ACTION SPECTRUM FOR THE APPEARANCE OF THE 520 mμ DIFFERENCE BAND IN ILLUMINATED *CHLORELLA* CELLS

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ABSTRACT An action spectrum of the 520 mμ difference band in *Chlorella* is determined using dim illumination. Pigment (or pigments) absorbing most strongly at and above 680 mμ, probably the so-called "long-wave forms" of chlorophyll *a* appear to be the primary sensitizer of the 520 mμ effect.

The appearance of an absorption band at 510 to 525 mμ in illuminated cell suspensions of *Chlorella pyrenoidosa* (and of certain other algae) has been reported by many investigators, beginning with Duysens (1-7). Chance (5), studying the time course of the 520 mμ change during illumination of aerobic and anaerobic cell suspensions of *Chlorella,* found that the 520 mμ band was complex; one component of it could be observed also in the dark, upon admission of oxygen to an anaerobic suspension of *Chlorella.* He suggested that this component, centered at 518 mμ, is due to an oxidation product of a carotenoid. It is not a necessary concomitant of photosynthesis, since it does not occur in a carotenoid-deficient mutant of *Chlamydomonas,* which is still capable of photosynthesis. Coleman and Rabinowitch (7), studying the intensity dependence of the 520 mμ effect, also found that the absorption change in the 510 to 525 mμ region is complex. They suggested that part of it (at the long-wave side of the band) may be due to the reduction of chlorophyll *a* (or of a special form of chlorophyll *a*) to a compound of the type of "eosinophyll" (the pink product of the "Krasnovsky reaction"—photoreduction of chlorophyll *a in vitro* by ascorbate (8)), while another part could be due to a carotenoid. The "negative band" (loss of absorption), appearing at 480 mμ, may be correlated with the latter component of the "positive band" at 520 mμ.

In all these investigations, the difference bands were produced either by white light or by broad bands of colored light. We have now measured the action spectrum for the absorption change at 520 mμ, using narrow spectral bands (band width, 3.3 mμ) from a "large" Bausch & Lomb monochromator with a grating blazed for maxi-

mum intensity in the red. The intensity of the 520 mμ difference band was studied with a sensitive difference spectrophotometer developed in our laboratory from Duysens' original instrument (9). A Corning blue-green glass color filter (4-72) was placed in front of the photomultiplier to absorb scattered red light and fluorescence. The intensity of the illuminating light was about 480 erg/cm² sec. as measured by an Epply thermopile. The cells were grown, harvested, and resuspended in a carbonate buffer, using the methods developed by Emerson and coworkers (*cf.* Govindjee and Rabinowitch (10)).

The sample was illuminated at right angle to the measuring beam for about 30 seconds, with 2½ minute dark intervals between successive illuminations. The change in absorption at 520 mμ was followed on a Brown recorder. A tracing obtained in this way is shown in Fig. 1. It is similar in shape to those obtained by Chance upon

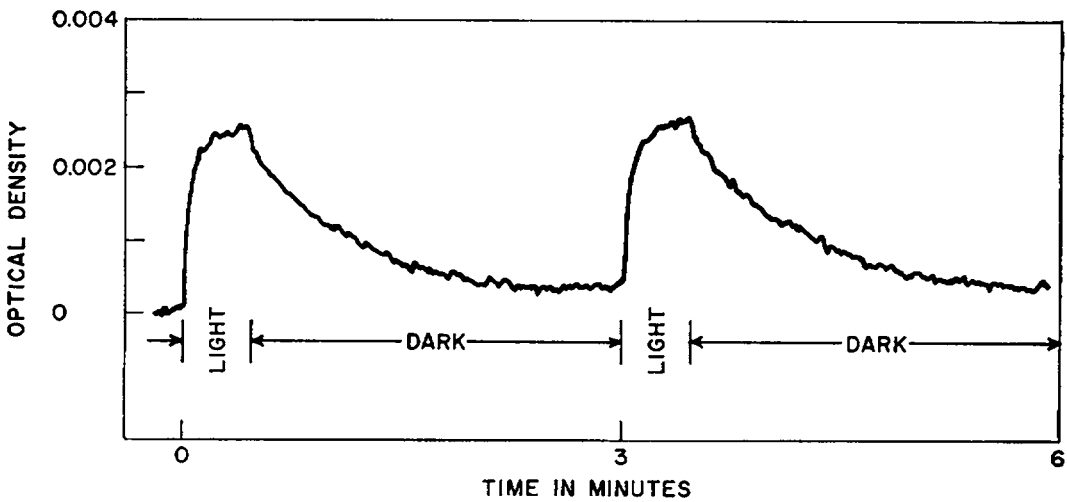

FIGURE 1 Spectrophotometric recording of the absorption change at 520 mμ upon illumination of a cell suspension of *Chlorella* with 710 mμ light (480 erg/cm² sec.).

