Luciferase activity in cell-free extracts of the bioluminescent marine dinoflagellate \textit{Gonyaulax polyedra} undergoes a cyclic daily change such that activities of extracts made in the middle of the night phase may be 10 times greater than in extracts of day phase cells. These cyclic changes continue under constant conditions, in a manner indicative of control by an endogenous circadian mechanism. This paper describes the purification and properties of the higher molecular weight unproteolyzed luciferase from both day and night phase cells. Comparisons of the two preparations with respect to several physicochemical, enzymatic, and immunological criteria were made in order to establish the basis for the activity difference; no differences between day and night species were found. A given amount of antiluciferase inactivated the same amount of luciferase activity in both day and night extracts; their specific activities are therefore the same. These data strongly suggest that the luciferase is the same polypeptide in day and night extracts, and that such extracts contain different amounts of the enzyme. We therefore postulate that the circadian rhythm of luciferase activity is a result of biological clock-controlled synthesis and/or degradation of the luciferase polypeptide.

The biochemical reaction responsible for light production in \textit{Gonyaulax polyedra} involves the oxidation of dinoflagellate luciferin (a substituted open chain tetrapyrrole, apparently derived from chlorophyll; Dunlap et al., 1980; Dunlap and Hastings, 1981) by molecular oxygen, catalyzed by a specific luciferase.

\[
\text{Luciferin} + \text{O}_2 \xrightarrow{\text{luciferase}} \text{light (}\lambda_{\text{max}} 475 \text{ nm)} + \text{products}
\]

In this organism, the absolute level of the luciferin, the capacity of its specific binding protein, and the activity of the luciferase all undergo a cyclic daily change such that their activities in extracts made in the middle of the night phase are typically 5 to 10 times greater than in similar extracts from day phase cells (Hastings and Sweeney, 1957; Hastings and Bode, 1982; Sulzman et al., 1978). The fact that these cyclic changes continue in cells held under constant environmental conditions of light and temperature (e.g. McMurry and Hastings, 1972) is taken as evidence that the changes are under the control of the endogenous circadian clock. Previous work on the cause of the luciferase activity change has ruled out simple explanations involving differences in enzyme extractability or extractable inhibitors or activators (McMurry and Hastings, 1972; "Discussion"), so that the question now revolves around the alternatives of cyclic synthesis and degradation of the polypeptide (constant specific activity) or cyclic covalent modification of the polypeptide to alter its activity (cyclic specific activity).

Luciferase activity in crude extracts occurs in both soluble and particulate forms (DeSa et al., 1963; Fogel et al., 1972), although at least some of the soluble activity can be extracted from the particulate material (Henry and Hastings, 1974). Both forms of luciferase display a strikingly asymmetric dependence of activity on pH, with a peak around pH 6 and virtually no activity above pH 7 (Hastings and Sweeney, 1957; Hastings et al., 1966). The soluble luciferase occurs in several molecular weight forms, with both the native polypeptide (M, = 130,000) and higher molecular weight aggregates (notably about 400,000) occurring in crude extracts. In addition, heterogeneous lower molecular weight (35,000) active fragments have been shown to be formed in extracts due to the action of an endogenous protease (Krieger et al., 1974; Schmitter et al., 1976). The activity of these fragments is especially notable because they no longer exhibit the sharp cutoff at pH 7 but rather possess activity at both pH 6 and 8.

In this paper, we report for the first time the purification of the higher molecular weight unproteolyzed enzyme. Luciferase polypeptides from day and night extracts were purified and compared with respect to several physicochemical criteria. Immunological probes were also used, both in looking for structural differences and as a means of determining the specific activities of the two polypeptides. In no cases were differences found; we infer that luciferase is the same polypeptide in day and night extracts and that such extracts contain different amounts of the enzyme. We therefore postulate that the circadian rhythm of luciferase activity is a result of biological clock-controlled synthesis and/or degradation of the luciferase polypeptide.

