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ENDOGENOUS AND PHOTOPERIODIC DIURNAL RHYTHMS OF *IN VIVO* LIGHT ABSORPTION AND SCATTERING IN THE GREEN ALGA *ULVA LACTUCA* L.[1]

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The *Ulva* thallus is two cells in thickness. Each cell contains one large chloroplast covering the outer face of the cell (Taylor, 1937). Held to the light, *Ulva* appears as a translucent, homogenous green sheet. Britz, while at the Chesapeake Biological Laboratory during the summer of 1970, observed the *in vivo* visible light absorption spectra of locally collected *U. lactuca* and noted the absorbance varied considerably over time at all wavelengths in a seemingly rhythmic pattern (unpublished data) (see Fig. 2A). This paper presents a closer examination of these observations under controlled conditions.

Methods and Materials

At the end of November, 1970, free floating specimens of *U. lactuca* were collected along shore near the mouth of the Patuxent River, Solomons, Maryland. These specimens were cultured in the laboratory through the end of April, 1971, at which time fresh specimens were obtained from the same location. Provasoli ASW-8 nutrient medium was used with the substitution of Chesapeake Bay water for sea water in the formula (Provasoli, 1958). Specimens were grown in beakers or flasks covered with clear plastic Petri dish covers in a water bath at $24 \pm 1^\circ$ C. Overhead illumination was provided by two 20W cool white fluorescent and two 40W incandescent lights. Light intensity at the water bath surface was approximately 200 ft-c. A light-dark cycle of 16 hours of light and 8 hours of darkness was employed, the cycle being synchronized to a natural photoperiod with D + 8 at 0600 EST. Considering the possibility that epiphytic bacteria could be involved in normal thallus morphogenesis (Provasoli, 1958) no attempt was made to culture *Ulva* axenically. Epiflora were assumed to be removed by careful washing of the thalli. Bacterial growth in the nutrient medium was held down by changing the containers and medium weekly. At the same time thalli were cleaned and washed by hand.

Initial measures of light absorption were obtained from spectra of live *Ulva* thalli recorded against a bleached thallus reference using a Bausch and Lomb Spectronic 505. Due to light scattering and reflection, it should be emphasized that the optical density values recorded cannot be construed as absolute measures of light absorption. For a more detailed discussion of the problems and methodol-

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ogy of *in vivo* spectroscopy the reader would be advised to consult Butler (1964). More specifically, Mestre (1935) related the problems involved in using a bleached thallus reference for absorbance measurements in *Ulva*. For the purpose of this work, however, it was considered sufficient to use relative values of light absorption, since a rhythmic phenomenon provides its own internal standard. It was assumed, furthermore, that the thick cell walls of the *Ulva* thallus would diffuse effectively the transmitted and scattered light, acting to some extent like opal glass in providing an internal correction for scattered light effects (Butler, 1964). Recognizing the above distinctions, the recorded optical density values are referred to as Relative Optical Density (ROD) values.

ROD measurements were made on 13 mm diameter *Ulva* thallus circles cut with a cork borer and placed between two square glass sample holders cut from microscope slides. Caution was exercised to avoid wrinkles and air bubbles in the light path. The sample holder was placed in the "optical bench trough" resting flush against the cuvette holder on the phototube side. Eight measurements of ROD were obtained for each thallus by rotating the sample holder 90° for each face. An average ROD was then calculated. In general, one thallus circle would be monitored throughout a particular experiment. This technique served to eliminate minor variations in ROD from thallus to thallus. Relative standard deviations of the mean ROD were of the order of a few per cent.

For all ROD measurements a reference cell was used consisting of a thallus circle bleached 24 hours in dimethylsulfoxide (DMSO). The bleached thallus was soaked for several minutes in distilled water, rinsed twice, and placed between two glass plates of a sample holder which was then sealed with clear nail polish. The ROD spectrum of the reference thallus versus a water blank revealed a smooth, monotonically increasing curve with decreasing wavelength. This was interpreted as being due to light scattering. At 350 and 800 nm ROD values were 0.85 and 0.51, respectively. Between 560 and 760 nm the curve was approximately linear.