weak illumination of "anaerobic" cells ("phase 3" in his nomenclature).[1] In the weak light used in our experiment, the absorption changes were found to increase linearly with the intensity of illumination. Therefore, it appeared legitimate to "normalize" them (*i.e.* relate them to unit light intensity). Fig. 2 (solid line) shows the intensity of the 520 mμ band for unit *incident* quantum flux. This spectrum

[1] Although our cell suspensions were not gassed with N_2, we presume that they were also in the anaerobic state, since we used a dense unstirred suspension of cells and illuminated them with dim light only one-sixth of the total elapsed time, so that respiration must have brought the oxygen concentration around the cells to a very low level.

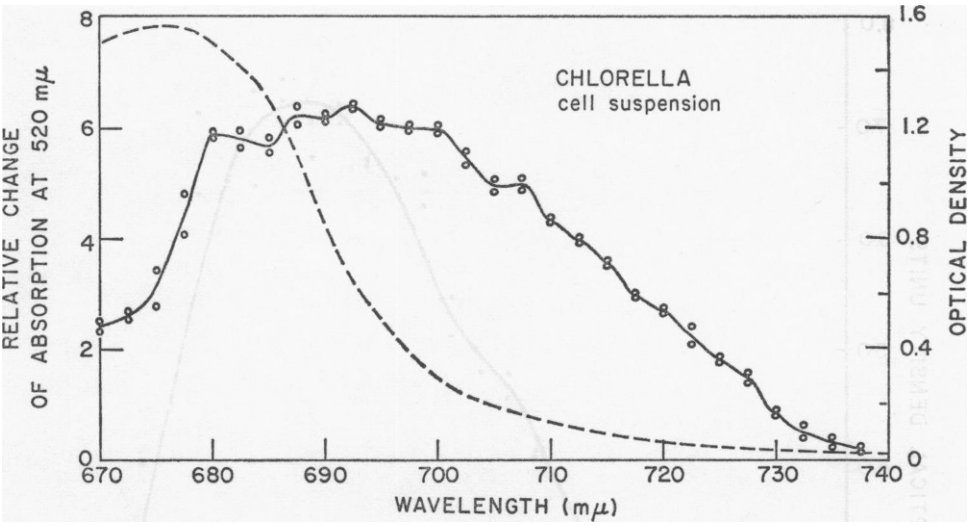

FIGURE 2 Action spectrum of the 520 mμ absorption change calculated for equal incident quantum flux (solid line); and the absorption spectrum of *Chlorella* suspension measured with three barrier layer cells placed on three sides of the cuvette to catch scattered light (dotted line).

shows a broad peak centered at about 693 mμ—considerably beyond the absorption peak of chlorophyll in the cells. This is particularly striking because this spectrum is strongly weighted in favor of shorter wavelengths by stronger absorption of the latter. (Most of the incident light in the 680 mμ region is absorbed, while most of the 705 mμ light is not.) To calculate the action spectrum for equal numbers of *absorbed* quanta, the absorption spectrum of the cell suspension was measured in the same vessel and under the same conditions under which the difference spectrum was determined. In this determination, three selenium barrier layer photocells were placed on three sides of the cuvette, covering these sides completely, in order to detect, in addition to transmitted light, a large part of the scattered light.

This simple system collects most of the scattered and transmitted light and thus approximates an integrating sphere. The efficacy of this method shown was checked by comparison of the absorption spectrum of *Chlorella,* obtained in this way (Fig 2, dotted line), with that obtained by means of an integrating sphere (*cf.* Latimer (11)).

