The Activity of Ribulose Diphosphate Carboxylase in Extracts of *Gonyaulax polyedra* in the Day and the Night Phases of the Circadian Rhythm of Photosynthesis

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**ABSTRACT**

The ribulose 1,5-diphosphate carboxylase from *Gonyaulax polyedra* Stein. has a half-life of about four hours in buffer, but can be stabilized by the addition of 50% glycerol. The optimum pH is 7.8 to 8.0 and the optimum Mg2+ concentration is 3 mM. Heavy metal ions (Cu2+, Hg2+, Ni2+, Zn2+), EDTA, pyrophosphate, and adenosine triphosphate were strongly inhibitory. Ribulose 1,5-diphosphate carboxylase from *Gonyaulax* was not cold-sensitive or activated by light activation factor from tomato or *Gonyaulax*. No difference in the activity of this enzyme was detected when extracts prepared at the maximum and the minimum of the circadian rhythm of photosynthesis were compared. The *Km* of HCO3- was also the same (16 to 19 mM).

In the marine plankton dinoflagellate *Gonyaulax polyedra* Stein., luminescence (7, 10, 19, 23), photosynthesis (8, 20, 22), and cell division (24) all appear to be controlled by a single oscillator (15, 19, 21). If a physiological process is to be driven in a rhythmic mode, there must be some biochemical connection between it and the controlling oscillator. With the hope of detecting the nature of such a link in the photosynthetic rhythm in *Gonyaulax*, a series of studies was initiated in this laboratory to examine *Gonyaulax* for biochemical changes accompanying the rhythm in photosynthesis. For this purpose, cells and cell extracts have been compared at the maximum and the minimum of the rhythm in photosynthetic capacity. Biochemical differences were particularly sought. Results of these studies showed a surprising uniformity over time in a number of processes related to photosynthesis (20–22). Previous studies of RuDPCase2 however appeared to indicate rhythmicity in the activity of this enzyme (20, 21). Unfortunately, the activity of RuDPCase in crude extracts of *Gonyaulax* was extremely short lived. Consequently, the nature of RuDPCase activity in *Gonyaulax* in general and differences in activity with phase of the circadian rhythm have been reinvestigated. The results of these studies are reported here.

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1 This research was supported in part by Grant GB 8418 from the National Science Foundation.

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3 Abbreviations: BSA: bovine serum albumin; LAF: light-activating factor; RuDP: ribulose 1,5-diphosphate; RuDPCase: ribulose 1,5-diphosphate carboxylase.

**MATERIALS AND METHODS**

Cultures of *G. polyedra*, a strain isolated in 1960, were grown in Fernbach flasks containing 1 liter of enriched seawater medium (23), and were allowed to reach the stationary growth phase. Cells were illuminated at 22 ± 1°C for 12 hr each day with cool fluorescent tubes of 300 ft-c intensity. If more than one flask of cells was to be used, the cells were mixed together and redistributed at least 1 day before the beginning of the experiment.

The cells were harvested by centrifugation at 18 to 20°C using a Sorvall RC-2B centrifuge. The sedimented cells were washed once with 5 ml of 50 mM tris-HCl, pH 8.0, and centrifuged again. To the sedimented cells was added cold buffer containing 40 mM tris-HCl, pH 8.0, 0.21 mM EDTA, 8 mM MgCl2, 6 mM β-mercaptoethanol in 20% glycerol. If ion effects were to be studied, no EDTA or MgCl2 was added to the buffer. The cells were broken in a French pressure cell with an internal pressure of 3400 p.s.i. To the resulting brei was added enough cold glycerol to give a 50% glycerol mixture (v/v). The mixture was then centrifuged at 2°C for 20 min at 20,000g. The orange supernatant containing 95% of the RuDPCase activity was stored at −20°C. Breakage of the cells was timed to occur either at the middle of a light period (Day extracts) or at the middle of a dark period (Night extracts).

The amount of protein present in the cell extract was usually determined spectrophotometrically both by the method of Murphy and Kies (16), using BSA as a standard and by the method of Warburg and Christian (25). Protein was also determined by the method of Lowry et al. (13), using BSA as a standard. The values obtained spectrophotometrically were consistently higher than those obtained using the Folin reagent. However, the presence of glycerol and mercaptoethanol complicated the Folin determinations, so that spectrophotometric analyses were a reasonable alternative for routine experiments and could be corrected for the difference between methods.

