting active and inactive forms of chlorophyll a with the inactive form absorbing at longer wave lengths. While other explanations may be necessary to account for the supplementary light effect of Emerson, Chalmers and Cederstrand (3, 4), detailed information about the spectra and the relative photosynthetic ability of the several forms of chlorophyll a as they exist in plants is essential to an understanding of this striking phenomenon.

Evidence for the coexistence of 2 forms of chlorophyll a in green plants has been given by Krasnovsky and his co-workers (16, 18, 23) on the basis of absorption spectroscopy. A shift in the position of the red absorption peak of chlorophyll a in chloroplast suspensions caused by partial photochemical bleaching was also discovered by the Krasnovsky group (17) and attributed by them to the selective destruction of one of the two forms of chlorophyll a.

The experiments here reported were started with the hope of using this selective photobleaching as an aid in deriving the absorption curve of the more readily bleached form by subtracting the curve for the sample after partial bleaching from the original curve.

Chlorella was used for these experiments because its derivative absorption spectrum has a marked discontinuity, showing very clearly the presence of 2 overlapping components in its red absorption bands (10). Preliminary experiments showed that the chlorophyll components of Swiss chard chloroplast material bleached at an equal rate (1). Chlorella, however, gives absorption spectra of different shapes before and after bleaching, as here described.

The action spectra obtained by Haxo and Blinks (15) indicate that plants contain an active and an inactive form of chlorophyll a. The approximate absorption spectra for the active and the inactive forms have been derived (13) for *Porphyra naiadum* and for *Delesseria decipiens* from the data of Haxo and Blinks. The present paper gives more precise curves for the components of the red band of chlorophyll in Chlorella.

PROCEDURE

Chlorella pyrenoidosa Chick, Emerson, Starr Collection no. 252 was grown for 10 days in Sorokin and Myers medium (22) on a shaker over a battery of fluorescent lights. The cells were concentrated about 5 times by centrifugation and resuspension in 0.1 M phosphate buffer at pH 7. This dense suspension was forced through a needle valve (11) to liberate the chloroplasts and break them into small particles. All treatments and manipulations of the preparation after breaking the cells were carried out in the dark or in very dim light. The broken mixture was centrifuged 5 minutes at 3500 × G to remove any remaining whole cells and the larger cell fragments. The resulting supernatant, although nearly clear, shows some Tyndall effect and therefore is actually a fine colloidal suspension of cellular material the color of which is due to the chloroplast fragments. Because this preparation was so nearly clear it was possible to record its absorbance curve with adequate precision by using spectral slit widths of about 3 mμ in the Beckman recording spectrophotometer.

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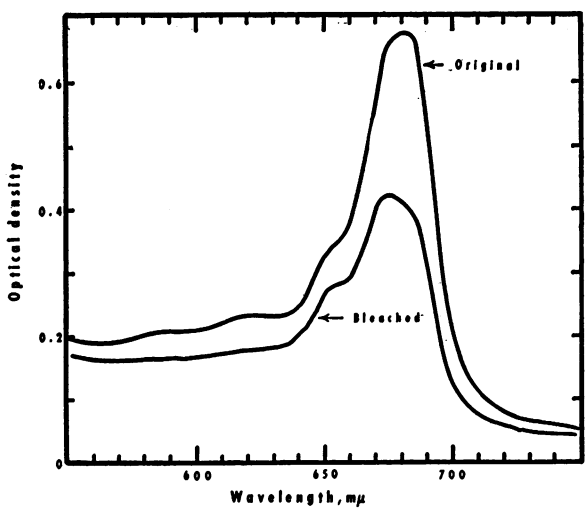

