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Regulation of the Photosynthesis Rhythm in *Euglena gracilis*

I. CARBONIC ANHYDRASE AND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE DO NOT REGULATE THE PHOSPHOSYNTHESIS RHYTHM[1]

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ABSTRACT

A circadian rhythm of O_2 evolution has been found in *Euglena gracilis*, Klebs strain Z. The rhythm persists for at least 5 days in constant dim light and temperature, but damps out in constant bright light. The phase of this rhythm can be shifted by a pulse of bright light and the period length is not changed over a 10 C span of growth temperature.

The O_2 evolution rhythm is found in both logarithmic and stationary phase cultures, but CO_2 uptake is clearly rhythmic only in stationary phase cultures.

The activity of glyceraldehyde-3-phosphate dehydrogenase was not rhythmic as previously reported (Walther and Edmunds [1973] Plant Physiol. 51: 250-258). Carbonic anhydrase activity was rhythmic when the cultures were maintained under a light-dark cycle with the highest enzyme activity coinciding with the fastest rate of O_2 evolution. However, the rhythm in carbonic anhydrase activity disappeared under constant conditions. Changes in the activities of these two enzymes are therefore not responsible for the rhythmic changes in photosynthetic capacity.

Circadian rhythms in photosynthesis have been observed in several algae and higher plants (12). The mechanism(s) responsible for the rhythms of O_2 evolution and CO_2 fixation have never been resolved, even though most aspects of the light and dark reactions have been investigated (3, 11, 17-19, 22). This problem has been reinvestigated using the single cell alga *Euglena gracilis* in an attempt to explain how the O_2 evolution rhythm is regulated.

Daily oscillations in photosynthesis have been reported for *Euglena* (22), but it was not established that a persistent circadian rhythm was involved. This investigation shows by several criteria that the rhythm of O_2 evolution in *Euglena* can be circadian. A careful description of this rhythm is given here since this work is the basis for several forthcoming papers dealing with the mechanism of the photosynthetic rhythm.

Several investigators have tried to determine if rhythmic changes in the activity of the Calvin cycle enzymes are responsible for the photosynthesis rhythm (3, 11, 19, 22). The activity of ribulose-1,5-bisP carboxylase is not rhythmic in *Euglena* (4, 22) or other algae (3, 11). Walther and Edmunds reported that changes in the activity of glyceraldehyde-3-P dehydrogenase might generate the photosynthetic oscillations in *Euglena* (22), but their results are contrary to the findings for *Acetabularia* (11). We have repeated this work using *Euglena*.

Carbonic anhydrase activity has been correlated with photosynthetic rates in several algae (8-10, 13, 16). The possibility that oscillations in carbonic anhydrase activity are responsible for the photosynthesis rhythm has also been investigated.

MATERIALS AND METHODS

Growth Conditions. Axenic cultures of *E. gracilis* Klebs strain Z were grown in Cramer and Myers inorganic medium (5). Filter-sterilized air was bubbled through the culture (12-14 liters in 5-gallon carboys) at a rate of approximately 1 liter/min. The cultures were magnetically stirred and kept in a growth chamber at 25 C. The cells were synchronized for approximately 7 days by growth in a light-dark cycle of 10 hr light (2×10^5 ergs cm^{-2} sec^{-1}, 10,500 lux at midcarboy height) and 14 hr dark. Cool-white Sylvania fluorescent tubes were used to provide illumination. Cultures were transferred to constant dim light (2×10^3 ergs cm^{-2} sec^{-1}, 430 lux) at 25 C for rhythm studies. Cells were removed for sampling during the late phase of logarithmic growth (approximately 90,000 cells/ml) unless otherwise specified. Irradiances were measured with a Yellow Springs Instrument radiometer and a Li-Cor model LI-185 photometer.