The "true" action spectrum, *i.e.* the intensity of the 520 mμ difference band as a function of the wavelength of the exciting light, for equal number of *absorbed* quanta, calculated from the two curves in Fig. 2, is shown in Fig. 3. The drop of the curve beyond 720 mμ is uncertain, because absorption in this region is very low and large errors in its determination are inevitable. Fig. 3 suggests that one or

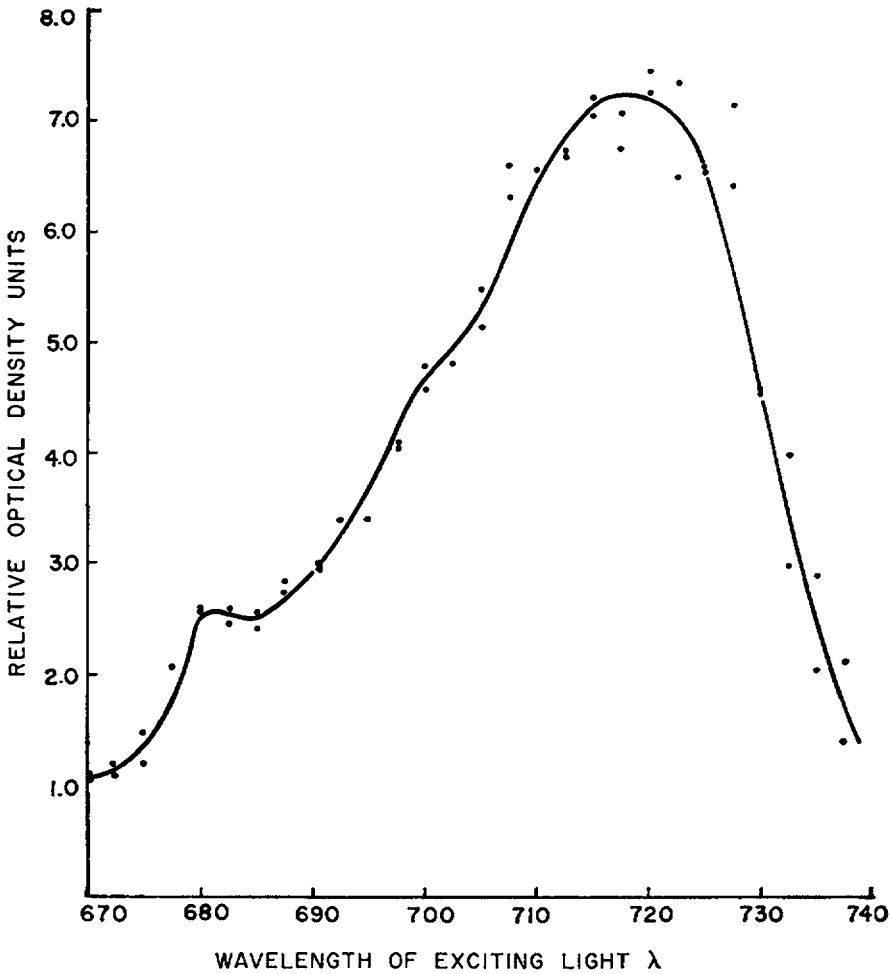

FIGURE 3 Same action spectrum as in Fig. 2, recalculated for equal absorbed quantum flux. The drop beyond 720 mμ is uncertain, and may be due to large errors of absorption measurements at those wavelengths.

several absorption bands in the 680 to 740 mμ region are most effective in sensitizing the absorption change at 520 mμ.[2]

Emerson and others (12, 13) found photosynthesis to be increasingly inefficient in

[2] These results were very surprising. We initially thought that they could represent an artifact, caused by the most strongly absorbed part of the exciting light being absorbed in the first layer of the suspension and not reaching the region of the suspension traversed by the measuring beam. However, measurements with beams of different cross-sections passing through various parts of the cuvette, convinced us that, in our apparatus, this artifact was too small to substantially affect the results.