Extractable chlorophyll *a* and *b* concentrations were obtained by a modification of the method of Reger and Krauss (1970). Thallus circles 13 mm in diameter were cut with a cork borer, placed in 5 ml of prechilled 20% DMSO in methanol, and left to extract in the dark at 6° C. Extraction of pigments was complete within two hours and the pigments were stable for at least 24 hours under the same conditions as extraction. Using a separatory funnel, the pigments were transferred to prechilled anhydrous ethyl ether. The ether solution was adjusted to 5 ml total volume and dried for several minutes over anhydrous Na_2SO_4. After the extract had warmed to room temperature, optical densities at 642 and 662 nm were recorded on a Bausch and Lomb Spectronic 505 and the values applied to the simultaneous equations of Smith and Benitez (1955). All pigment extraction work was done in either very dim white or green light.

To measure a possible photoperiodic change in optical dichroism, the differential absorption of vertically and horizontally polarized light was measured in a Beckman DK-2 Spectrophotometer. Substantial horizontal polarization of light by the optics of the Bausch and Lomb Spectronic 505 made this instrument unsuitable. A cradle for the glass sample holder was designed to fit a Beckman DK-2 light path. Polaroid *J* filters were inserted in the light beam to produce either horizontal or vertical polarization. A bleached *Ulva* reference cell was used. ROD

values were calculated from the relative transmittance at 682 nm using a correction factor derived to account for the differences in transmittance between horizontal and vertical polarizing orientations of the filter.

Results

Figure 1 shows a typical light absorption spectrum for an *Ulva* thallus at mid-photophase. At all wavelengths ROD values varied rhythmically with periods