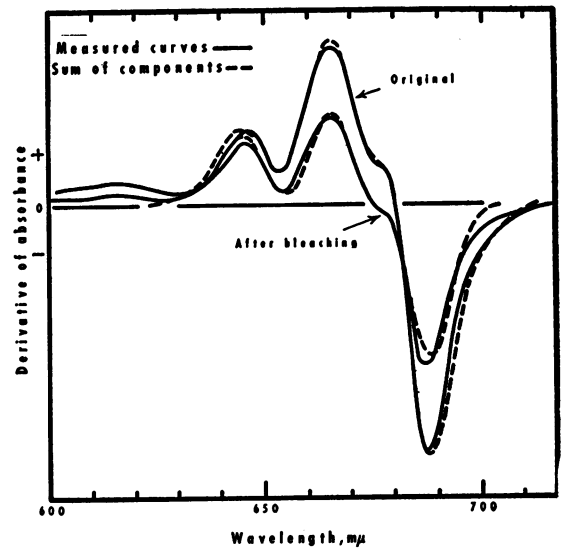

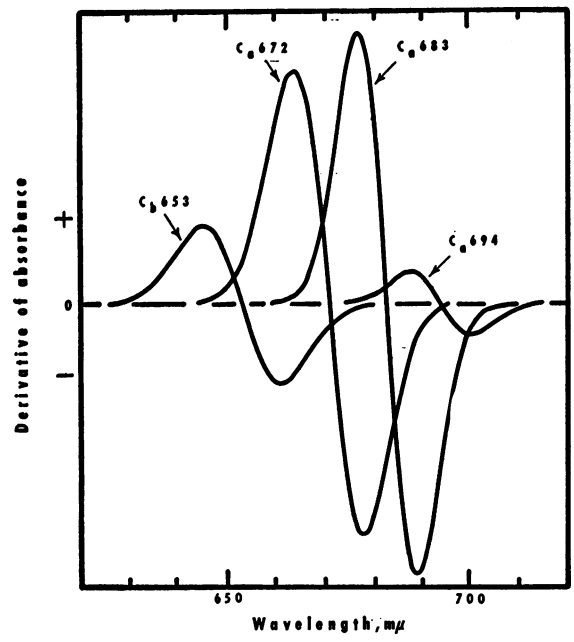

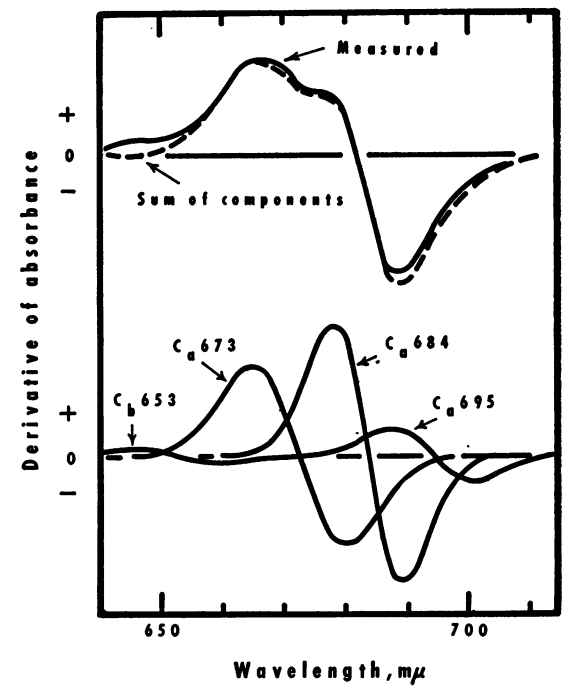

FIG. 1 (*top, left*). Absorption spectra of centrifuged Chlorella homogenate before and after partial bleaching for 15 minutes with red light.

FIG. 2 (*top, right*). Derivative spectra of the same Chlorella material as figure 1. The sums of the component curves used to fit the experimental results are given as dotted lines.

FIG. 3 (*bottom, left*). Component curves used to fit derivative spectra of the original material of figures 1 and 2. The sum of these curves is compared with the recorded derivative spectra in figure 2.

FIG. 4 (*bottom, right*). The difference between the derivative spectra of bleached and unbleached Chlorella of figure 2 and the analysis of the difference curve into its components.

Derivative spectra (7) show the band shapes much more clearly but the absorbance curves present the picture in more conventional form. Both types of measurements were used for this work. Without the derivative data the small differences between some absorbance curves, while visible, would not attract attention. To avoid the complication of carotenoid absorption the measurements were restricted to the orange and red part of the spectrum.

The partial bleaching was accomplished by illumination with a strong red light from a tungsten source through 5.5 cm of water and Corning filter no. 2418 transmitting beyond 620 mμ. With the filter removed the white light intensity at the position of the cuvette was about 100,000 ft-c.

For Experiment 1 only the derivative curves were measured before and after bleaching for 11 minutes. This bleaching time was divided into 5- and 6-minute exposures with a brief cooling period between to prevent the temperature from exceeding 30° C.

For Experiment 2 both derivative absorption spectra and the usual absorption spectra in the Beckman DK-2 were recorded with the same sample of green supernatant before and after bleaching for 10 minutes and again after an additional 5 minutes to make a total of 15 minutes of bleaching.

