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ANALYSIS OF THE RED ABSORPTION BAND OF CHLOROPHYLL *a* *IN VIVO**

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SUMMARY

A precise and sensitive integrating spectrophotometer has been constructed in the shape of a dodecahedron with one photoelectric cell on each side. With the help of this instrument and a computer, the red chlorophyll *a* absorption band of algae and chloroplasts was resolved (after subtracting the chlorophyll *b* band) into two Gaussian components, with peaks at 668 and 683 nm**. The half-width of the two-band envelope is 32 nm; the half-width of each component, about 18 nm. In the blue-green alga, Anacystis and the red alga, Porphyridium (both containing no chlorophyll *b*), the two-component bands seem to be in the same positions, but are considerably wider. (However, preliminary analysis suggests that the red band in Anacystis can be interpreted instead as the sum of three components—two belonging to chlorophyll *a*, and a third one probably due to allophycocyanin.) The relative heights of the two chlorophyll *a* components vary, in all plants used, only between 0.7 and 0.9, the 668-nm band always being the weaker one.

Broadening of chlorophyll *a* absorption curves by the so-called "sieve effect" may to some extent change the analysis presented here, by causing the component bands *in vivo* to deviate from the Gaussian shape; this effect calls for further investigation but is unlikely to affect the qualitative conclusions.

A comparison of the absorption spectrum so analyzed with that of the "Pigment systems I and II" (DUYSENS, FRENCH *et al.*) suggests that in Chlorella, a large portion of chlorophyll *a* 668 nm belongs, together with a large part of chlorophyll *b*, to System II, while a large part of chlorophyll *a* 683 nm must be identified with System I, although some of it probably belongs to System II. The simple identification of chlorophyll *a* 668 nm with System II, and chlorophyll *a* 683 nm with System I, as previously suggested, appears to be untenable. In red and blue-green algae, larger parts of both chlorophyll *a* 668 nm and chlorophyll *a* 683 nm seem to belong to System I.

INTRODUCTION

The shape of the red absorption band of chlorophyll *a in vivo* first led ALBERS AND KNORR[1] to suggest the presence of several components within the band envelope.

* This work is based on Ph.D. thesis of C. N. CEDERSTRAND (1965).
** The term "nanometer" (nm) is used for 10^{-9} cm instead of millimicron (mμ).

More convincing evidence for such complex character of the band came from FRENCH's experiments[2] with a "derivative spectrophotometer" (see also refs. 3–5). THOMAS AND GOVINDJEE[6,7] observed a complex structure of the red band by the "wet filter paper" technique.

With the aid of a curve analyzer, BROWN AND FRENCH[8] reconstructed the derivative absorption curve of *Chlorella pyrenoidosa* as a sum of the derivatives of four Gaussian curves, $y = a \exp[-bx^2]$; one they assigned to chlorophyll *b* and three to chlorophyll *a*.

The construction of a new precise integrating spectrophotometer and the use of a computer have permitted us to analyze the structure of the red absorption band of chlorophyll in live cells without resorting to differentiation. The results are presented in this paper. No evidence for the presence of more than two components in chlorophyll *a* was obtained.

MATERIALS AND METHODS

The algae used were *Chlorella pyrenoidosa*, *Anacystis nidulans* and *Porphyridium cruentum*, grown in inorganic media in culture flasks through which air containing 5% CO_2 was bubbled. They were continuously illuminated with white light and harvested after 4–7 days, after a 20–80-fold increase in cell population (see ref. 9 for the chemical composition of the growth media and culturing of the algae).

The spinach (*Spinacea oleracea*) was grown from seed supplied by the Burpee Seed Co. (American Spinach 5058). The plants were grown in a greenhouse in natural light. As soon as the leaves reached full size, but before they started to wrinkle, about 200 g were harvested and ground up with a little sand in a medium containing 0.4 M sucrose, 0.05 M Tris buffer, and 0.01 M NaCl (pH 7.2). The suspension was squeezed through eight layers of cheesecloth to remove the pulp, and centrifuged for 30 sec to remove the sand; the supernatant was then recentrifuged for 10 min at 1000 × *g* to form a chloroplast pellet. The pellet was resuspended in the buffer. A temperature of 5° was maintained throughout the procedure.