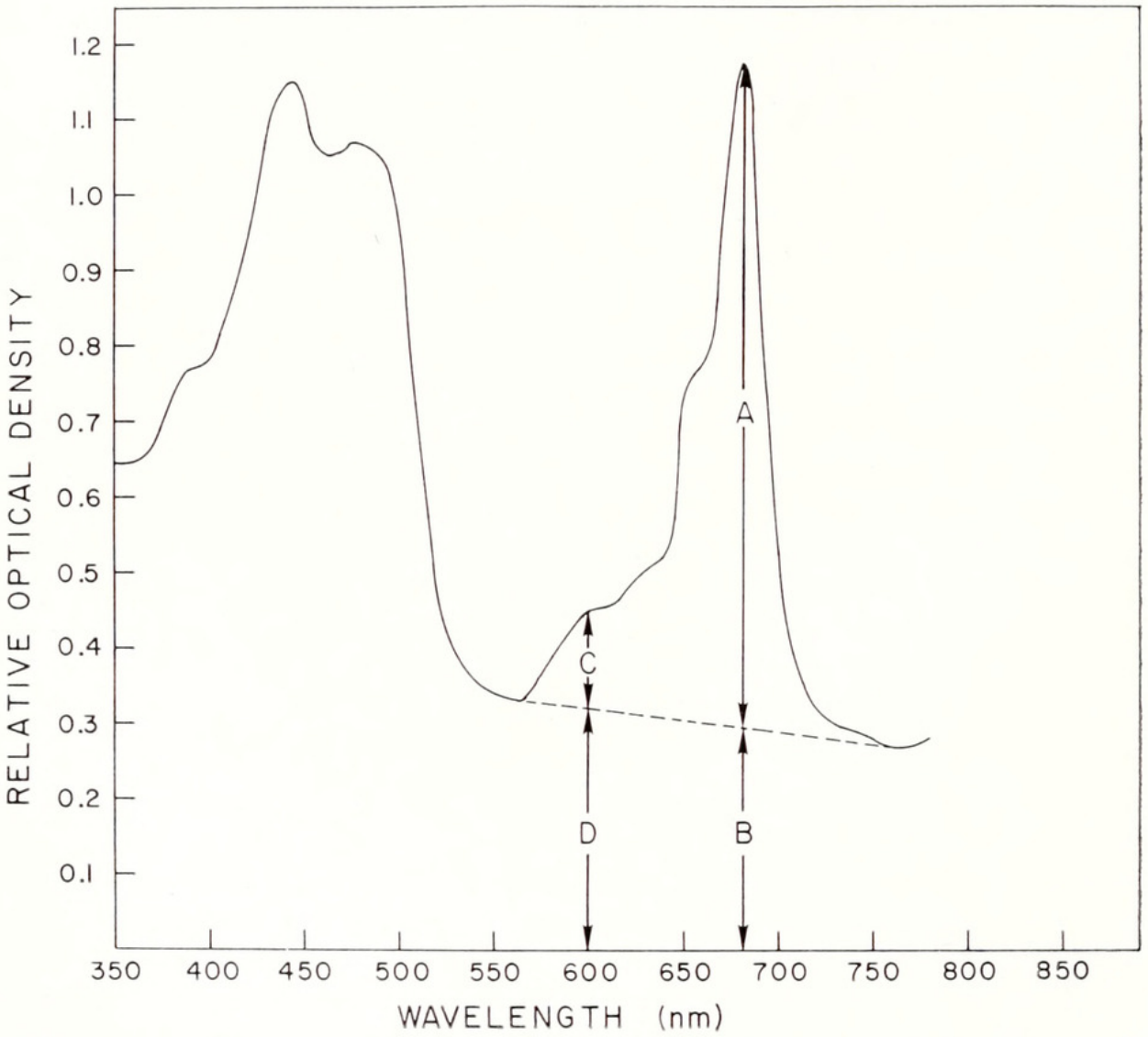

Figure 1. Typical *in vivo* absorption spectrum of mid-photophase *Ulva lactuca* L. recorded with a Bausch and Lomb Spectronic 505. See explanation under Results.

of 24 hours. However, the time of maximum ROD varied with wavelength. For example, ROD maxima at 432, 470, 493, 560, 600, 654, 682, and 760 nm occurred at L + 9, L + 9, L + 9, L + 14, L + 14, L + 12, L + 9, and D + 2, respectively. Values of ROD at 760 nm, where pigment absorption should be negligible, are defined to represent relative light scattering. The above observations were then interpreted as resulting from the addition of two different rhythms—light absorption by the photosynthetic pigments and light scattering. The light absorption rhythm seemed to have a maximum around mid-photophase, while light scatter-

ing was maximal during early scotophase. At a particular wavelength, the time of the observed ROD maximum would depend on the relative amplitude of the two rhythmic components.

On the basis of measurements conducted over three days, mid-photophase ROD values at 432, 470, and 493 nm were, respectively, 2.15, 1.97, and 1.89 times the scotophase minimum values. Although these comparisons are complicated by overlapping pigment absorption bands and light scattering, the constancy of change at three wavelengths where first chlorophyll *a*, then chlorophyll *b*, and finally the carotenoids, respectively, would be expected to be major absorbers suggests the light absorption rhythm involves both chlorophylls *a* and *b* and perhaps carotenoids.

High ROD values at 560 and 760 nm (see Fig. 1) suggested the bleached thallus reference was not correcting entirely for light scattering. Thus, in the portion of the ROD spectrum between 560 and 760 nm a further correction was made by connecting these points graphically with a straight line (dashed line in Fig. 1) designated the Light Scattering Baseline (LSB). This assumed a linear light scattering curve, such as seen for the ROD spectrum of the bleached thallus reference, and neglible pigment absorption at 560 nm. The latter assumption, however, is probably not correct, since the ROD_{560} rhythm is not in phase with the ROD_{760} rhythm—indicating the presence of substantial pigment absorption. The portion above the LSB (A and C in Fig. 1) was designated the Pigment Absorption Component (PAC), while the portion below the LSB was designated the Light Scattering Component (LSC). PAC values are defined to be relative measures of light absorption corrected for light scattering. It was noted that PAC rhythms at 600, 654, and 682 nm were in phase and had maxima at L + 8. The ability of the LSB correction to resolve a single light absorption rhythm at different wavelengths (where the ROD rhythms were out of phase) was taken as evidence for the validity of the procedure, at least at wavelengths where the amplitude of the PAC rhythm was large with respect to the amplitude of the LSC rhythm.

