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Characterization of Photosynthetic Rhythms in Marine Dinoflagellates

II. PHOTOSYNTHESIS-IRRADIANCE CURVES AND *IN VIVO* CHLOROPHYLL *A* FLUORESCENCE[1]

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ABSTRACT

Using data from light-dark cultures of *Gonyaulax polyedra* entrained to a 24-hour cycle, whole cell absorption curves and photosynthesis-irradiance curves were constructed for various circadian times. While whole cell absorbance and half-saturation constants of photosynthesis showed no statistical difference that could be directly related to the photosynthetic rhythm, the initial slope of the photosynthesis-irradiance curve was a time-dependent parameter which altered in direct proportion to the change in photosynthetic capacity. The results indicated a temporal change in the relative quantum yield of photosynthesis, and the circadian rhythmicity of light-limited photosynthesis was established under constant conditions. Circadian rhythmicity was detected in room temperature chlorophyll fluorescence yield. Low temperature fluorescence kinetics also showed fluctuations. The results suggest that regulation of photosynthesis by the biological clock of *Gonyaulax* may be mediated through the membrane-bound light reactions and a partial explanation of the underlying mechanism is proposed.

Once one accepts the idea that photosynthesis can be regulated by a biological clock, the question is how is the overt rhythm in photosynthetic capacity mediated. Photosynthesis could be regulated either through the membrane-bound light reactions and/or through the more loosely membrane-associated dark reactions involved in CO_2 fixation. Observations of conformational changes in chloroplast shape or thylakoid orientation (9, 23), periodicity in shapes of photosynthesis-irradiance curves (16-18, 22), lack of periodicity in dark reaction enzyme activity (3, 8), and periodicity in the activity of the photosystems (16, 17), have led some workers to suggest that the circadian oscillator regulates photosynthesis through some modifying interaction with the photosystems imbedded in the thylakoid membrane. In contrast, the reported periodicity in glyceraldehyde 3-P-dehydrogenase in *Euglena* and the lack of periodicity in the relative quantum yield of photosynthesis observed in photosynthesis-irradiance curves have been cited by workers who favor mediation by the circadian oscillator through the dark reactions of photosynthesis (25).

The difficulty in trying to make an independent interpretation of the results from several of these studies lies in their experimental design. Often only synchronized cultures entrained to light-dark cycles have been used to look for rhythmicity in photosynthetic parameters other than photosynthetic capacity (3, 16-18, 25). In these cases, it is difficult to discern whether the observed rhythmicity is derived from a circadian oscillation or the cell division cycle. Similarly, the observation of a diurnal periodicity in a light-dark cycle is not sufficient to demonstrate control by a biological clock. Rather, the rhythmicity of a cell parameter must also free run under constant conditions, be independent of cell division, and have a period of about 24 hr to qualify as a true circadian rhythm.

Cultures of the marine dinoflagellate *Gonyaulax polyedra* provide an almost ideal system for studying photosynthetic rhythms. Unlike *Euglena*, whose diurnal periodicity in photosynthesis stops after only one cycle under constant conditions (25), the photosynthesis rhythm in *Gonyaulax* persists for several days and even weeks in continuous dim illumination (ref. 7; B. Sweeney in preparation). Previous observations and the present study show that under the low light levels used to follow the free running photosynthesis rhythm, cell division is virtually halted in *G. polyedra*, while viability, cell size, pigmentation, and general photosynthetic activity are unaffected. Thus, any diurnal periodicity in a photosynthetic parameter which continues in constant illumination can be correlated with the rhythm in photosynthetic capacity. It was a search for such photosynthetic parameters that formed the basis of the present study.

MATERIALS AND METHODS

G. polyedra Stein clone 70A (UCSB[2] code No. 5M20) was cultured under conditions previously described (14). Likewise, details of the procedures for photosynthetic measurements utilizing an O_2 electrode have been documented (14).