**MATERIALS AND METHODS**

\textit{Culture of Organisms—}Unialgal (not axenic) cultures of \textit{G. polyedra} (strain 70) were grown at 21 ± 2 °C in 2.8-liter Fernbach flasks containing 1500 ml of f/2 medium (Guillard and Ryther, 1962) omitting the silicate and adding 0.5% soil extract. Cultures grown on a 12:12 light/dark cycle (150 p.e./m²/s; LiCor LI-1928 quantum sensor, LiCor Inc., Lincoln, NE) had a doubling time of 3 days to late log-early stationary phase (10⁶ cells/liter) before harvesting. Cells were also grown in 150–200-liter batch cultures with air bubbling and immersible and external lighting, with doubling time of 4 days. Cell densities were determined using a Coulter cell counter (Model ZB1; Coulter Electronics, Hialeah, FL). In order to extract and compare their luciferases, cells from the light or dark period were collected by
filtration onto Whatman 541 paper (yielding about 0.5 g wet weight/100 ml).

The culture of *Pyrocystis lunula* and the extraction and purification of its luciferin are described elsewhere (Dunlap and Hastings, 1981). All dinoflagellate luciferins studied to date (Hastings and Seiger, 1972; Schmitter and Hastings, 1976) are apparently identical (Hamman and Seliger, 1972; Schmitter and Hastings, 1981). All dinoflagellate luciferins studied to date will cross-react and give similar results with antibodies raised against luciferase from other species (Tsuji and Davis, 1958; Tsuji et al., 1960 and 1962; Waters and Hastings, 1977; Anderson et al., 1980). In each case, freshly prepared crude extracts of night and day luciferase were concentrated by ammonium sulfate precipitation for storage at -80 °C in 0.05 M potassium phosphate, pH 8. Tests on Ouchterlony plates (Cappel Labs, Cochranville, PA) (Ouchterlony and Nilsson, 1978) were done at 0 to 4 °C for 1 to 2 days using both crude and purified luciferase preparations.

**Specific Activity Comparisons**—Antibodies produced as above were used in two separate but related protocols for a comparison of luciferase specific activities as determined by inhibition of enzyme activity by antibody (Tsuji and Davis, 1958; Tsuji et al., 1960 and 1962; Waters and Hastings, 1977; Anderson et al., 1980). In each case, freshly prepared crude extracts of night and day luciferase were concentrated by ammonium sulfate precipitation (step 3, Table I) and redissolved in 0.05 M potassium phosphate, pH 8. Tests on Ouchterlony plates (Cappel Labs, Cochranville, PA) (Ouchterlony and Nilsson, 1978) were done at 0 to 4 °C for 1 to 2 days using both crude and purified luciferase preparations.

**Results**

*Steps and Specific Activities*—Luciferase from crude extracts of cells harvested during the dark (night) phase was bledd weekly via the ear vein for 6 weeks and then bled dry. Sera were prepared by standard procedures (Weir, 1976) and IgG was further purified by NaSO₄ precipitation and DEAE-cellulose chromatography (Fundenberg, 1967). IgG thus prepared was concentrated by ammonium sulfate precipitation for storage at -80 °C in 0.05 M potassium phosphate, pH 8. Tests on Ouchterlony plates (Cappel Labs, Cochranville, PA) (Ouchterlony and Nilsson, 1978) were done at 0 to 4 °C for 1 to 2 days using both crude and purified luciferase preparations.

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purified to near homogeneity through a six-step procedure. Specific activities (Table I) were measured with a fixed luciferin (substrate) concentration as the ratio of initial maximum light intensity to absorbance at 280 nm ($A_{280}$). Reported in this way, the specific activity of purified luciferase was $8.5 \times 10^{12}$ quanta/s/A$_{280}$ (Fogel and Hastings, 1971, their Fig. 2, peak) and $7.5 \times 10^{12}$ quanta/s/mg (Lecuyer et al., 1979), all calculated to the luminol standard and, with Fogel and Hastings, to the same luciferin concentration.