RuDPCase activity was determined by following the fixation of 1C from NaH14CO3 into acid-stable products in the presence of RuDP (21). The assay mixture usually contained 0.13 mM RuDP, 1.3 mM NaH14CO3 (0.5 μC), and 25 mM tris-HCl, pH 8.0, in a volume of 0.4 ml. If effector molecules were added to the assay mixture, the compounds were included in this volume. The reaction was initiated by the addition of 0.1 ml of extract (containing 0.2 to 0.6 mg of protein) in the 50% glycerol buffer described above, which contained 21.5 mM tris-HCl, 0.11 mM EDTA, 4.3 mM MgCl2, and 3.2 mM β-mercaptoethanol. MgCl2 and EDTA were omitted from this mixture when the effects of ions were being investigated. Each set of assays included a blank which had no RuDP in the reaction mixture. After the
tubes were incubated for 10 min at 25 C, the reaction was stopped by the addition of 0.5 ml of 70% ethanol, 5% acetic acid. Each sample was assayed in duplicate or triplicate. Aliquots of 0.2 ml were dried on filter paper and counted using a liquid scintillation technique. The reaction was linear both with respect to time and to amount of extract assayed.

“Light-Activating Factor” (LAF) was prepared from both tomato leaves and Gonyaulax according to the procedure of Wildner and Criddle (26). Assay mixtures studied under light conditions were illuminated with a tungsten lamp or with 325-nm light from a Bausch and Lomb Spectronic 20.

The rate of photosynthesis in living cells was determined by incubating 15- or 20-ml cell suspension of Gonyaulax for 30 min at 25 C in saturating light for photosynthesis (1000 ft-c) in the presence of HCO3 (0.5 µC). Cells were killed with formaldehyde and 5-ml aliquots were harvested immediately by filtration on Whatman filter paper No. 524. Filters were washed three times with sea water, dried, and counted by liquid scintillation technique. Dark fixation was determined in identical samples wrapped in foil, and the appropriate correction was applied to the values for fixation of C in light. The number of cells present was determined by visual counting.

RuDP was purchased from Sigma as the dibarium salt and converted to the disodium salt using Dowex 50 (100 mg Dowex/mg RuDP). ATP and biotin were purchased from Nutritional Biochemical Co. All solutions were prepared in deionized water which had also been distilled from all glass apparatus. Dialysis tubing was pretreated by boiling successively in 10 mM EDTA and 0.5 mM acetic acid twice, followed by thorough rinsing and storage in distilled water at 7 C.

RESULTS

Stability of RuDPCase.—When Gonyaulax cells were broken and extracted in buffer in the absence of glycerol, RuDPCase activity decayed rapidly with a half-life of 3.5 to 4.0 hr. In an attempt to stabilize the activity, a variety of compounds including NaHCO3, RuDP, BSA, sucrose, and hydrophobic organic solvents, were added to the extraction medium. The addition of MnCl2 after cell breakage increased RuDPCase activity, but did not affect the stability of the preparation. In the presence of 10 mM MnCl2, both 0.2 mM sucrose and BSA (5 mg/ml) increased the half-life of RuDPCase activity by several hr. However, glycerol, a successful stabilizing agent for other enzymes (3), proved much more effective. In its presence, Gonyaulax extracts exhibited a constant specific activity for many hours and showed significant RuDPCase and luciferase activity for several weeks after preparation. This stability was similar for both the day and night extracts. In the presence of glycerol, MnCl2 acted as a competitive inhibitor for NaHCO3; thus, MnCl2 was not usually included in the preparations described in this paper. Although Clandon’s reagent and β-mercaptoethanol could be used interchangeably during cell breakage, a mercaptoan was necessary to maintain RuDPCase activity.

Attempts to purify the extract further met with limited success. If the extract was subjected to (NH4)2SO4 fractionation, active RuDPCase was found in the precipitated material obtained between 50 to 75% saturation. However, almost all the original activity was lost irreversibly when the (NH4)2SO4 precipitate was suspended and dialyzed for several hours against buffer, in the presence or absence of glycerol, mercaptoethanol, EDTA, or MgCl2. This irreversible loss of activity upon dialysis was also observed with unfractionated extract. The presence of EDTA or a mercapta in the dialyzing buffer resulted in complete loss of activity.

Further attempts to purify the extract by Sephadex chromatography in a glycerol-free buffer resulted in large losses of activity upon elution, even with added MnCl2. A few active RuDPCase obtained had a half-life shorter than that observed in the initial extract (without glycerol), even when glycerol was added to the collecting tubes used during the Sephadex fractionation. Addition of small molecular weight compounds, including ATP and a variety of divalent cations, to the dialyzed or Sephadex fractionated extracts caused no restoration of RuDPCase activity. Also, no activity was restored upon recombination of the dialyzed extract and the concentrated dialysis buffer using both day and night extracts.