Photosynthesis Measurements. Photosynthetic capacity was calculated from O_2 evolution measurements in a system using a light intensity of 5×10^6 ergs cm^{-2} sec^{-1} (9,000 lux; sample size 1.6 ml). An unfiltered Unitron Koehler research illuminator, model LKR, was used as a light source, and an O_2 monitor, model 53 from the Yellow Springs Instrument Company, was utilized for the O_2 measurements. A constant temperature circulator maintained the cuvette temperature at 25 C. All values were corrected for respiration, and cell counts were done with a hemacytometer. Replicate determinations of photosynthetic measurements for the same cell culture were reproducible within 4 to 10% of the mean. CO_2 uptake was measured using a Beckman model 865 IR CO_2 analyzer. Cells were placed in a 125-ml double side arm flask and stoppered. The flask was shaken in a water bath at 25 C and the air in the closed system was circulated through the analyzer and back to the culture flask with a Masterflex peristaltic pump. The rate of CO_2 removal from the closed system was used as a measure of CO_2 fixation. The light intensity for the measurements was 4.5×10^6 ergs cm^{-2} sec^{-1} (8,500 lux), using a 300-w incandescent bulb.

Isolation of Enzymes. Both enzymes were isolated in an identical manner. An aliquot of the cell culture was centrifuged at 2,000*g* for 5 min. The cell pellet was washed with distilled H_2O and centrifuged in a clinical centrifuge for 10 min. For

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every 0.1 ml of packed cell volume, 1 ml of 10 mM Tris-HCl buffer (pH 7.8 for carbonic anhydrase assays and pH 8.4 for glyceraldehyde-3-P dehydrogenase assays) plus 1 of 2.5 mM Veronal buffer (pH 8.2) were added to the cells. Both buffers also contained 5 mM 2-mercaptoethanol. The cells were broken by three 30-sec pulses (4-ml volume) from a Sonifier cell disruptor, model W185D, used at voltage setting 2. The sonication was performed in an ice-water bath (0 C), and 1-min cooling periods were used between each pulse. An aliquot of the sonicate sap was added to 100% acetone and the Chl was determined by the method of Arnon (2). The remainder of the sonicate sap was centrifuged for 20 min at 40,000*g* at 4 C.

Assay of Glyceraldehyde-3-P Dehydrogenase. The assay of Wu and Racker (23) was used to measure the rate of enzyme activity. The oxidation of NADPH was recorded at 340 nm with a Gilford model 240 spectrophotometer and the rate is expressed as mmol of NADPH oxidized/min/mg Chl. Final concentrations of the assay components were 50 mM Tris-HCl (pH 8.4), 0.2 mM NADPH, 9 mM $MgCl_2$, 6 mM ATP, and 10 mM 3-phosphoglyceric acid. The assay was started by adding 0.5 ml of the enzyme extract to 1 ml of the reaction mixture. The reaction was saturated with respect to the substrates and was linear with time. The rate was proportional to the amount of extract added to the reaction mixture. Three to five replicates were performed for each determination and the rates were reproducible within 6% of the mean.

Assay of Carbonic Anhydrase. The reaction was followed by measuring the change in pH of the buffer from 8 to 7.5, and the rate is expressed as the number of whole pH units changed/min/mg of Chl (14, 15). The uncatalyzed rate of the reaction using boiled enzyme was subtracted from the catalyzed rate. The reaction mixture contained 0.75 ml of 25 mM Veronal buffer (pH 8.2) with 20 μg/ml bromothymol blue, 0.50 ml of crude extract, and 0.50 ml of CO_2-saturated water. A Corning model 12 pH meter was used with a Sargent-Jena miniature combination electrode, and the reaction mixture was magnetically stirred and maintained at 2 C with a refrigerated water bath. The assay was linear with time over the pH range measured, and the rate was proportional to the amount of enzyme extract added to the reaction mixture. Rates were reproducible within 3% of the mean.

RESULTS

Photosynthetic Capacity Rhythm. A rhythm in photosynthetic capacity, as measured by the rate of O_2 evolution, was found when photosynthesis measurements were made with saturating light (5×10^6 ergs cm^{-2} sec^{-1}, Fig. 1). The rate was highest in the middle of the light phase and lowest at the end of the dark phase. The peak of the maximum is sharp, but the rate decreases slowly throughout the last 14 hr of the cycle. The rhythm persists for at least five cycles when cultures are transferred to constant dim light conditions (2×10^3 ergs cm^{-2} sec^{-1} light).