Measurements of cell absorption were made at room temperature with a single beam spectrophotometer on line with a PDP-8/I computer (4). For comparison of absorption properties at various times in the circadian cycle, cells were harvested during exponential growth by centrifugation (5 min, 3,000 rpm, room temperature) and resuspended in fresh growth medium. Aliquots of the cell suspension were diluted with growth medium to adjust cell concentrations to 8×10^4 cells/ml $\pm$ 3%. Absorption spectra were determined for 0.5-ml aliquots to which 70 mg of calcium carbonate had been added. Comparable 0.5-ml samples of growth medium with 70 mg of calcium carbonate were used for base line determinations.

In vivo room temperature steady-state fluorescence (F_m) in the absence and the presence of DCMU (F_{DCMU}) was measured for cell suspensions of *G. polyedra*. Cells were collected at

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[2] Abbreviations: P_{max}: photosynthetic capacity; LD; light-dark cycles; LL: continuous illumination; ct: circadian time; UCSB: University of California, Santa Barbara algal culture collection; PSU: photosynthetic unit; P-I: photosynthesis-irradiance curve.

various times in the circadian cycle, centrifuged (2 min, 300 rpm), and resuspended in a small volume of fresh medium. The suspension was adjusted to a concentration of 10^4 cells/ml or approximately 0.45 μg Chl *a*/ml. The cell suspension was aliquoted into four 2-ml samples and 0.025 ml 10^{-4} M DCMU added to two of the samples. All four aliquots were stoppered and dark-adapted for 30 min. At the end of the incubation period, sufficient DCMU had entered the cells to allow DCMU-induced steady-state fluorescence to plateau. Measurements were made with a Perkin-Elmer model 2A spectrophotofluorimeter, using ratio recording of reference and sample signals to provide stable monitoring of Chl *a* fluorescence emission. Sample and reference sensitivity settings were maximized. Slit widths of 8 nm were used for both the excitation wavelength of 440 nm and the emission wavelength of 680 nm. Fluorescence emission was recorded for 10 to 30 sec for each sample and replicate values were averaged. Data were plotted as a ratio of steady-state fluorescence in the presence and absence of DCMU (F_m/F_{DCMU}) *versus* circadian time.

Low temperature Chl *a* fluorescence measurements were determined using a vertical cuvette and Dewar system (4). A concentrated cell suspension (approximately 10^6 cells/ml) was dark-adapted, a 0.3-ml aliquot removed and frozen to liquid N_2 temperature, and the Chl *a* fluorescence kinetics was monitored. A white light source was supplied from a Unitron Lamp passed through a heat-reflecting filter. The collimated light passed through a filter combination consisting of a Coliflex 4303 filter, a Coliflex 5030 filter, and a Baird-Atomic 437 filter to define the exciting light at a wavelength of 437 nm. The exciting light was incident on the top surface of the frozen sample and fluorescence at 690 nm (defined by a filter combination consisting of a Corning 9830 glass filter, a Toshiba VR-65 glas filter, and a 690 nm Balzers interference filter) was measured from the bottom surface. The output was converted to voltage (EMI 9558C photomultiplier with S20 response), amplified, and measured on a strip chart recorder.

RESULTS

The well defined system in *G. polyedra* was used for extended studies on the mechanism of regulation of photosynthetic capacity by a biological clock. First, the *in vivo* whole cell absorption properties of *G. polyedra* in samples taken during the middle of the light and dark period (Fig. 1) were compared. Although thylakoids of this species display a circadian rhythmicity in orientation which reaches extremes at these two times of day (9), the degree of thylakoid appression did not appear to affect the light-absorption capabilities of *Gonyaulax*.

Using data from LD cultures entrained to a 24-hr cycle, photosynthesis-irradiance (P-I) curves were constructed for ct 3, ct 6, ct 9, and ct 12. In measurements made over 6 days, P_{max} at ct 6 averaged 184 ± 21 × 10^{-7} μmol O_2/cell·hr. Photosynthetic measurements at other times and irradiances were plotted as percentages of ct 6-P_{max} determined for that day (Fig. 2). As previously shown P_{max} is a time-dependent parameter in photosynthesis in *G. polyedra*, the maximum rate of O_2 evolution occurring at midday (14). The half-saturation constants for the various P-I curves ranged from about 1,800 μw/cm^2 (ct 3 and ct 9) to about 2,200 μm/cm^2 (ct 6 and ct 12), but there were no statistical differences that could be directly related to the change in P_{max} (Table I).