Because of this instability of the more purified RuDPCase, most experiments were performed using the crude cell extract which had been stabilized with glycerol. Differences between extracts from day- and night-phase cells which might include variations in the concentration of soluble substances affecting RuDPCase activity, could be more easily detected in such unpurified extracts.

Optimal Conditions for RuDPCase Assay. RuDPCase activity in both the day and night extracts was measured as a function of pH. As shown in Figure 1, a narrow pH optimum of pH 7.8 to 8.0 was observed in both extracts. Although CO2, rather than HCO3-, is reported to be the biological substrate (5), no Gonyaulax RuDPCase activity in vitro was observed below pH 6.0 where more CO2 would be available. This may reflect an instability of the enzyme at low pH, however, rather than a preference for HCO3- as the substrate.

The thermal properties of RuDPCase were studied in both day and night extracts. As shown in Figure 2, a 15-min preincubation of the extracts at temperatures lower than 8 C resulted in negligible loss of enzymatic activity, but preincubation at 35 C completely inactivated the enzyme. These results are in sharp contrast to observations by Kawashima et al. (11) who
which had been stored was assayed to determine the stability of the RuDPCase activity. Tobacco extracts were prepared and assayed as described in the text. Each set of values was compared to a control for that phase which contained no added salt.

Table I. Ion Effects on RuDPCase Activity

<table>
<thead>
<tr>
<th>Salt</th>
<th>Assay Concentration</th>
<th>Relative Activity</th>
<th>Salt</th>
<th>Assay Concentration</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>Day</td>
<td>Night</td>
<td>mM</td>
</tr>
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<td>2</td>
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</tbody>
</table>

found that tobacco Fraction I protein was inactivated at 0 °C but was very stable at 25 °C.

A series of cations and anions was added to the RuDPCase assay mixture to detect any ion effects on the enzyme reaction. As shown in Table I, the only cation which significantly increased RuDPCase activity was Mg²⁺, although Mn²⁺ also activated the enzyme in the absence of glycerol. The heavy metals (Cu²⁺, Hg²⁺, Ni²⁺, Zn²⁺) all poisoned the enzyme such that little, if any RuDPCase activity could be detected in their presence. In the anionic series, no strongly activating species were found; however, both EDTA and PP₃ were strongly inhibitory. In all these studies very similar behavior was observed for both day and night extracts.

Because of the possible physiological implications of Mg²⁺, K⁺, and PP₃ effects, a range of concentrations of these ions was studied in both day and night extracts. The effects of MgCl₂ (Fig. 3) reached a maximum at a concentration of about 3 mM and resulted in RuDPCase activity as great as 2.3 times that of the control. The largest KCl activation (only 1.2 times the activity of the control) occurred at about 10 mM. The PP₃ inhibition was first evident at a concentration of 0.1 mM and was complete at 2 mM. In each of these studies the effects on day and night extracts were identical within experimental error.

Studies by Champigny and Bismuth (4) indicated an effect of ATP on RuDPCase of isolated chloroplasts. Because effects of biotin-ATP combinations have been observed with other carboxylases (1), and because it appeared that some small molecular weight component(s) with an ultraviolet maximum at 260 nm was lost upon dialysis of Gonyaulax extracts, the effects of ATP and biotin on RuDPCase activity were studied. ATP concentrations of 0.1 and 0.4 mM with or without equimolar biotin had little effect on the activity of RuDPCase, while 5 mM ATP or ATP and 5 mM biotin inhibited activity completely. At intermediate concentrations (0.8 mM) the inhibitory effects of ATP and biotin together were overcome by the addition of 1.6 mM MgCl₂. The addition of ATP and biotin to dialyzed extracts resulted in no activation of either the day or night phase extracts.

The Michaelis constant for HCO₃⁻ was determined for both day and night extracts (Fig. 4). The average Kₘ for extracts in the day phase was 19 ± 3 mM, while extracts in the night phase exhibited a Kₘ of 16 ± 3 mM. Thus, no significant difference in affinity for HCO₃⁻ was observed between the enzymes in the two phases.

Fig. 2. Temperature effects on RuDPCase activity. Gonyaulax extracts which had been stored at −20 °C were incubated for 15 min at the temperature indicated. The extracts were then assayed for 10 min at 25 °C as described in the text. The complete assay mixture was the same as in Figure 1 at pH 8.0.