Photosynthesis Rhythm. A light saturation curve for photosynthesis is shown in Figure 2. The rhythm of photosynthetic capacity reported in Figure 1 was measured in saturating light (5×10^6 ergs cm^{-2} sec^{-1}). However, a rhythm of O_2 evolution can also be found when measurements are made with a subsaturating light intensity (*e.g.* 2×10^5 ergs cm^{-2} sec^{-1}, the intensity used for entrainment). The only discernible difference between the two conditions is a lower maximum photosynthetic rate with the subsaturating condition.

Criteria for Circadian Rhythmicity. The rhythm of O_2 evolution can be designated as circadian by several criteria (20), including the persistence of the rhythm in constant environmental conditions. The exact period length of this rhythm has not been determined, but it approximates 24 hr. Constant bright light quickly damps the rhythm after one cycle (Fig. 3A). A 1-hr pulse of bright light, given at the part of the cycle correspond-

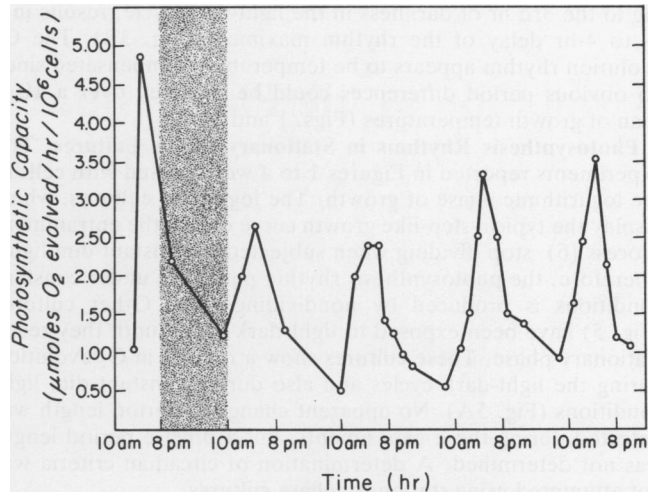

FIG. 1. Persistence of photosynthetic capacity rhythm in constant conditions. The division-synchronized culture was grown in 10-hr light and 14-hr dark (shade) and then exposed to constant dim light (2×10^3 ergs cm^{-2} sec^{-1}) at 25 C after the last dark period.

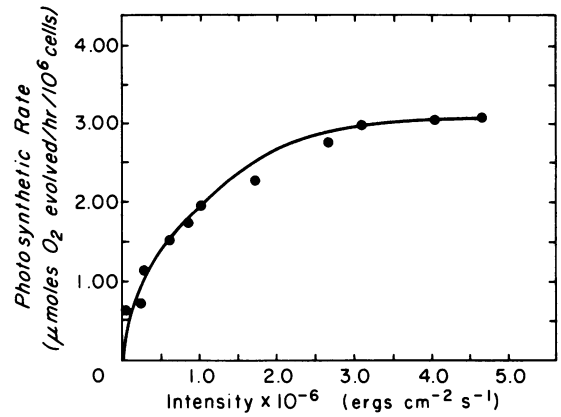

FIG. 2. Light saturation curve for photosynthesis. The rate of O_2 evolution was measured at various light intensities. Cells at the maximum of the photosynthetic rhythm were used for the measurements.

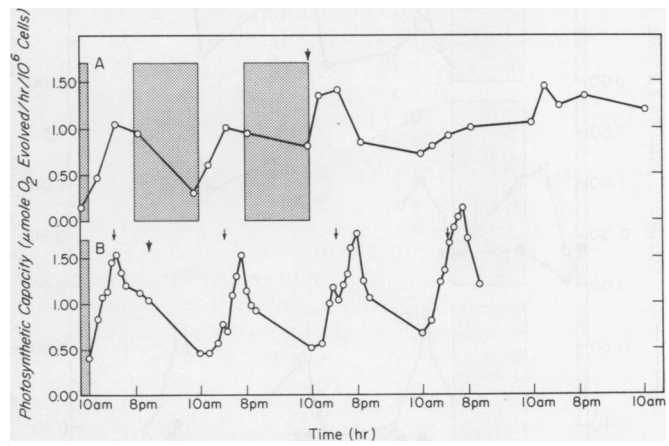

FIG. 3. Effects of bright light on the O_2 evolution rhythm. A: effect of constant bright light on the rhythm. A culture was exposed to continuous bright light (1.75×10^4 ergs cm^{-2} sec^{-1}) at the arrow. B: effect of a bright light pulse on the phase of the rhythm. A culture was exposed to continuous dim light (2×10^3 ergs cm^{-2} sec^{-1}) after the last dark period shown. A bright light pulse (1.75×10^4 ergs cm^{-2} sec^{-1}) was administered to the culture for 1 hr (heavy arrow) and the phase of the rhythm was determined for 3 days. The maximum rate of O_2 evolution was expected to occur at 3 pm (light arrows) if no phase shift had occurred.