Spectrophotometer

The construction of the spectrophotometer[10] used in this work, was first suggested by the late R. EMERSON.

Monochromator. The light source was a GE (18A/T10/1P-6V) ribbon-filament lamp (6 V, 18 A); the slits had a bilateral design similar to that of BARNES AND BRATLAIN[11]. The limiting *f*-number of the monochromator was 10.6; Bausch and Lomb flat grating with 600 grooves per mm, ruled over an area of 52 mm × 52 mm, and blazed for maximum reflection at 500 nm, was used. The linear dispersion of the instrument was 1.0 nm/0.001 inch at the exit slit. The wavelengths could be read with an accuracy of ± 0.1 nm. We used 1.0-nm bandwidths in most of our experiments.

Integrating sphere. The "sphere" used was a dodecahedron. It had 12 light detectors (silicone photocells), one on each face. The reflectivity of the "sphere", coated with MgO, was close to 99.5%. The absorption cell (a cylinder 22 mm in diameter with 5 mm axial path length) was placed in the center of the "sphere"; light entered the latter from below.

In the usual integrating spheres, a single photosensitive device is placed behind

a baffle somewhere in the sphere; repeated diffuse reflections from the walls are supposed to make the light received by this device a correct average of the light scattered in all directions. Studies by LATIMER[12] in our laboratory showed, however, that the averaging is far from perfect and that the results strongly depend on the placing of the photocell in the sphere. LATIMER selected for his experiments a location which seemed to best approach the average. The new instrument integrates twelve light samples, one from each wall of the dodecahedron, thus approaching the ideal of a complete photosensitive internal surface. One advantage of this method over that of a single sampling cell is that its sensitivity is one order of magnitude higher, permitting the use of much narrower spectral bands. Orienting experiments confirmed that the averaging was relatively reliable: eliminating any of the twelve cells did not change the result significantly, showing that none of them was distorting the total result by preferential scattering of light (or avoidance of scattering) in its direction.

Amplifier. The light entering the monochromator was interrupted at 400 cycles/sec by a sector disc, and the output from the photocells was fed into a 400-Hz amplifier with a gain of 80 dB. A synchronous detector was used to discriminate against 400-Hz noise.

Measuring procedure

The absorption curves presented here are plots of the absorbance, $A = \log I_0/I$ as a function of wavelength, λ, (or wavenumber, $1/\lambda$). Since a single-beam spectrophotometer was used, I_0 and I had to be determined separately. I_0 was determined with water (or bleached algal suspension) in the absorption vessel, readings being made every 5 nm. The vessel was then filled with a suspension of cells or chloroplasts and I-readings taken every 1.0 nm (in the red region, where the bandwidth of the measuring beam was 1.0 nm). The data were transferred to punch cards and computation of the absorbance performed on a 7094 IBM computer.

Since I_0-readings were available only every 5.0 nm, intermediate values were obtained by linear interpolation. That this causes little loss in accuracy is shown by Fig. 1.

EXPERIMENTAL RESULTS

Absorption bands of chlorophyll in vivo

The main feature of the absorption curves is the complex structure of the red band *in vivo*, as evidenced by shoulders and peaks on the band envelope (*cf.* Curve 1, Fig. 2). These details represent changes in absorbance of the order of only 0.2%; it is important to make sure that they correspond to true changes in absorbance. Curve 1 in Fig. 2 (left) was obtained with live *Chlorella pyrenoidosa* cells, Curve 2 with a pigment extract. The amount of fluorescence from the extract must have been five to ten times greater than that originating from the live cells, yet no fine structure appears in the curve.

There was also no suggestion of fine structure in the spectrum of the extracted cells (Fig. 2, left, Curve 3), or in a suspension of extracted cells in the extract (Fig. 2, right). On the other hand, a doublet structure of the red band was consistently observed in live cells grown under the same conditions. Nevertheless, a correction for