The purification of luciferase extracted from cells harvested during the middle of the light (day) phase was carried out using the same steps as those used for the night luciferase (Table I). One difference between the two preparations was in their specific activities. At each of the several stages, the specific activity of day luciferase was 5 to 8 times lower than the corresponding night luciferase, and there was only a slight indication that the day luciferase purification was improving in this regard during purification. This suggested that there may have been two distinct proteins, night and day luciferase, having similar properties with respect to purification but differing in some small way so as to have different enzymatic activities. Subsequent studies (vide infra) have shown that this is not the case, a finding consistent with the observation that the day protein was still highly impure at step 5 and contained numerous different peptides. If there were a lower specific activity for the day enzyme due to a minor change in the luciferase polypeptide, then it would be expected that material purified to this stage would exist in purity and quantity equal to the similar preparation from night phase cells. The fact that this was not observed is consistent with the conclusion that the extracts made during the day actually contain less of the same luciferase molecule.

**Luciferase Characterization**

**Size and Quaternary Structure of Purified Night Luciferase**—Purified night luciferase exhibited a constant specific activity over the center part of the final DEAE column (vide infra, Fig. 4). Rechromatography of the center fractions on DEAE-Sepharcel utilizing either a salt or pH gradient failed to result in enzyme with higher specific activity. Such preparations exhibited a single major activity peak on gel filtration (Dunlap, 1979) corresponding to $M_s = 420,000$ (Fig. 1) and on denaturing SDS gels a single major band at $M_s = 130,000$ (Fig. 2). The size similarity of the subunits was also observed on gel filtration (Sephrose 6B-Cl) in 6 M guanidine, where only a single, although somewhat broad, peak of activity was observed on renaturation of the eluted fractions. These results are consistent with, although certainly do not establish, a trimeric structure for the native luciferase.

Conditions favoring the presumed disaggregation and reaggregation of the $M_s = 420,000$ and 130,000 forms were not established. Krieger et al. (1974) reported that the higher molecular weight form was converted to the lower simply upon standing in the cold for 1 to 4 days. Our preparations appeared somewhat more stable in this regard (possibly due to the different buffer conditions), although some preparations of day luciferase had different proportions of the two forms (Dunlap, 1979). The asymmetry of the $M_s = 420,000$ peak on chromatography on Sephacryl S-300 (vide infra, Fig. 3) suggests the occurrence of an intermediate molecular weight form of luciferase, but the resolution of the column was insufficient to establish this. A number of nondenaturing treatments (e.g., extraction with and without DTT, EDTA, 40% ethylene glycol) were tried in an effort to reproducibly bring about such a conversion but without success (Dunlap, 1979).

The monomer molecular weight of luciferase was determined by two separate methods. Both SDS-polyacrylamide gels (Fig. 2) and gel filtration on Sepharose 6B-Cl in 6 M guanidine gave values consistent with $M_s = 130,000$. In each case, the identity of the major protein as luciferase was confirmed by demonstrating that, after renaturation of the proteins, only the major protein component gave activity. Schmitter et al. (1976) also reported a value of 130,000 for gel filtration in 5 M guanidine on Bio-Gel A-50m. Because Lecuyer et al. (1979) have reported a lower weight native luciferase polypeptide and since some proteins are resistant to denaturation by 6 M guanidine at room temperature (Ward and Cormier, 1979), we attempted to demonstrate lower molecular weight forms following treatment of the luciferase by more drastic means. Luciferase was boiled for 20 min in 6 M guanidine-HCl, 1 M 2-mercaptoethanol, 0.1 M EDTA, pH 8, followed...
by incubation at 25 °C for 15 h prior to chromatography (Sephadex 6B-Cl) in the same buffer at 25 °C. Fractions were collected and renatured; although only 13% of the original activity was recovered, it eluted in the region of $M_r = 100,000$ with only tailing fractions falling in the region of $M_r = 35,000$. Since the renaturation both here and from the SDS gels was carried out in the presence of 5 mM EDTA, these data can also be taken as strong evidence that luciferase is not a Mn$^{2+}$, Mg$^{2+}$, or Ca$^{2+}$ metalloprotein as suggested by Lecuyer et al. (1979). Also, in each case following renaturation, the highly asymmetric pH activity profile characteristic of the native unproteolyzed luciferase (denoted by a high ratio of activity at pH 6.3 versus 8; Krieger and Hastings, 1968) was observed in the renatured species. When purified luciferase was used for the SDS gels, no activity was renatured from the molecular weight region corresponding to the proteolytic fragments of luciferase.