![Graph showing temperature effects on RuDPCase activity](https://www.plantphysiol.org/aop/PlantPhysiol/content/50/3/448/F2.large.jpg)

Fig. 3. Mg²⁺ activation of RuDPCase. Gonyaulax extracts from light and dark phases were prepared in the absence of EDTA and MgCl₂. The extracts were assayed at pH 8.0 as described previously (legend to Fig. 1), with MgCl₂ added to the assay mixtures to give the desired Mg²⁺ concentrations.

![Graph showing Mg²⁺ activation of RuDPCase](https://www.plantphysiol.org/aop/PlantPhysiol/content/50/3/448/F3.large.jpg)
FIG. 4. Double reciprocal plot showing RuDPCase activity as a function of HCO$_3^-$ concentra-
tions. Gonyaulax extracts from light and dark phases were prepared normally and assayed at pH 8.0 within 2 hr of preparation (for assay mixture see legend to Fig. 1). Deviations from normal procedures included assay concentrations of 0.2 mm RuDPCase and a constant 0.5 µC per assay with the varying HCO$_3^-$ concentrations provided by the addition of unlabeled NaHCO$_3$ to the assay mixtures.

FIG. 5. Comparison of $^{14}$C fixation in whole cells and RuDPCase activity in extracts. $^{14}$C fixation in whole cells was determined as described in the text. Extract preparations were timed so that breakage of the cells coincided with the $^{14}$C fixation determinations. RuDPCase activity at pH 8.0 was determined 1, 2, and 12 hr after cell breakage according to the procedure described in the text, using the complete assay mixture given for Figure 1. Cultures in this study were maintained on a light-dark 12:12 cycle.

made at the peak of the day phase, at the minimum of the following night phase and at the next day's maximum. Cell extracts were assayed using 2 mm HCO$_3^-$, a concentration which corresponded to the HCO$_3^-$ concentration in the culture medium.

Figure 5 shows a typical set of results obtained in a study of cells maintained on a light-dark cycle. A well defined photosynthetic rhythm was exhibited in the whole cells. However, the RuDPCase activity in the extracts exhibited almost constant activity. The activity in these extracts was invariant with respect to the number of cells broken, the amount of protein in the extract and total activity recovered. These results are in disagreement with those reported earlier (20, 21) which indicated a rhythmic behavior in the activity of the extracts. In the present study, the Gonyaulax extracts were much more stable with respect to the activity of RuDPCase, and a higher HCO$_3^-$ concentration was used in assays. Because the studies in this paper were carried out under conditions allowing for optimal enzymatic activity, the constant RuDPCase activity observed most probably reflects a constant amount of enzyme. Thus, the photosynthetic rhythm does not appear to be related to a change in amount of RuDPCase in the cell.

**Effect of Light-Activating Factor.** The presence of LAF as described by Wildner and Criddle (26) was considered as a possible control for expression of the photosynthetic rhythm. LAF was prepared both from *Gonyaulax* and from tomato leaves and was added to *Gonyaulax* extracts from both the day and night phases. LAF from *Gonyaulax* cells activated RuDPCase from the night phase by less than 8% and produced less than a 5% effect on day extracts. LAF from tomato leaves caused a light-activated inhibition of RuDPCase activity of 55 to 65% in extracts from either phase. Addition of LAF to dialyzed extracts did not restore RuDPCase activity. Thus, it is unlikely that a light activation factor accounts for the rhythmic photosynthetic behavior of *Gonyaulax*.

Occasionally, extracts stored for some hours at −20°C showed as much as four times more activity than when freshly prepared. Since this activation was not reproducible, it was not investigated further.

**DISCUSSION**

The similarity of behavior between RuDPCase activity in extracts from day- and night-phase *Gonyaulax* cells is the most striking feature of this study. The identity of response to pH, temperature, and small molecular weight effectors, coupled with a constant relative activity of RuDPCase studied under optimal assay conditions, indicate that the enzyme in both phases is the same and present in the same concentration. There is no evidence in this work for differential amounts of
enzyme which would account for the rhythmic changes observed in the rate of \(^{14}C\) fixation in the intact cells.