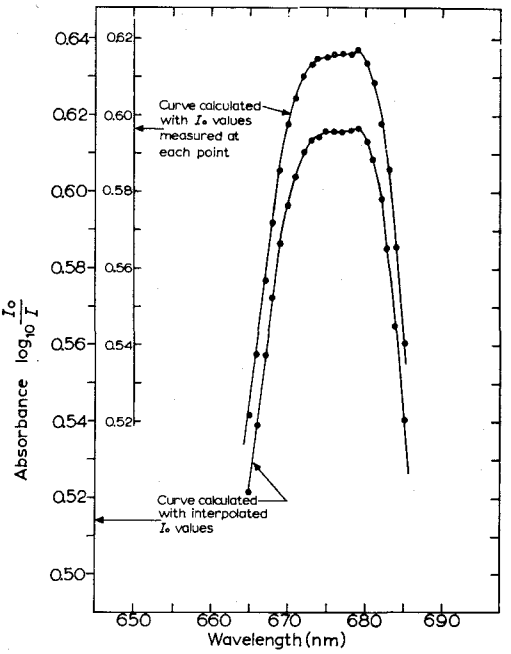

Fig. 1. Absorption curve of *Chlorella pyrenoidosa*, calculated with I_0-values measured each 1.0 nm (upper curve), and with I_0-values interpolated between points measured each 5.0 nm (lower curve).

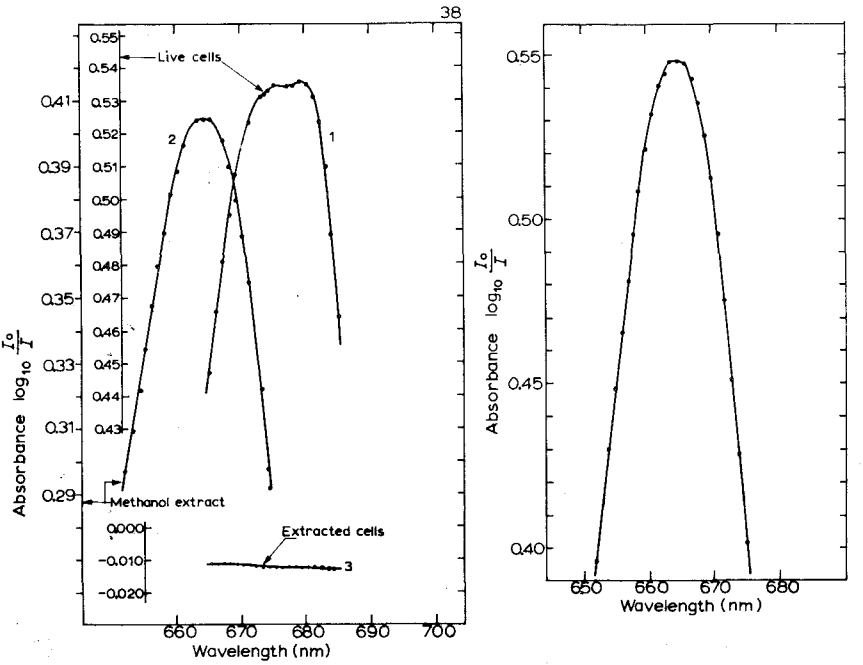

Fig. 2. Left: Absorption curve of *Chlorella pyrenoidosa* cells (1); of methanol extract from the cells (2); and of Chlorella cells after extraction (3). Right: Spectrum of extracted cells suspended in the extract.

fluorescence should be made in more precise evaluation. Since we deal with "weak" light, this correction may amount, in the peak of the fluorescence excitation spectrum (at about 670–675 nm), to about 3% of absorbed light, dropping rapidly towards the longer waves. Another correction is needed to take into account the so-called "sieve effect" which causes an apparent flattening of the absorption band (see DISCUSSION). Neither correction is likely to affect the general conclusions reached in our analysis.

Resolution of the absorption bands into components

To resolve an absorption band, one must postulate a certain shape of the component bands. The simplest choice is to assume that the shape of each component band is similar to that of the chlorophyll *a* absorption band in solution, and look for a function most closely matching the latter (see under DISCUSSION for a reason why this assumption may be not exactly correct). With absorption plotted as a function of wavenumber, the Gaussian error curve, $y = a \exp [-bx^2]$, has often been used for this kind of analysis in the past[8]. It does in fact provide a close fit for the red chlorophyll *a* absorption band in ether (see Fig. 3). A slight deviation appears in the long-wavelength end of the band, and a stronger one at its short-wavelength end, where a vibrational sub-band overlaps with the o-o electronic band. In the case of Chlorella (Fig. 4), good matching of the band envelope could be achieved by superimposing three appropriate Gaussian components. Their amplitudes, bandwidths and locations were first chosen arbitrarily, and the computer was used to sum the three components. The calculated curves were compared with the experimental absorption curve. After each comparison, an adjustment was made in one of the nine variables, and a new series of summation curves calculated. This procedure was repeated several thousand times, until a best possible fit was obtained. Similar analysis was performed