In Figure 2B typical values of relative light absorption, represented by PAC_{682}, and light scattering, given by ROD_{760} are plotted over three normal light-dark cycles and 57 hours of continuous darkness following the last scotophase.

The rhythm of PAC_{682}, while dampening, continued clearly for at least three cycles of continuous conditions with a periodicity that seemed to remain close to 24 hours. The ROD_{760} rhythm also appeared to continue, albeit rather erratically toward the end. It did not appear to change its phase relation to the PAC_{682} rhythm. At the conclusion of the period of continuous darkness the thallus was transferred to a normal light-dark cycle where over a period of a week its regained a normal rhythm pattern.

In an attempt to elucidate the basis of the light absorption rhythm, chlorophyll *a* and *b* concentrations were determined three times over a 24 hour period. Five 13 mm diameter thallus circles were extracted at each time, and the average content per thallus of chlorophyll *a* and *b*, respectively, was as follows: 21.5 ± 0.7 and 9.2 ± 0.4 μg at D + 7.7, 22.5 ± 0.2 and 9.5 ± 0.2 μg at L + 8.2, and 22.8 ± 1.0 and 9.3 ± 0.3 μg at L + 15.4. Cholorphyll *b* concentrations did not seem to change, but cholorophyll *a* concentrations did appear to increase gradually about 6% over the 16 hour light period. However, comparison of the maximum and

minimum chlorophyll *a* values by t-test showed no significant difference to approximately 85% certainty. Even if significant, the chlorophyll *a* concentration increase was neither large enough to account for the amplitude of the light absorption rhythm nor was the change in chlorophyll *a* concentration in phase with the rhythm.

Alternatively, it was considered that the light absorption rhythm might be brought about by a rhythm of cholorphyllide *a* and *b* incorporation as chlorophyll *a* and *b* by the thylakoid membranes. Ethyl chlorophyllides *a* and *b* have absorb-