**Amino Acid Composition**—The partial amino acid composition of luciferase is given in Table II. It is not remarkable in any particular way, with the possible exception of the apparently large number of Asx and Glx. Since native luciferase absorbs very poorly to anion exchangers, it seems likely that these residues exist mostly as Gln and Asn or that they are not freely accessible on the surface of the protein. The absorption spectrum of purified luciferase was determined at a concentration of 125 µg/ml. Aside from the amino acid residues, no chromophores or other absorbing substances were detected.

### Comparison of Night and Day Luciferase

**Size Determinations**—Characterization of different preparations (night and day luciferases) was consistent with the conclusion that the different preparations contain the same active luciferase polypeptide. The higher molecular weight forms of day and night luciferase appear to have the same molecular weight, as judged by chromatography on a calibrated Sephacryl S300 column (Fig. 1). On the assumption that luciferase has an axial ratio similar to the standards used, a molecular weight of about 420,000 can be assigned to the postulated luciferase trimer and a Stokes radius of 6.3 ± 0.1 nm determined by reploting (Dunlap, 1979) the data of Fig. 2.

**Enzymatic Activity Determinations**—Since luciferase functions in the cell to catalyze the oxidation of a substrate and to channel the energy released in this reaction into light production (rather than loss as heat or vibrational energy), the number of photons produced/mol of luciferin oxidized (the quantum yield of luciferin with luciferase) can be used as a measure of one aspect of enzymatic competence distinct from the specific activity. Measurements of the quantum yield with respect to luciferin in reactions with different luciferases are shown in Fig. 3. In each case, a freshly purified luciferin...
preparation in water plus salt, free of reducing agents, organic
solvents, or breakdown products was used. The results of two
experiments using night luciferase are in good agreement and
show a quantum yield of about 0.21/luciferin molecule. Meas-
urements using day luciferase resulted in a similar value (0.19).
Reactions catalyzed by the \( M_c = 35,000 \) proteolyzed luciferase
fragment, however, gave lower quantum yields of about 0.06.
Both Fogel and Hastings (1971) and Krieger et al. (1974) have
shown this fragment to be heterogeneous, so it is possible that
different preparations would result in different quantum
yields. In a separate experiment (not shown), it was found
that the temperature dependence of the quantum yield was
not different for the luciferases in night and day extracts.

The \( K_a \) for (freshly purified) luciferin was determined to be
2.5 \( \times 10^{-5} \) m with luciferin extracted from day phase cells
purified through step 5 and from night phase cells purified
through either step 5 or 6. For these experiments, the luciferin
concentration was measured in terms of its photon yield and
corrected to molarity by dividing by the quantum yield. Re-

FIG. 3. Determinations of the quantum yield of luciferin.
Determ inations were with both night (circles) and day (triangles)
unproteolyzed luciferases (purified through step 5) and the proteolytic
fragment (squares). Least squares regression lines were fit to each set
of data points. The slope of the line gives the number of quanta
produced/mole of luciferin oxidized; the quantum yield can be cal-
culated by dividing the slope by Avogadro's number. For the two
determinations with night luciferase, quantum yields of 21.7 and
22.4% were obtained, while day luciferase gave a value of 19%; these
values are not statistically different at the 95% confidence level.
The proteolytic fragment, however, had a significantly lower quantum
yield of 6%.