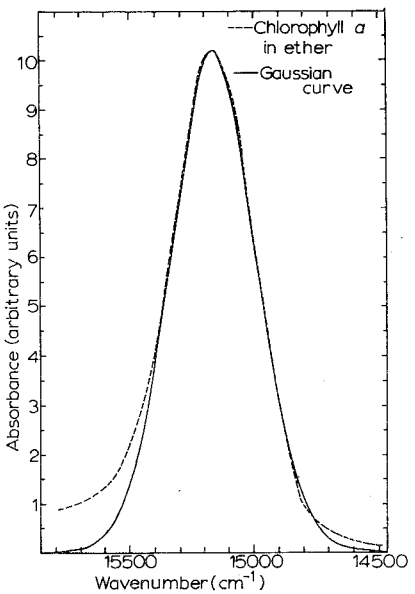

Fig. 3. Comparison of the chlorophyll *a* absorption band in ether (dashed curve) with a Gaussian error curve (solid curve).

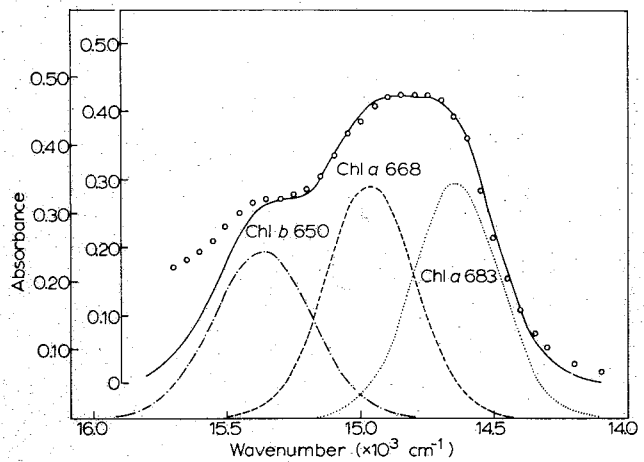

Fig. 4. Resolution of the red chlorophyll absorption band of Chlorella into three Gaussian components. The solid line gives the sum of the Gaussian components; the circles indicate the measured absorption values. The dash–dot curve belongs to chlorophyll *b*; the dotted curve to chlorophyll *a* 683 nm and the dashed curve to chlorophyll *a* 668 nm.

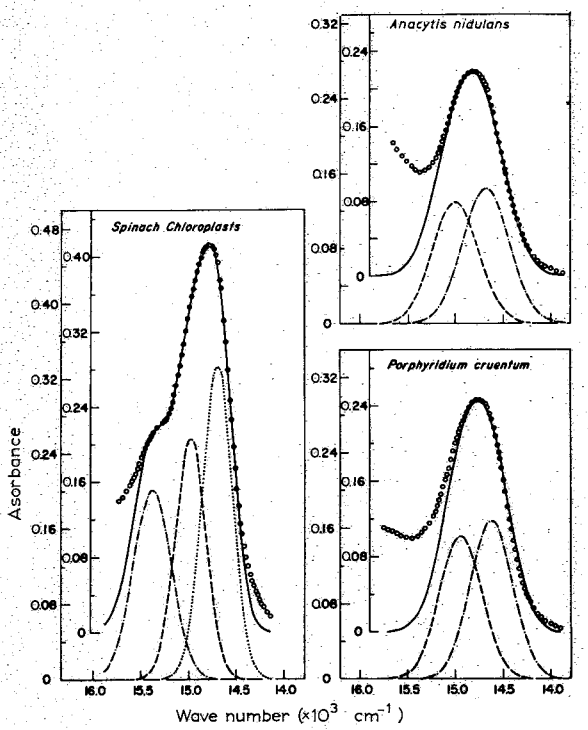

Fig. 5. Absorption curves of several algae, and of spinach chloroplasts, resolved into Gaussian components. Circles show the absorption measurements, broken lines the postulated components and solid lines the sum. In Anacystis and Porphyridium, the large deviations at shorter wavelengths are due to the phycobilins.

with the absorption curves of spinach chloroplasts, and of the cells of *Anacystis nidulans* and *Porphyridium cruentum* (Fig. 5). A summary of the results is given in Table I.