The initial slope of the P-I curves was a time-dependent parameter of photosynthesis and did alter in direct proportion to the change in P_{max} (Table I). Slopes were determined by simplified linear regression analyses, which assumed a zero intercept and utilized photosynthesis values between 0 and 3,000 μw/cm^2. χ^2 analyses for linearity provided a confidence limit greater than 90% for ct 3, ct 6, and ct 9. Summed data for ct 12 from several days did not fit well the criteria for linearity, although individual daily experiments displayed linear slopes significantly different from slopes determined at other circadian times. Standard deviations were determined and the variance tested for the slopes

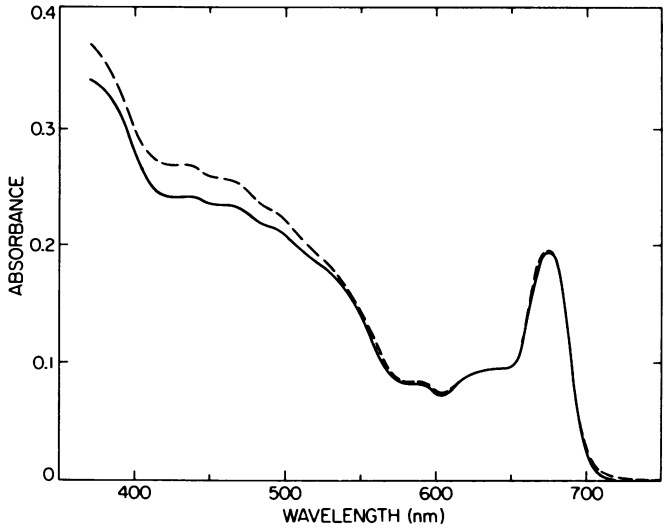

FIG. 1. Room temperature absorption spectra of *G. polyedra* recorded midday at ct 6 (- - -) and midnight at ct 18 (——). Cells were harvested from cultures entrained to a 12:12 hr light-dark cycle, where the irradiance of the light period was about 1,000 μw/cm^2. Replicate 0.5-ml samples were measured, each containing 8 × 10^4 cells/ml ± 3% and 70 mg calcium carbonate. Comparable 0.5-ml samples of growth medium with 70 mg of calcium carbonate were used for base line determinations.

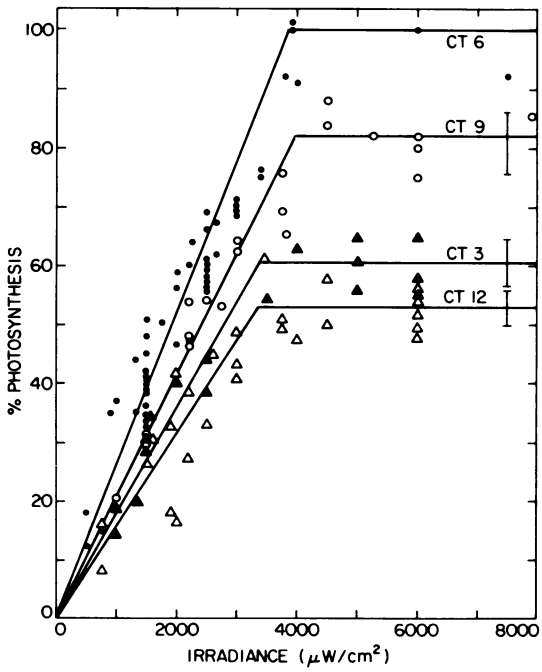

FIG. 2. P-I curves for *G. polyedra* determined at ct 3 (▲), ct 6 (●), ct 9 (○), and ct 12 (△). Cells were harvested from cultures entrained to a 12:12 hr light-dark cycle, where irradiance of the light period was about 1,200 μw/cm^2. Measurements were made over 6 days and P_{max} at ct 6 averaged 360 ± 42 × 10^{-8} μmol oxygen/cell·hr. Photosynthesis was plotted as percentages of ct 6-P_{max} determined for that day. Standard deviations of photosynthetic capacity at various circadian times are indicated by error bars. Slopes were determined by simplified linear regression analyses, which assumed a zero intercept and utilized photosynthesis values between 0 and 3,000 μw/cm^2.