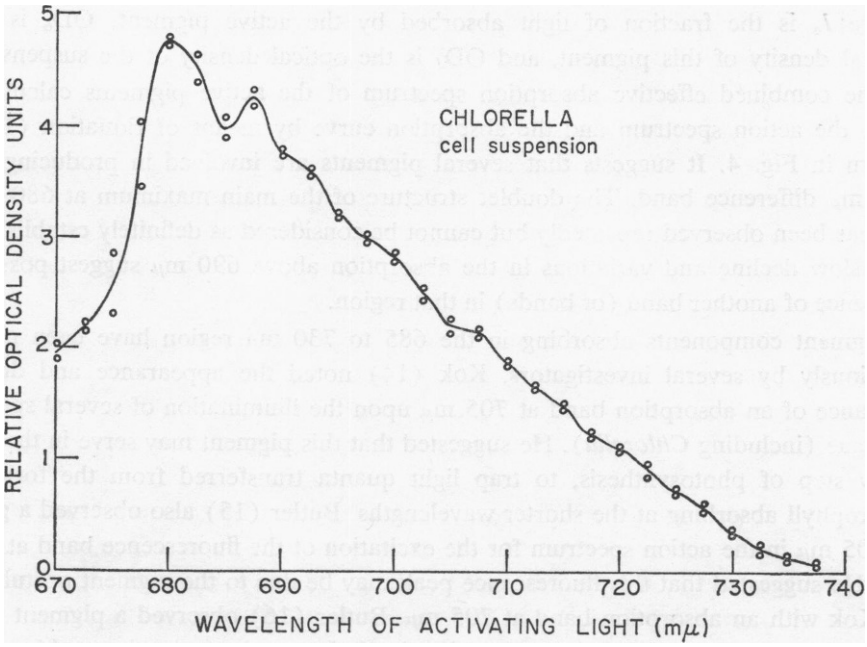

FIGURE 4 Combined, weighted absorption spectrum of the active pigments, calculated by multiplying the curve in Fig. 2 (solid line) by $OD/(1-e^{-OD})$.

the far red region above 680 mμ. More recent experiments (10, 18, 20) suggested that this inefficiency may be due to the incapacity of light in this part of the spectrum to sensitize one of the two essential photochemical reactions in photosynthesis. The difference band at 520 mμ could be associated with the photochemical step which can be sensitized by the far red light. It is remarkable that the 520 mμ difference band does not occur in red algae (19, 4). This suggests that, if it is related to a step in photosynthesis, this step is somehow different in the photosynthesis of red algae compared to that of green. Perhaps, however, Chance is right in suggesting that the 520 mμ difference band is not associated with photosynthesis at all.

Since the absorption change at 520 mμ is proportional to the amount of light absorbed, the effective absorption spectrum of the active pigments can be determined from our data. This is the sum of the absorption spectra of the active pigments, each multiplied by the efficiency of the respective pigments in producing the 520 mμ effect.

Such an expression can be derived by using the formula 1 for the fraction of light absorbed by a given pigment in the presence of other pigments (17).

$$I_a = \frac{OD_a(1 - e^{-OD})}{OD} \tag{1}$$

where I_a is the fraction of light absorbed by the active pigment, OD_a is the optical density of this pigment, and OD_J is the optical density of the suspension.

The combined effective absorption spectrum of the active pigments calculated from the action spectrum and the absorption curve by means of Equation (1) is shown in Fig. 4. It suggests that several pigments are involved in producing the 520 mμ difference band. The doublet structure of the main maximum at 680-688 mμ has been observed repeatedly but cannot be considered as definitely established. The slow decline and variations in the absorption above 690 mμ suggest possible existence of another band (or bands) in that region.

Pigment components absorbing in the 685 to 730 mμ region have been noted previously by several investigators. Kok (14) noted the appearance and disappearance of an absorption band at 705 mμ upon the illumination of several species of algae (including *Chlorella*). He suggested that this pigment may serve in the primary step of photosynthesis, to trap light quanta transferred from the form of chlorophyll absorbing at the shorter wavelengths. Butler (15) also observed a peak at 705 mμ in the action spectrum for the excitation of the fluorescence band at 720 mμ. He suggested that this fluorescence peak may be due to the pigment postulated by Kok with an absorption band at 705 mμ. Butler (16) observed a pigment with an absorption band at 730 mμ involved in stimulating seed germination and in other light responses of plants. Whether any of these pigments contribute to the action spectrum observed in this paper, remains uncertain.

We wish to thank Dr. Rajni Govindjee and Dr. Govindjee for their helpful suggestions, J. Spencer and N. Kalra for their assistance in making the measurements, and Mrs. Elaine Fox for growing the algae used in this investigation.

After this work was described in a paper presented at the meeting of the Biophysical Society in Washington in 1962, its conclusions were confirmed by Kok and coworkers (Kok, B., Cooper, B., and Yang, L., *Plant and Cell Physiol.*, 1963, suppl., 373). Müller *et al.* using chloroplasts illuminated with constant light, obtained action spectra for the 515 mμ effect similar to those described above. In flashing light, the action spectrum of the instantaneous change at 515 mμ had a maximum at shorter wavelengths (Müller, A., Fork, D. C., and Witt, H. T., *Z. Naturforsch.*, 1963, **18***b*, 142).

Received for publication, July 26, 1962.

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