The experimental derivative curves were matched by summation of hypothetical components with a curve analyzer (12). The component curves were the first derivatives of the probability or Gaussian curve, $y=ae^{-bx^2}$. The curve analyzer allows us to change the horizontal or vertical scale and wave length position of each component independently and to record the individual components, as well as their sum, on the same scale as the original data. Trials were made until the sum of the components fitted the experimental curves as closely as possible.

In working with a derivative of a Gaussian curve it is convenient to express the width of the curve as the distance in wave length units between the negative and the positive peaks of the derivative curve. This value has been multiplied by 1.18, a factor which gives the width at half-height for the corresponding integral curves.

Results

Experiment 2, being more complete, will be discussed first. The absorption spectra of the supernatant from disintegrated Chlorella cells are given in figure 1 before and after 15 minutes of bleaching by red light. Figure 2 shows the derivative absorption spectra of the same preparation and also gives the summation of the component curves used to fit the experimental data. The proposed component curves are given in figure 3. By changing their size but not their position or width, these curves may also be fitted to the derivative curve for the bleached preparation as shown in figure 2.

The original hope based on Krasnovsky's results was that partial bleaching might selectively reduce the amount of only a single component thereby making it possible to determine by direct subtraction the shape of the absorption spectrum for 1 form of chlorophyll a. However, the difference spectrum between the original and the partially bleached material had a complicated shape as shown in figure 4.

This difference curve between the original and the material bleached for 15 minutes appears to consist of chlorophyll b and the 3 different chlorophyll a components given in figure 3. The sum of these components is compared with the difference curve in figure 4.

While the difference curve does not represent a single component as had been hoped, it does show that the different components present in the original preparation are not equally photosensitive. If they had been bleached proportionally, the original spectrum, that for the bleached material, and that for their difference would all have the same shape and would differ only in a scale. Furthermore, from a comparison of the curve analyses of these 3 spectra we may conclude that no components absorbing in this wave length region were produced by photo-bleaching.

The data of Experiment 1 were analyzed similarly. In addition an independent analysis by a different procedure was kindly done by Dr. Joseph E. Hayes, Jr. of the National Heart Institute. The results of these 2 curve analyses are compared in tables I and II.

The peak position and half width of the probability

TABLE I

CHARACTERIZATION OF IN VIVO CHLOROPHYLL COMPONENTS

PEAK POSITIONS, Mμ						HALF-WIDTHS, Mμ					
EXP. 1			EXP 2		SELECTED NOMINAL VALUE	EXP. 1			EXP 2		SELECTED NOMINAL VALUE
BROWN	HAYES		BOTH ORIG AND BLEACHED CURVES	DIFFERENCE CURVE		BROWN	HAYES		BOTH ORIG AND BLEACHED CURVES	DIFFERENCE CURVE	
	Orig.	*Bl.*					*Orig.*	*Bl.*			
653	651	651	653	654	653	20	14	16	18	15	15
672	674	673	672	673	673	19	24	24	17	18	18
683	684	684	683	684	683	14	12	12	14	14	14
694	694	696	694	695	695	14	15	14	14	15	15

curves used to fit the data are given in table I. The agreement of peak position of the component curves selected on various occasions in this laboratory and also by Dr. Hayes for Experiment 1 are very close. In order to obtain an adequate fit the 683 component must be narrower than the 673 component. There is, however, some latitude in the possible width of the 673 component as shown in table I where either 24 or 17 mμ has been used. We prefer to keep the 2 components as nearly the same width as possible.

The relative amounts of these components, described in table I and used to fit the experimental curves, are given in table II. The relative amounts were obtained by summing the products of the vertical and horizontal distances between the peaks of each derivative component—a figure proportional to the area under each absorption band.

It is evident from the reduced percentage of the 683 component in all curves for the bleached material that the 683 component bleaches more rapidly than the 673 component, as was found by Krasnovsky. The 695 component was particularly sensitive to bleaching. The chlorophyll b peak at 653 mμ was the most stable of all.

Discussion

The derivative absorption spectra of Chlorella and of many other green algae have a plateau or shoulder near the wave length at which this curve crosses the zero line (10). (The wave length where the derivative is zero is the position of the absorption peak.) This plateau has always been observed in living Chlorella. It is present but less sharp in aqueous extracts prepared by breaking the cells in a needle-valve. An absorbance curve shows a small region of constant slope near the absorption peak corresponding to the plateau on the derivative curve.