Table 1. Comparison of photosynthetic parameters of G. polyedra measured at different times in the circadian cycle.

Circadian time	Photosynthetic capacity as % $(P_{max})_{ct\ 6}$	Relative slope of P-I curves*	Slope of P-I curves as % $(slope)_{ct\ 6}$	Irradiance of half-saturation of P_{max} ($\mu W/cm^2$)	Irradiance of half-saturation as % $(I)_{ct\ 6}$
ct 3	61.33±3.55	.01716±.00229	68.04±9.08	1844±293	92.2±14.65
ct 6	100±0.00	.02522±.00250	100±0.00	2000±188	100±0.00
ct 9	82.27±6.26	.02290±.00177	90.80±7.02	1799±115	90.0±5.75
ct 12	52.31±3.66	.01260±.00361	49.96±14.3	2230±528	111.5±26.40

*determined from linear regression analyses of photosynthesis values at irradiances up to 3000 $\mu W/cm^2$ and assuming a zero-intercept

measured at each circadian time. The slopes were found to be significantly different from each other at the 95% confidence limit.

To test the circadian nature of light-limited photosynthesis in *G. polyedra*, photosynthetic rates at saturating (5,000 $\mu w/cm^2$) and nonsaturating (2,500 and 1,500 $\mu w/cm^2$) light levels were followed through one LD cycle and for 2.5 days following transfer of the cultures to LL (Fig. 3). Circadian rhythmicity was observed at all light levels, and comparable amplitudes of about 1.8, 1.6, and 1.6 were noted for 5,000 $\mu w/cm^2$, 2,500 $\mu w/cm^2$, and 1,500 $\mu w/cm^2$, respectively. However, the amplitude of dark respiration declined from about 1.8 to the LD cycle to approximately 1.3, 1.2, and 1.1 in successive LL cycles. This is consistent with previous observations by Sweeney (unpublished) on the diel periodicity of respiration in *G. polyedra*, which suggested that respiration, unlike photosynthesis, was not regulated by a circadian oscillator. These results indicated a temporal change in the relative quantum yield of photosynthesis (O_2 evolved/relative quanta absorbed) which correlated with the circadian rhythmicity of P_{max}.

Since there is no circadian change in pigment content (14) and whole cell absorption, and yet the rate of photosynthesis varied, it seemed consistent that a circadian rhythm in re-emitted light or Chl *a* fluorescence should exist in *G. polyedra*. When room temperature Chl *a* fluorescence at 680 nm was measured in the presence and absence of DCMU over the circadian cycle, temporal changes in relative quantum yield were reflected as a change in the F_m/F_{DCMU} ratio (Fig. 4). Averaged from five preliminary experiments, F_m/F_{DCMU} values ranged from a minimum at ct 6 of 0.61 to a maximum at ct 18 of 0.825. The average daily amplitude was about 1.35. The greatest amount of Chl *a* fluorescence occurs when photosynthetic rates are the lowest, and vice versa. This is consistent with the idea that an underlying mechanism of the circadian rhythm in photosynthesis involves an interference with the energy transduction process in the thylakoid membranes. Preliminary Chl *a* fluorescence measurements at liquid N_2 temperatures indicated a large difference in the F_m/F_0 ratio determined at ct 6 and ct 20, but were not sufficient to indicate which parameter was the variable component. (F_0 is a minimum fluorescence value measured when all reaction centers of photosynthesis are open; F_m is a maximal steady-state value when all reaction centers are closed and includes the F_0 value.) The average of triplicate samples showed a mean F_m/F_0 value of 2.70 ± 0.07 at ct 20 and 1.77 ± 0.04 at ct 6. To understand more fully how energy transduction in the membrane-bound light reactions is controlled and regulated by a biological clock, it will be necessary to quantify more precisely the various fluorescence parameters on a cell basis and examine carefully temporal changes in low temperature Chl *a* fluorescence kinetics.