TABLE I

CHLOROPHYLL COMPONENTS *in vivo*

*Name of plant**	*Chlorophyll a*				*Ratio of short-wavelength to long-wavelength component*	*Chlorophyll b* (b) *or allophycocyanin* (p)	
	Short-wavelength component		*Long-wavelength component*			*Peak location (nm)*	*Half-width (nm)*
	Peak location (nm)	*Half-width (nm)*	*Peak location (nm)*	*Half-width (nm)*			
Chlorella pyrenoidosa I	668	17	683	18	0.99	651 (b)	18 (b)
Chlorella pyrenoidosa II	668	17	683	18	0.92	650 (b)	20 (b)
Spinacea oleracea (chloroplasts)	667	17	680	18	0.77	650 (b)	20 (b)
Anacystis nidulans	667	30	681	26	0.90	—	—
	664	23	678	23		643 (p)	23 (p)**
Porphyridium cruentum	669	23	684	24	0.91	—	—

* I and II refer to two different cultures of Chlorella.

** The first line under Anacystis refers to analysis into two, the second line to analysis into three components.

The short-wavelength component of chlorophyll *a* in Chlorella has a peak at 668 nm and a half-width of 17 nm; the long-wavelength component a peak at 683 nm and a half-width of 18 nm; the chlorophyll *b* band has a peak at 650–651 nm and a half-width of 17–20 nm. The red absorption band of the red alga Porphyridium also could be divided into two components (peaks at 669 nm, and 684 nm, half-widths 23 and 24 nm respectively), and the red absorption band of blue-green alga Anacystis, into one component at 667 nm (half-width, 30 nm) and one at 681 nm (half-width, 26 nm). An alternative analysis into three narrower bands is also possible.

DISCUSSION

The two main components

Our analysis of the absorption curves of freshly harvested Chlorella cells, and of spinach chloroplasts, consistently suggested the presence of one chlorophyll *b* and two chlorophyll *a* components (Figs. 4 and 5). BROWN AND FRENCH[8] had suggested four spectral components, while THOMAS[13] (see also ref. 14) found as many as eight irregularities in the absorption curves of chloroplasts from *Aspisdistra elatior* (obtained with a Beckman DK 2). We saw no need of assuming more than three components*.

* Some curves, presented by us at the 5th International Biochemistry Congress at Moscow in 1961 (refs. 3–5), showed more than three peaks; but these were later withdrawn because they were obtained with a "noisy" instrument.

More specifically, in analyzing the derivative absorption curve of Chlorella, BROWN AND FRENCH[8] postulated a third, weak band of chlorophyll *a* at 693 nm; they also broadened the chlorophyll *b* band and displaced it toward shorter wavelength—all in order to get a better fit of the sum of Gaussian components to the actual band envelope at its two ends. However, there is no reason to expect the absorption curve of each component to be more similar to an ideal Gaussian curve than is the red band of chlorophyll *a* in solution. Since the solution curves are not strictly Gaussian, we fully expect a sum of Gaussian components to mismatch the observed curve for live cells at the two ends (see Fig. 3). Therefore, to postulate a 693-nm component of chlorophyll *a* and to "correct" the long-wavelength tail by broadening and shifting the chlorophyll *b* band, as proposed by BROWN AND FRENCH[8], seems unjustified (even if other reasons[15] exist for postulating a weak chlorophyll *a* band *in vivo* somewhere near 700 nm). On the short-wavelength end of the red band, the presence of the first vibrational band of chlorophyll *a* in itself makes a mismatch inevitable. We therefore chose the position and the half-width of the chlorophyll *b* band in such a way as to obtain the closest fit in the immediate neighborhood of the chlorophyll *b* peak at 650 nm, and neglected deviations at the shorter waves (Fig. 4).