ing to the 3rd hr of darkness in the light-dark cycle, results in a 3- to 4-hr delay of the rhythm maximum (Fig. 3B). The O_2 evolution rhythm appears to be temperature-compensated since no obvious period differences could be detected over a 10 C span of growth temperatures (Figs. 1 and 4).

Photosynthesis Rhythms in Stationary Phase Cultures. The experiments reported in Figures 1 to 4 were started with cells in the logarithmic phase of growth. The log phase cultures, which display the typical step-like growth curve during the entrainment process (6), stop dividing when subjected to constant dim light. Therefore, the photosynthesis rhythm persisting under constant conditions is produced by nondividing cells. Other cultures (Fig. 5) have been exposed to light-dark cycles until they reach stationary phase. These cultures show a rhythm in O_2 evolution during the light-dark cycles and also during constant dim light conditions (Fig. 5A). No apparent change in period length was detected during these experiments, but a precise period length was not determined. A determination of circadian criteria was not attempted using stationary phase cultures.

Rhythm in CO_2 Uptake. A rhythm in CO_2 uptake was found (Fig. 5B), but only with stationary phase cultures. The cell density from log phase cultures was not great enough to measure CO_2 uptake or release directly with our experimental procedures. Consequently, cells from log phase cultures were concentrated by centrifugation to the same density as the stationary cultures. No discernible rhythm in CO_2 uptake was observed at 30 or 25 C growth temperatures, but a possible rhythm of low amplitude was observed at 20 C (Fig. 4). Subsequent experimentation failed to verify a rhythm at 20 C. A determination of circadian criteria was not attempted for the CO_2 uptake rhythm.

Absence of a Respiration Rhythm. The measurements for O_2 evolution and CO_2 uptake were both corrected for dark respiration. No rhythms in either O_2 consumption or CO_2 evolution were ever observed.

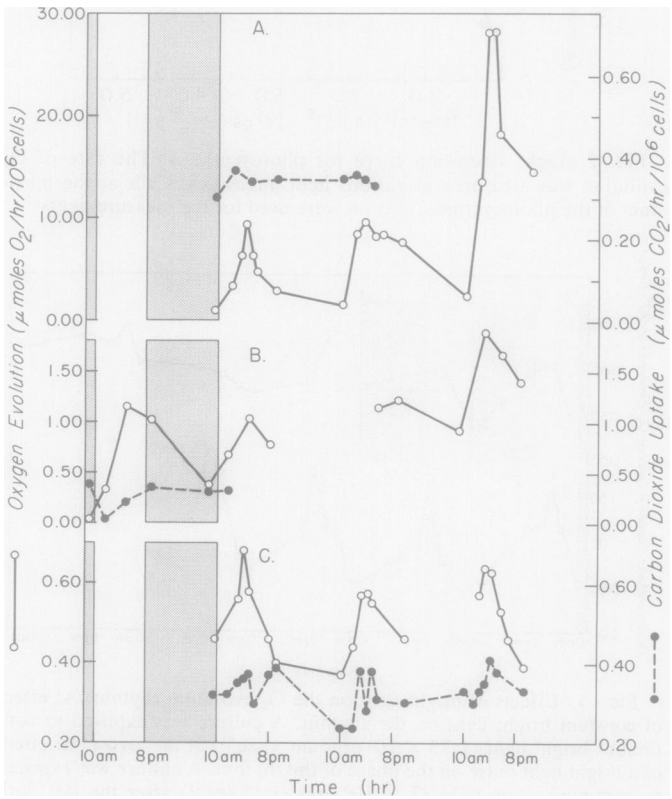

FIG. 4. Effect of temperature on the photosynthetic rhythm. Logarithmic cultures were used for O_2 evolution and CO_2 uptake measurements. Growth and measurement temperatures were: 30 C (A), 25 C (B), and 20 (C).