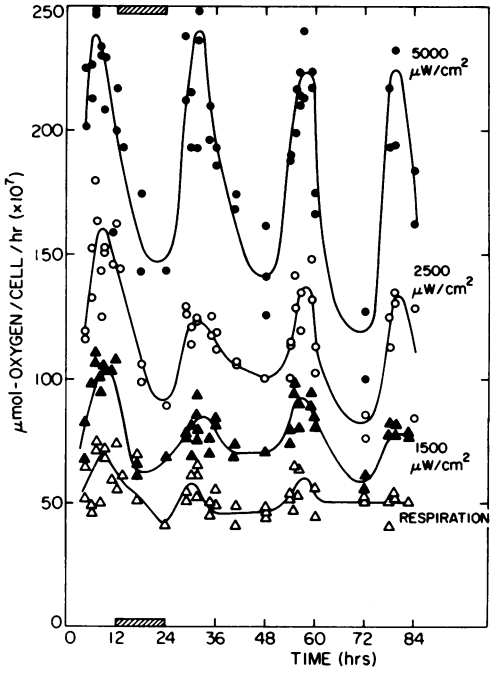

FIG. 3. Photosynthesis rhythms of *G. polyedra* measured at different irradiances. Photosynthetic capacity (●), light-limited photosynthesis at 2,500 $\mu w/cm^2$ (○, ▲, respectively), and respiration (△) are expressed as μmol oxygen/cell·hr. The light-dark cycle was 12:12 hr and the irradiance of the light period was 1,200 $\mu w/cm^2$. Dark period is indicated by hatched bar on abscissa. Cultures were transferred to constant dim illumination (500 $\mu w/cm^2$) at circadian hr 24. Cells were in midexponential phase of growth.

DISCUSSION

We examined the nature of the circadian rhythm of photosynthesis in *G. polyedra*, looking for possible underlying fluctuations in membrane parameters which could be correlated with the overt rhythm in photosynthetic capacity (P_{max}) (14). The photosynthesis rhythm could have been generated in three general ways: (*a*) rhythmicity in the number of cells in the culture capable of carrying out photosynthesis; (*b*) rhythmicity in the activity or concentration of enzymes regulating electron transport between the photosystems and/or dark reactions related to the fixation of CO_2; (*c*) rhythmicity in the coupling between photochemical events within the photosynthetic apparatus.

The first possibility was eliminated when rhythmicity of photosynthesis was demonstrated in single cells of *G. polyedra* (20). The second possibility is incompatible with the circadian changes observed in the photosynthesis-irradiance (P-I) curves (Fig. 2). Lowering the activity or concentration of a photosynthetic enzyme would cause the saturation of the light reactions to occur at a lower irradiance and thereby lower the value of P_{max}. However, at light-limiting irradiances, photosynthesis would proceed at a rate independent of circadian regulation an enzyme activity. Thus, it would be the half-saturation constants of photosynthesis, not the relative quantum yield, which would be expected