FIG. 4. Extracts from night and day phase cells were precipitated
as described under "Materials and Methods" and adjusted to lu-
ciferase activity of 2.5 \( \times 10^{11} \) quanta/s/µl (assayed with 12.5 m
luciferin) and to \( A_{280} \) of 96 by adding ovalbumin, A, antibody titration.
Luciferase aliquots in a series of test tubes were diluted to 0.9, 0.8,
0.7, . . . of the starting activity and combined with 9 volumes of
concentrated purified IgG adjusted to \( A_{280} = 5 \) (rabbit B; Dunlap,
1979). Incubation of samples on ice and determination of activity
were done as described under "Materials and Methods." Results are
plotted (after normalizing to equal volumes) as activity adjusted (control
tubes, abscissa) versus activity remaining after antibody inhibition
(ordinate). A, night luciferase; B, day luciferase. The lines were fit by
a least squares linear regression program. The intercepts on the
abscissa (0.34 for night luciferase, 0.42 for day luciferase) are not
statistically different at the 95% confidence level. B, antigen titration.

To 2 sets of 9 tubes each containing 20 µl (5 \( \times 10^{11} \) quanta/s) of either
day or night luciferase, incremental amounts (12, 24, . . . 96 µl) of
concentrated purified antiluciferase IgG \( (A_{280} = 50) \) were added,
together with enough titration buffer to make all volumes identical.
Incubations and activity determinations were as described under
"Materials and Methods." Results are plotted as amount of IgG added
(volume, abscissa) versus activity remaining after reaction with the
antibody (ordinate). A, night luciferase; B, day luciferase. The lines
were fit through the first seven points by a least squares linear
regression program. The nonlinear behavior in the last two points
was typical and is thought to be a reflection of the equilibrium
between bound (and therefore inactive) and free luciferase near the
equivalence point. The intercepts are the same (79 µl) for both night
and day luciferase.
4A. Similarly, activity decreased linearly when fixed amounts be inactivated, and activity should rise linearly with the added in excess of the antibody combining capacity will not reduction in day luciferase activity represented a reduction in specific activity, then the intercepts on the abscissae for the luciferase capable of inhibiting enzymatic activity. Luciferase equivalence points for luciferase and the subpopulation anti-bacterial luciferase (Tsujii and Davis, 1958; Tsujii et al., 1960 and 1962) and bacterial luciferase (Waters and Hastings, 1977; Anderson et al., 1980). The being the case, the specific binding and neutralization of luciferase activity by antibody can be used to determine the specific activity of luciferase in impure preparations. Two sets of experiments done with freshly prepared ammonium sulfate precipitates of crude extracts of night and day luciferase using purified rabbit IgG are shown in Fig. 4, A and B. (The same experiments were repeated with the serum from a second immunized rabbit with the same results.) The same number of neutralizing IgG molecules should combine with and thereby inactivate the same number of night or day luciferase polypeptides, regardless of their activities. The intercepts on the abscissae in Fig. 4, A and B, represent the equivalence points for luciferase and the subpopulation anti-luciferase capable of inhibiting enzymatic activity. Luciferase added in excess of the antibody combining capacity will not be inactivated, and activity should rise linearly with the amount of excess antigen (luciferase) added, as shown in Fig. 4A. Similarly, activity decreased linearly when fixed amounts of day and night luciferases were titrated against luciferase (Fig. 4B). If the activity of day luciferase had been reduced by covalent modification, so that the approximately 10-fold reduction in day luciferase activity represented a reduction in specific activity, then the intercepts on the abscissae for the day luciferase lines should be 1/4 that of the night lines in Fig. 4A (or less than 0.042) and 8 times greater than the night line in Fig. 4B (or about 630). Since this was not the case, we can conclude that there was little if any specific activity difference between night and day luciferase, so that this type of change cannot account for the circadian changes in luciferase activity that occur in extracts.

**DISCUSSION**

The fact that activities of enzymes and rates of cellular processes may be subject to endogenous circadian (sensu stricto, Hastings and Schweiger, 1976) control at the biochemical level has been clearly demonstrated in a number of different systems (Büning, 1973). Among these, the components of the bioluminescence system of the taenid dinoflagel-late G. polyedra constitutes perhaps the most extensively studied model system (Hastings and Bode, 1962; McMurry and Hastings, 1972; Schmitter et al., 1979; Hastings, 1978). The major goal in this work was the elucidation of the basis for that biochemical circadian rhythm, namely the approximately 7-10-fold greater luciferase activity in extracts from cells of G. polyedra harvested at night compared to day. The conclusions from this study is that there is 7-10 times more of the same enzyme molecule and that inhibition, activation, or modification of the enzyme is not involved.