Because of the identity of the enzyme in the two phases, it is likely that control of the photosynthetic rhythm is exerted through variations in simple physiological parameters which affect RuDPCase activity. Either an increase or a decrease of only 0.2 \(pH\) unit from the optimum (Fig. 1) can lower activity by 30%. A small change in Mg\(^{2+}\) or K\(^{+}\) concentration also causes a significant effect in RuDPCase activity. A rhythmic movement of small molecules accompanied by small \(pH\) fluctuations within the chloroplasts where RuDPCase is localized could then account for the observed differences in \(^{14}C\) fixation between day and night phases. For example, enzyme present in the day phase could be activated by an increase in Mg\(^{2+}\) or K\(^{+}\) concentrations at the \(pH\) optimum, while RuDPCase activity in the night phase could be decreased by a small decrease in \(pH\) accompanied by less favorable ion concentrations. A cumulative, or possibly synergistic, effect of these variations on RuDPCase activity could easily cause a 3-fold difference in overall photosynthesis and thus account for the observed rhythmicity.

It is quite feasible that each of the rhythmic manifestations in _Gonyaulax_ is mediated through changes in concentrations of small molecules which affect the biological activity of various enzyme or transport systems, rather than through control of transcription or translation of the macromolecules involved. A study of the _Gonyaulax_ luciferase, the enzyme responsible for rhythmic luminescence, supports this conclusion (15). The luminescence rhythm is not caused by a change in the amount of luciferase present, but may be related at least in part to changes in a simple effector molecule which alters the activity of the enzyme. The control of regulatory enzymes by small molecular weight molecules has recently been discussed with respect to the _Escherichia coli_ threonine deaminase (9) and the rabbit muscle phosphofructokinase (2) which undergo "ligand-induced maturation" in the presence of appropriate effectors. Thus, this type of control may be quite important in a diversity of regulatory systems. In _Gonyaulax_, there is evidence that a single oscillator may direct all the observed circadian rhythms (21, 22). This study suggests that such a multiplicity of control could be mediated by an oscillation in the amount or intra- cellular distribution of small molecules or ions.

The instability of the _Gonyaulax_ RuDPCase is one of the more puzzling properties of the enzyme. RuDPCase from a variety of sources including spinach, tobacco (18) and possibly two green algae (12) and the chemosynthetic bacteria _Thiobacillus thioparus_ and _Thiobacillus neapolitanus_ (14) is a complex enzyme composed of a number of subunits. If the _Gonyaulax_ enzyme is similar in structure, its inactivity after dialysis and Sephadex fractionation may reflect a dissociation of the enzyme into its subunits, especially in the presence of EDTA and mercaptans which might cause disruption of the quaternary structure by removal of structural metal ions. Stabilization by glycerol may reflect the prevention of spontaneous dissociation by the increased viscosity of the buffer or by providing a more stable polyol environment which favors the complex protein. If RuDPCase can be readily broken into subunits, one might expect a rupture of quaternary structure accompanied by a loss of activity when the _Gonyaulax_ cells are broken by the French press (17). However, comparison of RuDPCase activities in _Gonyaulax_ extracts prepared by the French press and by sonication showed that the sonicated extracts had lower \(^{14}C\) incorporation per mg protein, lower total activity, and less stability of RuDPCase activity, even in 50% glycerol. Thus, it seems unlikely that a significant amount of enzyme activity is lost by dissociation using the French press. Frieden (6) has suggested that reversible dissociation and re-association of complex regulatory enzymes may act as a form of metabolic control. Although this is an attractive idea, there is little evidence for reversible dissociation in the _Gonyaulax_ RuDPCase. If this form of regulation accounted for the rhythmic photosynthetic behavior, different relative activities would be expected in the day and night phases unless reassociation occurred during preparation of the extracts. RuDPCase, when inactivated by aging in extracts not treated with glycerol, did not appear to regain activity in the presence of material found in the soluble cell extract. If reversible behavior does occur _in vivo_, again it may be related to the small molecule effectors in a manner related to the "ligand-induced maturation" discussed above. This may then reflect a completely different conformational state from that of the "laboratory-induced" inactivated enzyme described in this work. It is worthy of note that RuDPCase from _Gonyaulax_ is different from that from other plant sources. It is not inactivated by cold as is the enzyme from tobacco (11), shows little effect of LAF (26), and is much more unstable.

In conclusion, the data reported here indicate a consistency in properties and activity of the glycerol-stabilized RuDPCase in soluble extracts of dark and light phase _Gonyaulax_. The differences in over-all \(^{14}C\) fixation between the two phases may be explained by fluctuations in physiological conditions which cause RuDPCase activity to vary. This type of control can easily be extended to include other rhythmic systems found in _Gonyaulax_.

**LITERATURE CITED**

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