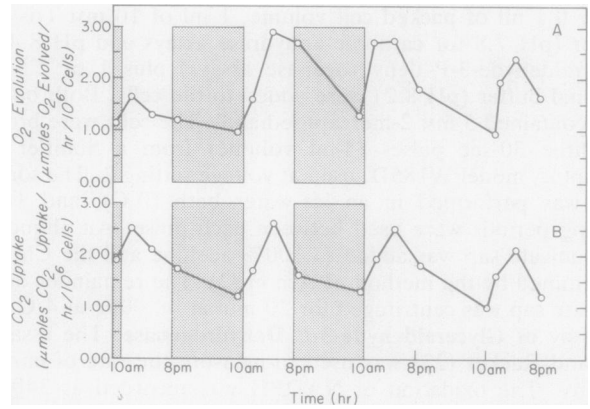

FIG. 5. Photosynthetic rhythms for a stationary phase culture. Photosynthetic capacity as measured by (A) O_2 evolution and (B) CO_2 uptake.

Glyceraldehyde-3-P Dehydrogenase Activity Is Not Rhythmic. A previous report (22) for *Euglena* indicated that glyceraldehyde-3-P dehydrogenase activity was rhythmic. The phase of this enzyme rhythm was correlated with the observed oscillations in photosynthetic rate. We were unable to find rhythmic activity of this enzyme during the entrainment cycle or constant dim light (Fig. 6). Although Figure 6 shows only 1 day of constant conditions, subsequent experiments demonstrated that no rhythm was present even after 4 days in constant dim light.

Carbonic Anhydrase Activity Is Not Rhythmic. Carbonic anhydrase has been implicated as a regulatory enzyme in photosynthesis (8–10, 13, 16). Rhythmic changes in carbonic anhydrase activity were observed with the greatest enzyme activity found at the same time as the highest rate in photosynthesis. The changes in enzyme activity were found only when the light-dark cycle was operating, and disappeared when the culture was transferred to constant conditions (Fig. 7). The small fluctuations of enzyme activity seen in constant conditions did not show a consistent pattern from experiment to experiment.

DISCUSSION

A true circadian rhythm in O_2 evolution had been found in *Euglena*. This rhythm is classified as circadian by several criteria including persistence in constant environmental conditions, temperature compensation (Fig. 4), and susceptibility to phase shifting by light (Fig. 3). This conclusion is contrary to that of Walther and Edmunds (22), who reported that the photosynthesis rhythm in *Euglena* does not persist in constant conditions. Variations in culture conditions might account for this difference in our results. The cultures used by Walther and Edmunds (22) for O_2 evolution measurements were not aerated during growth and were supplemented with 20 mM sodium acetate. Although the effects of sodium acetate on photosynthesis have not been tested in our system, acetate is known to repress the synthesis of Chl and fructose 1,6-diphosphatase in *Euglena* (1).

The two parameters used most frequently for measuring net photosynthetic rates, O_2 evolution and CO_2 uptake or fixation, are rhythmic in *Euglena*. A hypothesis tested by several investigators to explain these rhythms involves a rhythmic change in the activity of one or more Calvin cycle enzymes.

The only Calvin cycle enzyme reported to have rhythmic activity is glyceraldehyde-3-P dehydrogenase. Walther and Edmunds (22) have reported an oscillation in the activity of this enzyme which they suggest may be responsible for the photosynthesis rhythm in *Euglena*. We were unable to find any rhythmic activity of this enzyme (Fig. 6). A possible explanation for the

difference in results between our laboratory and theirs is based on the fact that our enzyme preparation was more active and required three orders of magnitude more NADPH to reach saturation. When the assay procedure used by Walther and Edmunds was repeated with our enzyme extract, the enzyme activity was not saturated. The major discernible differences in the preparation of the enzyme extract were buffer composition, culture density, and duration of sonication. In addition, the data reported by Walther and Edmunds include only two 24-hr cycles with no standard deviations for the data points. It is therefore not easy to determine if the oscillations they reported are significant. It is difficult to see from the data presented in Figure 6 how glyceraldehyde-3-P dehydrogenase could control the rhythms of O_2 evolution or CO_2 uptake. Our data are corroborated by the data of Hellebust *et al.* (11) for *Acetabularia,* an organism known to have a photosynthesis rhythm. None of the Calvin cycle enzymes in that study was found to have rhythmic activities.