This plateau is considered to be direct evidence for the simultaneous existence of 2 overlapping forms of chlorophyll a in vivo. The plateau is caused by the positive peak of the longer wave length component falling on or very close to the same wave length as the negative peak of the shorter wave length form. By varying the proportion of the components, the plateau can be moved along the curve. Bleaching at first destroys the longer wave length component to a greater extent than the shorter form thus causing the plateau to move toward longer wave lengths. If the proportions of the 2 components change in such a way that the plateau moves across the zero line, a shift in the absorption peak equal to the width of the plateau results. If, however, both components bleach at an equal rate, the positions of the plateau (less distinct in Swiss chard than in Chlorella) and of the absorption peak remain constant, as was found with Swiss chard chloroplasts (1). To study this situation quantitatively some sort of curve analysis is essential.

As far as we know, there is no simple physical theory that gives the universal shape of a single isolated absorption band of a dissolved pigment. Probability curves have been used successfully to fit approximately an isolated band and to analyze a complex band in terms of its individual components (24). For bands that cover an appreciable range of the spectrum, a frequency scale is preferable to a wave length scale on theoretical grounds. For narrow bands such as those of chlorophyll this extra conversion step would make too small a difference to be worth while.

Analysis by almost any assumed band shape for components is preferable to visual inspection for location of individual peak positions in complex spectra. For estimation of the band widths of individual components the shape used is more important.

In the analysis of spectra of solid particles, the distorted shape due to the flattening reported by Duysens (2) and the light scattering found by Latimer (19), even when largely avoided by attention to suitable spectrophotometry (21), might still have residual effects on band shape large enough to make the exact shape of the assumed component curves a matter of less critical importance. Analysis by probability curves is admittedly a rough-and-ready procedure, but it nevertheless appears to be the best method available giving an approximation to the actual shape of individual curves corresponding to real chemical molecules of different types in a complex mixture.

Evidence for the reality of the component at 695 mμ is not conclusive. By including this component in the curve for the original material before bleaching we get a closer fit. The 695 mμ component is not present in the bleached material. The independent curve analysis of the data from Experiment 1 also used a 695 component. In attempting to fit an intermediary (10 min) bleached curve (not illustrated by a figure, but measured during Experiment 2 and reported in table II) the 695 mμ component appeared to be present but in lesser amount than in the original. If the 695 mμ component is real, it is easily bleached. Old cultures of Euglena contain a large 695 mμ component (8). This led us to suppose that a similar component might occur in lesser amounts in other algae. However, it may also be that component curves of a different shape could give an adequate fit without a 695 component.

Table II

Relative Amounts of in vivo Chlorophyll Types in Disintegrated Chlorella Preparations

Component*	Chlorophyll b 653	Chlorophyll a 673	683	695
Amount of each component as % of total				
Experiment 1				
Original	16	43	36	5
Bleached 11 min	23	44	29	4
Experiment 1 Hayes analysis				
Original	9	71	17	3
Bleached	15	69	13	3
Experiment 2				
Original	15	40	40	5
Bleached 10 min	18	42	37	2
Bleached 15 min	22	42	35	0
Difference, Original — Bl, 15 min	2	44	46	8

* Exact wave length and half-width used for the different curve analyses are given in table I.

The derivative absorption spectra of a large number of algae have been recorded in this laboratory. The shapes of these curves vary widely although there are similarities between closely related species. In some species the physiological age of the culture greatly influences the shape of the absorption spectrum. With Chlorella, however, the shape of the absorption curve does not change appreciably during growth. We have some evidence that these same 3 chlorophyll a component absorption spectra may be combined in various proportions to match experimental absorption curves in other algae.

The measurements here presented define the shapes of the individual component curves for chlorophylls a and b in Chlorella.

An eventual use of such curves will be to clarify the chemical nature of the different forms. However, we wish to avoid any specific interpretation of the chemical nature of the components of chlorophyll a in living plants until the number and spectral characteristics of the different chlorophyll components in various plants have been determined.

Summary

When a suspension of broken Chlorella cells, clarified by centrifugation, is partially bleached by red light, the shape of the absorption curve changes and the peak position moves towards shorter wave lengths. This shift is attributed to differing photosensitivity of the different forms of chlorophyll which have different but overlapping absorption spectra in situ. The red peaks of the individual components appear to have the following peak positions and widths in mμ at half-height: chlorophyll b: 653 (15); chlorophyll a: 673(18), 683(14), 695(15). The fraction of the total absorption due to each component and their relative sensitivity to bleaching by red light have been estimated.

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