Figs. 4 and 5 and Table I show that the locations of the two chlorophyll *a* components are practically the same (within approx. ± 4.0 nm) in all the spectra resolved and their relative intensities (integrals of the band areas) do not differ from those in Chlorella by more than 10 %. The greatest deviation was found in spinach chloroplasts, where the height of the 668-nm band was only 75 % of that of the 685-nm band. (Perhaps, in this case, some of the 668-nm component was leached out in the preparation of the chloroplasts.)

The chlorophyll *b* band is located, in green algae and in spinach chloroplasts, at about 651 nm.

The width of the component bands and the presence of allophycocyanin

The greater width of the two components in the phycobilin-containing algae, compared with the green ones, seemed suggestive of the presence of an additional pigment, perhaps the known long-wavelength form of phycocyanin ("allophycocyanin"), with a band at about 640 nm (ref. 16). Preliminary analysis of the red band of Anacystis as sum of three Gaussian components showed that good approximation can be obtained by postulating an allophycocyanin band at 642 nm, and two chlorophyll *a* components at 664 and 678 nm, respectively, each with a half-width of about 23 nm (Fig. 6 and Table I). This is still broader than the corresponding components in Chlorella (17–18 nm), but the difference is much less than when the allophycocyanin component is omitted. This example shows that the computer analysis should not be taken as final, since alternative postulates could give a similarly good approximation. It seems, however, that no analysis could fail to reveal, in all cells studied, two approximately equally intense chlorophyll *a* components, one at 665–669 nm and one at 678–683 nm.

The "sieve effect"

Another possible source of error in the above analysis must be acknowledged —the so-called "sieve effect"[17–19], which may cause a band that has a Gaussian shape

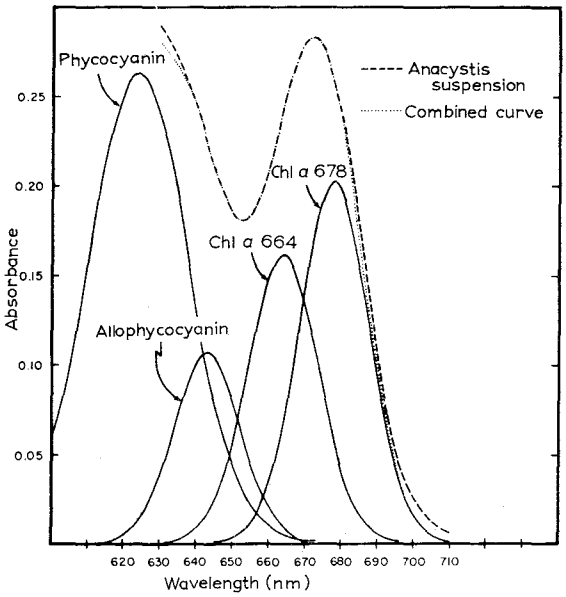

Fig. 6. Resolution of the red chlorophyll absorption band of Anacystis into three Gaussian components: chlorophyll *a* 664 nm, chlorophyll *a* 678 nm and allophycocyanin; the phycocyanin band is also shown.

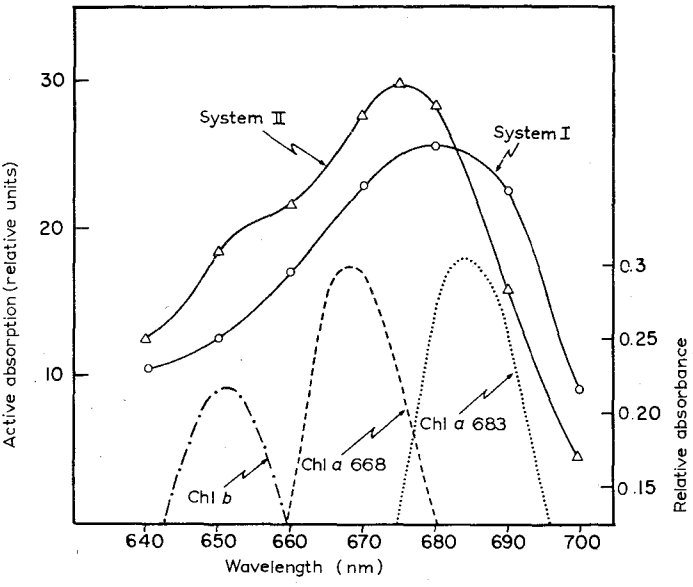

Fig. 7. Absorption spectra of Pigment systems II and I in Chlorella derived from the measurements of FRENCH, MYERS AND MCCLOUD[23] by multiplying their action spectra with $A/(1-\exp[-A])$ (assuming an absorbance of 1.0 at 680 nm). The peaks of the absorption bands of chlorophyll *b*, chlorophyll *a* 668 nm and chlorophyll *a* 683 nm are shown for comparison.