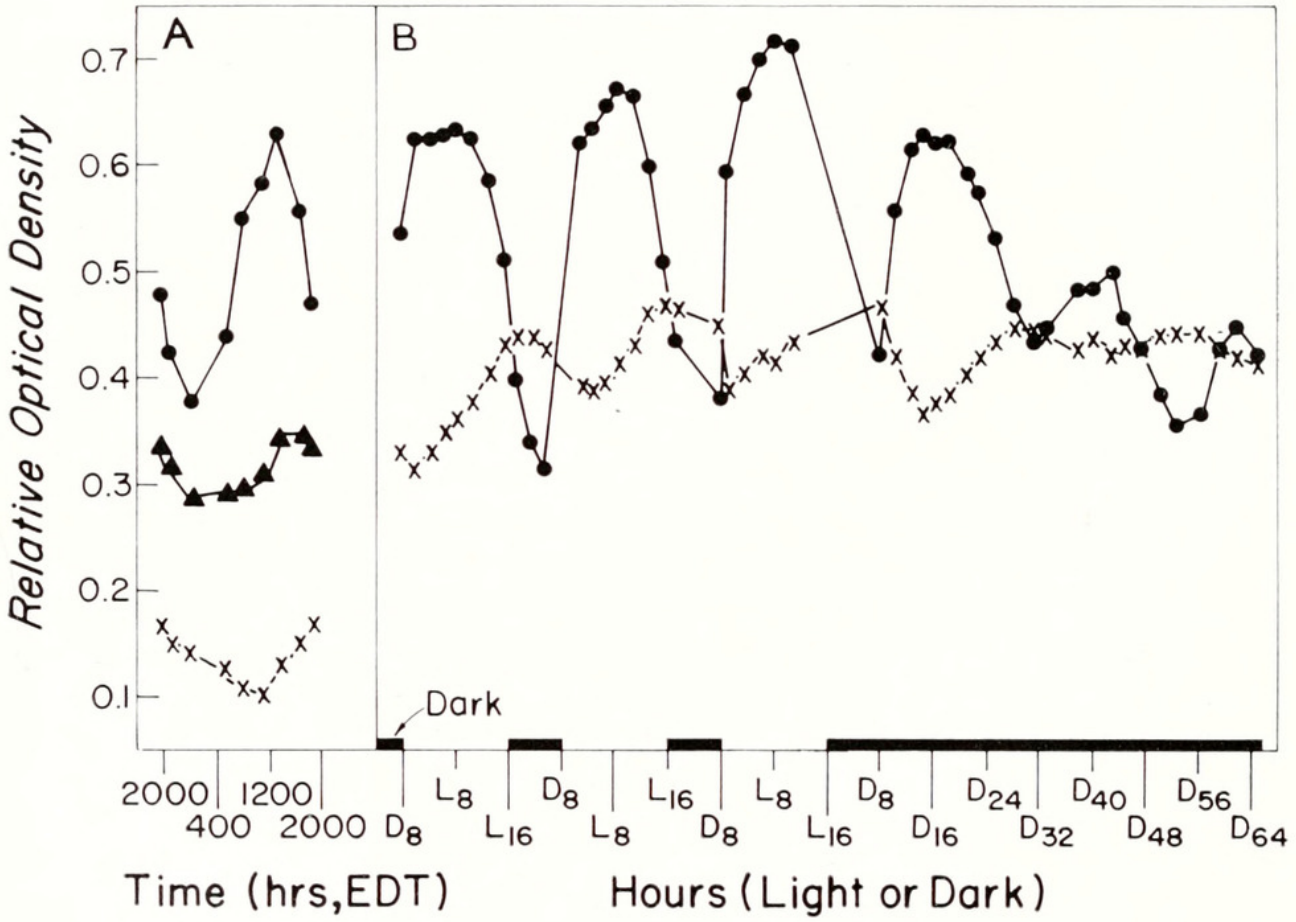

FIGURE 2. (A.) The change in ROD over July 13–14, 1970 (See Introduction) at 445 (●—●), 673 (▲—▲), and 615 nm (x—x). Measurements were made on a Coleman Model 6-A Spectrophotometer. *U. lactuca* was grown in glass-wool filtered, running bay water with a natural photoperiod from window light illumination. Light intensity at 1430 EDT was 5×10^3 erg/cm^2/sec; (B.) PAC_{682} (●—●) and ROD_{760} (x—x).

ance spectra very close to chlorophyll *a* and *b*, respectively (Holt and Jacobs, 1954). Electron microscopy of *U. mutabilis* F. (Løvlie and Bråten, 1968) showed the thylakoids were oriented largely in the plane of the thallus. If a similar situation existed in *U. lactuca*, then the potential for a detectable change in linear dichroism would exist. If a large proportion of chlorophyll *a*, for example, dissociated to chlorophyllide *a* during scotophase, then the average orientation of the porphyrin dipole moment should change from being approximately in the plane of the thallus to being randomly oriented, and the absorbance should decrease. For a light beam oriented perpendicular to the plane of the thallus, there should be no difference in the absorption of vertically and horizontally polarized light whether

the orientation of the dipole moment of the absorber is in the plane of the thallus or randomly oriented. If the light beam were incident at 45°, then the electric vector of the horizontally polarized light would have only one component with half the intensity in the plane of the thallus. If the dipole moment of the absorber were largely in the plane of the thallus, one might expect up to twice as much absorption of vertically polarized light as horizontally polarized light relative to random orientation.