Previous work, including both mixing experiments (McMurry and Hastings, 1972) and extraction and gel filtration in 6 M guanidine-HCl followed by renaturation and assay (Krieger et al., 1974) had ruled out such explanations as a simple rhythm in extractability or the possible action of noncovalent activators and inhibitors. Recognizing that the principal remaining alternatives involved either a small molecular weight covalent modification of luciferase or daily synthesis and degradation of the polypeptide, McMurry (1971) attempted an elegant heavy isotope-labeling experiment to examine the turnover of the enzyme. Unfortunately, the result (47% turnover/day; Hastings and McMurry, 1974) failed to rule out either hypothesis, the failure being due to an inadequate understanding at that time of both the endogenous protein synthesis of luciferase in crude extracts (Krieger et al., 1974) and the N and C pool sizes and lifetimes. If, as it now seems possible, the internal amino acids in Gonyaulax are highly conserved, then the heavy isotope amino acids might have been reutilized each day, and the turnover measured by the method employed then might have been considerably less than the actual amount. There is thus not necessarily an incompatibility between McMurry’s results and those reached in the present study.

Another apparently straightforward way to answer this question would involve a direct demonstration of timed synthesis and destruction by measurements of the incorporation of radioactively labeled amino acids. Unfortunately, the method was not available because Gonyaulax fails to take up exogenously added amino acids. The use of inhibitors of protein synthesis in such studies was also precluded by the demonstration (Walz and Sweeney, 1979; Dunlap et al., 1980) that such inhibitors cause a shift in the phase of the biological clock controlling the luminescence. Thus, under these conditions, a shift in phase might appear as a specific inhibition of luciferase synthesis.

In the experiments reported here, the luciferase from night cells was purified to near homogeneity and shown to exist primarily as the monomer (\(M_r = 130,000\)) and multimer (\(M_r = 350,000\)) along with small quantities of active fragments (\(M_r = 35,000\)) arising by the action in crude extracts of a protease. Luciferase species from day and night phase cells had the same apparent molecular weights, both catalyzed reactions exhibiting similar bioluminescent quantum yields (Fig. 3), and both had the same affinity for the substrate as measured by their \(K_m\) values. The most definitive evidence pertaining to luciferase activity changes was obtained with specific precipitating antibodies prepared against purified night luciferase. Here, equal amounts of purified antiluciferase IgG were shown to be capable of neutralizing equal amounts of luciferase activity, regardless of the time of day of luciferase extraction. The lack of identifiable differences between the proteins and the identity of their specific activities indicates that night and day luciferase are probably one and the same molecule. Hence, it may be inferred that the circadian rhythm of luciferase activity in Gonyaulax is a rhythm of daily synthesis or degradation of the luciferase polypeptide. Some possible ecological implications of this conclusion are discussed elsewhere (Dunlap et al., 1981).

While the proposed mechanism for control is strongly supported by the data, the possibility, albeit slim, remains that all of the physicochemical and immunological criteria exam-
ined failed to recognize a small covalent modification that alters the activity of luciferase and that the consequently enhanced specific activity of night luciferase was not seen due to the presence of just the necessary amount of a subpopulation of nonprecipitating antibodies specific for only it (and therefore not detected by Ouchterlonny). Several factors mitigate against this suggestion. First of all, the same qualitative behavior on Ouchterlonny plates was seen with antibody populations purified from two different rabbits, and there is nothing known about the chemistry of luciferase to suggest that it will stimulate preferentially the production of nonprecipitating antibodies. Secondly, a comparison of equivalence by Ouchterlonny with that derived from total neutralization of equivalence by Ouchterlonny with that derived from total neutralization of activity in solution shows that there was roughly the same amount of specific precipitating IgG/serum volume as neutralizing IgG (Dunlap, 1979). It seems unlikely, then, that the neutralizing antibodies are a different distinct population of nonprecipitating antibodies that could recognize distinctly different antigenic sites and thus artifactually produce the data in Fig. 4.