Although the enzyme carbonic anhydrase is not considered a Calvin cycle enzyme, it has been hypothesized that this enzyme might be associated with the transport of CO_2 through the cell (7-9, 21). Carbonic anhydrase activity was found to be rhythmic only when cultures were maintained in a light-dark cycle; therefore, the rhythmic behavior represents a forced oscillation. The rhythm did not persist when the cultures were maintained under constant conditions (Fig. 7) and thus rhythmic changes in the activity of carbonic anhydrase are not responsible for the O_2 evolution rhythm.

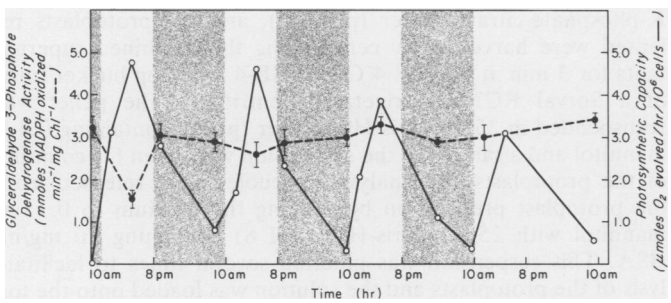

FIG. 6. Photosynthetic capacity and glyceraldehyde-3-P dehydrogenase activity. Standard deviations are shown for the replicates of each enzyme determination.

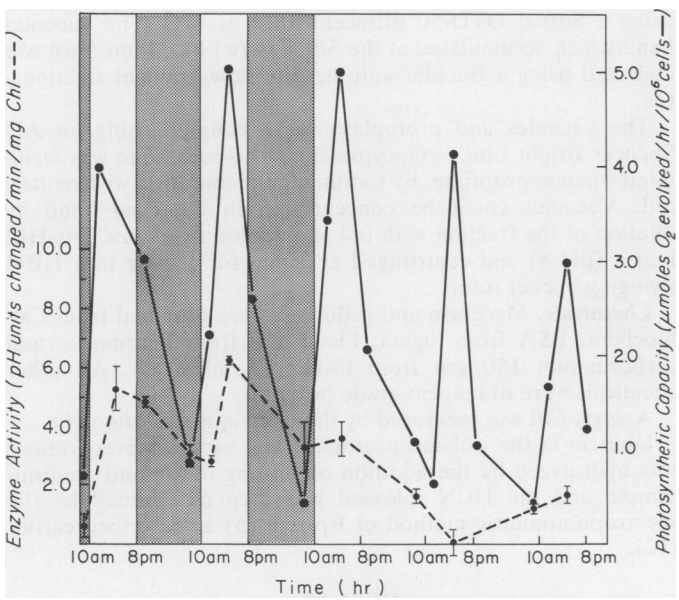

FIG. 7. Photosynthetic capacity and carbonic anhydrase activity. Standard deviations are shown for the replicates of each enzyme determination.

The reason for the CO_2 uptake rhythm is not known. This rhythm clearly is not generated by changes in the activities of the two enzymes reported in this paper unless isolation of the enzymes has adversely altered existing regulatory mechanisms. For example, the activities of the Calvin cycle enzymes could conceivably remain constant throughout the day and changes in the concentrations of NADPH or ATP might result in changes in the rate of CO_2 fixation. Oscillations in the light reactions could generate varying amounts of these substrates used in the Calvin cycle. It is not known if the rhythms in O_2 evolution and CO_2 uptake are controlled by the same mechanisms, but it appears that the O_2 evolution rhythm does not require the simultaneous operation of the CO_2 rhythm (Fig. 4). Possibly, biochemical or physiological differences between the log and stationary phase cells could explain why no CO_2 rhythm was observed in cells from log phase cultures.

Further investigations (unpublished data) have indicated that rhythmic changes in some component of the light reactions are responsible for the O_2 evolution rhythm.

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