in solution to become flattened in a suspension of colored particles, such as the chloroplasts, because of mutual shading of molecules within each particle. This effect cannot, however, convert a single band into a double band, such as is clearly observed in Chlorella cells, or change the intensity ratios of the two components.

Comparison of the calculated absorption band with the calculated spectra of the two pigment systems

The absorption bands of the two main chlorophyll *a* components *in vivo*, shown in Fig. 4, can be compared with the action spectrum of the Emerson enhancement effect[9,20,21], and the absorption spectrum of the Pigment systems I and II derived by DUYSENS[22] from the measurements of FRENCH, MYERS AND MCCLOUD[23].

The action spectrum of the Emerson effect in Chlorella shows, in the red, peaks at 650 nm and 670 nm (ref. 9, Fig. 2). This led us to suggest that chlorophyll *b* and chlorophyll *a* 670 nm belong, in Chlorella, to Pigment system II, and chlorophyll *a* 683 nm (to use the position of the absorption peak now determined) to System I. According to RUBINSTEIN AND RABINOWITCH[24,25], to convert the action spectrum of an effect into the absorption spectrum of the pigments responsible for it, the action spectrum curve must be multiplied by $A/(1-\exp[-A])$. Applying this correction to the action spectrum of the Emerson effect[9], we still obtain the two peaks at 650 and 670 nm.

The half-width of the 670-nm band in the action spectra of the Emerson effect n green cells[9,20,21] is about 19 nm, practically equal to the half-width of the 668-nm component determined by analysis of the absorption curve. This supports our belief that the components obtained by computer analysis have physical significance.

However, DUYSENS[26–28] has pointed out that the curves so determined do not represent the true absorption curve of System II, but rather the difference between the absorption curves of System II and System I. He suggested that the way to calculate the true absorption spectra of the two systems is to measure the action spectrum of photosynthesis in the presence of abundant background light belonging either to System I (background light approx. 700 nm), or to System II (background light approx. 650 nm—assuming that the larger part of chlorophyll *b* belongs to System II).

The result of DUYSENS' analysis[22], based on the experimental data of FRENCH, MYERS AND MCCLOUD[23] (also corrected according to RUBINSTEIN AND RABINOWITCH[24,25]), is shown in Fig. 7. A comparison of the band components with the calculated absorption spectra of the two pigment systems (Fig. 7) suggests approximately the same distribution of quanta absorbed in green plants by chlorophyll *a* 670 nm and by chlorophyll *b*. In phycobilin-carrying algae, however, a large part of chlorophyll *a* 668 nm, together with chlorophyll *a* 683 nm, must contribute quanta to System I.

The absence of a 670-nm peak in the action spectrum of the Emerson effect in red and blue-green algae[9,29] provides an even clearer argument against our earlier postulate that chlorophyll *a* 668 nm is "chlorophyll *a* in System II", since we now find that this chlorophyll *a* component is about as abundant in Porphyridium and Anacystis as it is in Chlorella. It is disappointing to give up the simple attribution of the two spectral components of chlorophyll *a in vivo* to the two pigment systems, but there seems to be no way of sustaining this attribution. Variations in the relative heights of the peaks at 650 and 670 nm in the action spectra of the Emerson effect,

which we had observed[20,21], in different Chlorella cultures, suggest that the distribution of chlorophyll *a* 670 nm between the two systems may vary, in one and the same species, with the history of the cells.

NOTE ADDED IN PROOF (Received May 5th, 1966)

One additional possibility has to be kept in mind. The two band components may be due not to the existence of two pigment forms, but to the splitting of the band of one and the same form into two components. HOCHSTRASSER AND KASHA[30] have shown that splitting in two approximately equal components may occur when a crystalline two-dimensional pigment layer consists of unit cells containing two molecules each (in an appropriate mutual orientation).

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