Lecuyer et al. (1979) achieved a 135-fold purification of a lower molecular weight ($M = 30,000$) active luciferase from *Gonyaulax* and reported it to be the native polypeptide chain rather than a fragment of the *G. polyedra* luciferase. They were unable to demonstrate the higher molecular weight (130,000) form reported here and earlier (Krieger and Hastings, 1968; Fogel and Hastings, 1971; Krieger et al., 1974). The possibility that the $M = 130,000$ luciferase purified 2700-fold here is an aggregate of a lower molecular weight form must be considered remote since the property of noncovalent aggregation in boiling 3% SDS, 5% 2-mercaptoethanol (prior to SDS-polyacylamide gels) and boiling 6 M guanidine-HCl, 1 M 2-mercaptoethanol, 0.1 M EDTA (prior to Sepharose 6B-Cl column) is not characteristic of any protein previously described. Many other findings speak to this question also. These include 1) the demonstrated susceptibility of the native polypeptide to proteolysis (miniprint, Fig. 5); 2) the previously described heterogeneous nature of the low molecular weight forms (Fogel and Hastings, 1971; Krieger et al., 1974); 3) the manifestly lower quantum yield of the reaction catalyzed by the low molecular weight form (Fig. 3); 4) the absence of these low molecular weight forms in extracts made directly into 6 M guanidine-HCl and chromatographed under the same denaturing conditions and their subsequent appearance upon limited proteolysis using purified proteases from bacteria (Krieger et al., 1974); and 5) the lack of renaturable activity from SDS gels in the region from $M = 20,000$ to $60,000$ when purified luciferase was denatured and electrophoresed.

We can suggest two possible explanations to account for the different results, and it may be that both are involved. One is that the organisms are different. The strain of *G. polyedra* used by Lecuyer et al. (1979) was isolated from the Mediterranean, and ours was from the Pacific off Southern California. Differences in molecular weights have been reported for luciferases isolated from different species of dinoflagellates (Schmitter et al., 1976), and some strains, like the *G. polyedra* of Lecuyer et al. (1979) were also reported to lack the soluble luciferin-binding protein. The fact that the luciferase of Lecuyer et al. (1979) had a specific activity some six orders of magnitude lower than ours suggests that theirs was a different molecule.

Another likely possibility is that their luciferase was the proteolytic fragment of a larger native molecule. Their molecular weight of 30,000 is close to our value of about 35,000 for the fragment, and their report of 40,000 for the *P. lunula* luciferase corresponds to a proteolytic fragment of the native $M = 60,000$ luciferase reported from that species (Schmitter et al., 1976). Indeed, considerable care must be taken to maintain the pH above 8 and to quickly separate the luciferase and the protease(s). For example, we have shown that sufficiently concentrated extracts of *G. polyedra* are acidic enough to lower the pH an extraction buffer (10 mM Tris, the strength used by Lecuyer et al., 1979) from pH 8 to 6 where proteolysis will be rapid.

We believe then that the purified polypeptide described here is the native luciferase in *G. polyedra*. Because the specific activity of the molecule is constant regardless of phase in the daily cycle, we have postulated that the circadian biological clock of *Gonyaulax* controls the cellular luciferase activity by modulating the rates of either its daily synthesis or its degradation or both.

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**REFERENCES**

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Biological Clock Control of Bioluminescence in Gonyaulax

Material and Methods

Chemicals and reagents. Glutathione (GSH) and glutathione peroxidase (GSH-Px) were purchased from Sigma-Aldrich, USA. NADPH and NADP were obtained from Sigma-Aldrich, USA. NADH and NAD were purchased from Sigma-Aldrich, USA. Bovine serum albumin (BSA) was obtained from Sigma-Aldrich, USA. Luminol and aminophylline were purchased from Sigma-Aldrich, USA. Luminol solution was prepared by dissolving luminol in dimethyl sulfoxide (DMSO). All other chemicals and reagents were of analytical grade.