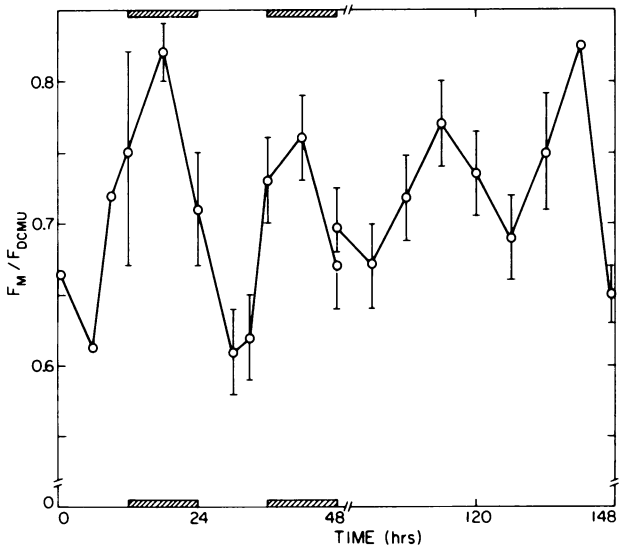

FIG. 4. Rhythm of room temperature *in vivo* Chl *a* fluorescence of *G. polyedra* in the absence and presence of DCMU and expressed as the ratio F_m/F_{DCMU}. Points represent averaged data from five preliminary experiments and per cent error is indicated by error bars. Light-dark cycle was 12:12 hr and the irradiance of the light period was 1,200 μw/cm^2. Dark periods are indicated by hatched bars on abscissa. Cultures were transferred to constant dim illumination (500 μw/cm^2) at hr 48 and measurements resumed at hr 96. Cells were in midexponential phase of growth.

to change in direct proportion to P_{max}. This is not the case in *G. polyedra*.

The results of the P-I curves can be best explained in terms of the activity of the light reactions, or more specifically, in terms of the proportion of total photosynthetic units which are functional in the thylakoid at any one time in the circadian cycle. (A photosynthetic unit, PSU, is defined here as the smallest portion of the thylakoid membrane which can carry out the light reactions of photosynthesis; a PSU is comprised of photosystems I and II surrounded by bulk light-harvesting pigments.) Since the absorption capabilities, cellular pigment content (14), and half-saturation constants do not change significantly, it can be assumed that the functional size and total number of PSU per cell are relatively constant. Thus, a change in photosynthetic potential must reflect a change in the activity of existing photosynthetic apparatus. If the activity of all of the photosynthetic units were partially reduced in their energy transduction process at times of lower P_{max} (*e.g.* by incomplete coupling of energy flow), one would predict that the resulting P-I curves would have features similar to those generated when enzyme substrate is limiting. A change in P_{max} would be accompanied by a change in half-saturation constants, not in the relative quantum yield. However, the P-I curves obtained can only be explained if a portion of the total number of photosynthetic units were completely inactivated. The effect would be to lower the relative quantum yield and directly affect the photosynthetic capacity of the cell (Fig. 2). (It should be noted that close examination of data previously reported to confirm no rhythmicity in photosynthesis at low light levels, does in fact reveal maxima of low magnitude that are in phase with the light-saturated photosynthesis rhythm [7].)

Since light energy not used in photosynthesis is re-emitted as fluorescence, inactivating photosynthetic units would generate a rhythm in Chl fluorescence yield that is 180 degrees out of phase with the photosynthesis rhythm (Fig. 4). Although only preliminary, the low temperature fluorescence measurements (which indicated an increase in the F_m/F_o ratio during the dark phase) suggest that an uncoupling site of the PSU may occur between the reaction center and the light-harvesting pigments.

Studies on *Acetabularia, Chlorella,* and *Scenedesmus* also suggest circadian regulation of the activity of the light reactions as a basic phenomenon underlying the photosynthesis rhythm in these organisms. In each case, the P-I curves resembled those constructed for *Gonyaulax* (16–18, 22). In both *Acetabularia* and *Synechococcus*, the photosynthesis rhythm was unrelated to pigmentation and correlated to changes in relative quantum yield. The same was presumably true for *Chlorella,* although some accumulation of Chl over the cell cycle resulted in a decrease in both the relative quantum yield and half-saturation constant with a decrease in P_{max} (18). Furthermore, in *Acetabularia* the photosynthetic rhythm was unrelated to the activity of nine enzymes of the reductive pentose cycle (8) and correlated to rhythmic changes in chloroplast shape (23). In *Scenedesmus*, the size of the PSU was shown to be constant over the cell cycle (15); while the rhythmicity in quantum yield was further related to a change in the activity of photosystem II (16, 17).