It was found, however, that at both D + 7.5 and L + 8.5 the ratio of 682 nm vertically polarized light absorbed to horizontally polarized light absorbed was equal to one for both 90° and 45° angles of incidence of the light beam. This indicated the rhythm of light absorption at that wavelength could not be accounted for in terms of an incorporation of oriented chlorophyll *a* into thylakoids from previously randomly oriented cholorphyllide *a*. It was assumed that similar results would be found for the case of chlorophyll *b* and cholorophyllide *b*.

Chloroplast movement is a well known phenomenon for many different plants (for a review, see Zurzycki, 1962). In *Ulva* the appropriate shifting of the chloroplast might decrease light absorption through a shading effect. However, *in vivo* light microscopy observations did not reveal any obvious change in chloroplast morphology between scotophase and photophase.

Discussion

Under constant conditions, the persistence of the light absorption and scattering rhythms and the maintenance of periods approximately 24 hours imply that these are endogenous circadian rhythms. Any postulated mechanism must account for what seems to be the participation of both chlorophyll *a* and *b* and perhaps carotenoids. The light absorption rhythm was shown not to be due to changes in pigment concentration or in the overall change of absorber dipole moment orientation. It is unlikely that changes in the pigment environment drastic enough to alter the extinction coefficient by factors of two or three would occur, since no changes in wavelengths of maximum absorption were observed. Although no obvious changes in chloroplast position were noted, the small size of the cells (about 10 μm in diameter), their bilayer arrangement, and the large chloroplast made it difficult to visualize the chloroplast position.

Løvlie and Bråten (1968) reported qualitatively what seemed to be a rhythm of cell division in *U. mutabilis*. Most division occurred shortly after the beginning of the dark period and involved the chloroplast shifting to the side of the cell during cleavage. It does not seem likely that this type of chloroplast movement would be responsible for the light absorption rhythm reported here where the decrease in absorbance begins soon after mid-photophase. In addition, for our *Ulva* cultures only about one-quarter of the cells would be dividing per day (on the basis of an observed doubling time of about four days). Even if the dividing cells absorbed no light at all after chloroplast movement, the decrease in absorbance would only be about one-fourth, instead of the one-half to two-thirds actually seen. It does seem possible, however, that the light scattering rhythm reported here could be related to the cell division of Løvlie and Bråten, since the two rhythms seem to be in phase. Further light microscopy is indicated to resolve the issue.

Murakami and Packer (1970a) described light induced reduction of thylakoid membrane thickness, increased ordering and flattening of the thylakoid discs, and chloroplast volume changes in the dark adapted thalli of *Ulva* and *Porphyra* sp. These changes were correlated with decreases in the 180° transmittance and increases in the 90° light scattering at 540 nm. Studies with isolated spinach chloroplasts revealed similar results (Murakami and Packer, 1970b). Though these changes are not endogenous rhythms, they do suggest that alteration of the thylakoid membrane structure can produce changes in light transmittance and scattering. The possibility that such changes could be involved in the rhythms reported here should be subject to investigation by electron microscopy. While Murakami and Packer do not report the presence of light absorption or scattering rhythms in their *Ulva* specimens, the possibility of such rhythms should be considered as a potentially influential factor in light scattering investigations of light induced chloroplast structural changes.

The adaptive significance of light absorption and scattering rhythms in *Ulva* remains to be investigated. It will be of interest to determine whether the rhythms are related to rates of or capacity for photosynthesis.

Summary

Techniques of *in vivo* spectroscopy were employed to demonstrate the presence of rhythms of light absorption and scattering in the green thalloid alga *Ulva lactuca* L. maintained in artificial nutrient medium under constant photoperiod. The absorbance during photophase at 682 nm, the chlorophyll *a in vivo* absorption maximum, was typically two to three times that during scotophase. Prephased endogenous rhythms (in continued darkness) were observed for a time period equal to three photoperiods. The absorbance rhythm did not correlate with changes in extractable chlorophyll *a* or *b* concentration. No changes in linear dichroism were observed, indicating the rhythm of light absorption could not be explained on the basis of orientation of individual absorber molecules. *In vivo* light microscopy did not reveal differences in chloroplast orientation. Alternative mechanisms are discussed.

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