The most recent models suggested to explain the mechanism of circadian rhythmicity have focused on the regulation capacity inherent in the structure-function relationship of biological membranes. Initially derived by Sweeney, from data accumulated on the photosynthetic rhythm in *Acetabularia*, a physiological membrane model was proposed that suggested that circadian rhythms are generated by a feedback loop, of which one component is active transport across organelle membranes and the other is the distribution of one or several molecules between organelles and the cytoplasm (21). Njus *et al.* (13) later suggested ions and membrane-bound ion gates as the active components of the feedback loop, with temperature compensation of circadian rhythmicity dependent on temperature adaptations of membrane lipids. Since additional information is now available on membrane and photosynthesis rhythms, a partial explanation of the photosynthesis rhythm in *Gonyaulax* can be proposed. Based on the membrane feedback model for circadian rhythms, the suggestion is made that circadian ion fluxes across the thylakoid membrane generate reversible conformational changes which couple/uncouple entire photosynthetic units in the membrane and thereby induce a circadian rhythmicity in the photosynthetic capacity of the cell.

Circadian oscillations in membrane properties of *Gonyaulax* have been documented and include rhythms in spheroplast membrane fragility (1), membrane electrical potential (1, 2), lipid composition (1), and thylakoid orientation (9). Chl *a* fluorescence, which also displays a circadian rhythmicity in *Gonyaulax*, is a sensitive probe of structural changes in the thylakoid and can be particularly responsive to changes in cation concentrations (11) and lipid composition (12). A specific cation and fatty acid effect on photosystem II activity in spinach chloroplasts also has been demonstrated *in vitro* (19). In *Gonyaulax*, the rhythmicity in relative quantum yield, as well as the lack of rhythmicity in pigmentation, half-saturation constants, particle size and number on the thylakoid freeze-fracture faces (B. Sweeney, in preparation), and ribulose di-P carboxylase activity (3), are all consistent with the thought that a change in the activity of the existing membrane-bound photosynthetic apparatus underlies the rhythm in photosynthesis.

The general effect of cations on the structural and energetic state of chloroplasts membranes has been well established. Specifically related to photosynthetic activity, protons are liberated as a result of photosystem II-dependent oxidation of water and transported into the thylakoid membrane or inner thylakoid space. Giaquinta and his co-workers (5) recently demonstrated in spinach chloroplasts that large proton accumulation and localization in the thylakoids generated a membrane conformational change that resulted in the uncoupling of ATP synthesis and electron flow. The uptake of protons has been shown to be

closely linked to K^+, Mg^{2+}, and water efflux, as well as ATP synthesis (11, 24). Of these, K^+ has been directly linked to the circadian oscillator. Not only is there a valinomycin-mediated K^+ uptake rhythm across the spheroplast membrane (1, 2) and a rhythm in the intracellular levels of K^+ (21), but K^+ has been directly implicated in the phase shift induced in the circadian rhythm in bioluminescence by valinomycin (21). Recent studies also define the reversible efflux of K^+ across the thylakoid and suggest that the equilibrium potential of K^+ can contribute significantly to the energetic state of the chloroplast (24).

It thus seems possible to suggest a working hypothesis in which the circadian oscillatory regulates the ion fluxes across the thylakoids of *G. polyedra* and thereby controls the energetic state of the membrane and thus its photosynthetic activity. This also would be consistent with rhythmicity observed in cell division and bioluminescence of *Gonyaulax*. All three cell processes are membrane-bound or membrane-associated and controlled by a single oscillator (6, 10). Ion fluxes between the chloroplast, nucleus, and cytoplasm and their effect on membrane properties could be evoked to explain the activity and phasing of each rhythm. Although photosynthesis has some potential for self-regulation (5, 24), inhibition of photosynthesis by DCMU does not affect the rhythmicity of bioluminescence (17). Therefore, photosynthesis itself can not be the driving force for circadian rhythmicity.

Further studies will be directed toward defining the site of photosynthetic inactivation in the photosynthetic unit, using techniques to measure fluorescence kinetics and individual photosystem activity.

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