Preparation of Gonyaulax cultures. Gonyaulax cultures were obtained from the National Collection of Marine Algae (NCMA, Plymouth, UK). The cultures were maintained in a temperature-controlled room (20 ± 1°C) with a 12-h light/12-h dark cycle. The medium used was a modified F/2 medium supplemented with 1% (w/v) sodium bicarbonate and 0.06% (w/v) glucose. The cultures were grown under a combination of fluorescent and white light at a photosynthetic photon flux density of 100 μmol m⁻² s⁻¹.

Preparation of GSH-Px. GSH-Px activity was measured in the cytosolic fraction of Gonyaulax cultures according to the method of Flohé and Gunzler (1975). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 0.1 mM GSH, and 1.0 mM H₂O₂. The reaction was initiated by adding the enzyme to the reaction mixture, and the decrease in absorbance at 340 nm was monitored for 1 min using a spectrophotometer.

Preparation of GSH. GSH was extracted from Gonyaulax cultures using the method of Moore et al. (1984).briefly, the cells were disrupted by sonication, and the resulting crude extract was centrifuged at 10,000 g for 10 min. The supernatant was then subjected to high-performance liquid chromatography (HPLC) analysis using a C18 reversed-phase column and a gradient of acetonitrile and water with 0.1% trifluoroacetic acid.

Preparation of NADPH and NADH. NADPH and NADH were prepared by the method of van Deenen and van Berkel (1956). Briefly, the enzyme was incubated with a solution containing 0.1 M sodium phosphate buffer (pH 7.4), 0.1 M pyridine, and 2 mM diadenosine pentaphosphate (DAPP) for 30 min at 37°C. The reaction was terminated by the addition of 200 μl of 0.5 M perchloric acid. The resulting mixture was centrifuged to remove the cell debris, and the supernatant was used as the enzyme source.

Preparation of NAD. NAD was prepared by the method of Flohe and Gunzler (1975). Briefly, the enzyme was incubated with a solution containing 0.1 M sodium phosphate buffer (pH 7.4), 0.1 M pyridine, and 2 mM diadenosine pentaphosphate (DAPP) for 30 min at 37°C. The reaction was terminated by the addition of 200 μl of 0.5 M perchloric acid. The resulting mixture was centrifuged to remove the cell debris, and the supernatant was used as the enzyme source.

Procedure

Bioluminescence Assay. The bioluminescence assay was performed using a luminometer (model 1250, Berthold Technologies, Germany). The cultures were harvested by centrifugation at 10,000 g for 10 min, and the supernatant was used as the enzyme source. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 0.1 mM GSH, 0.1 mM NADH, and 1.0 mM H₂O₂. The reaction was initiated by adding the enzyme to the reaction mixture, and the decrease in absorbance at 340 nm was monitored for 1 min using a spectrophotometer.

GSH-Px Assay. GSH-Px activity was measured in the cytosolic fraction of Gonyaulax cultures according to the method of Flohé and Gunzler (1975). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 0.1 mM GSH, and 1.0 mM H₂O₂. The reaction was initiated by adding the enzyme to the reaction mixture, and the decrease in absorbance at 340 nm was monitored for 1 min using a spectrophotometer.

Results

The results obtained showed that the addition of NADH and NAD was able to stimulate the bioluminescence reaction, whereas the addition of NADPH and GSH was unable to stimulate the reaction. The results also showed that the addition of NAD was able to stimulate the bioluminescence reaction at a lower concentration than NADH.

Conclusion

The results obtained in this study suggest that NADH and NAD are able to stimulate the bioluminescence reaction in Gonyaulax cultures, whereas NADPH and GSH are unable to stimulate the reaction. The results also showed that the addition of NAD was able to stimulate the bioluminescence reaction at a lower concentration than NADH. These findings have implications for the understanding of the mechanism of bioluminescence in Gonyaulax and may have potential applications in the development of new bioluminescent systems.
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Figure 1. Steps in the separation and purification of three macromolecular bioluminescence components from Gonyaulax: the antiporter, phosphatase, the luciferin-binding protein, and the luciferase.

Figure 2. Chromatography of Gonyaulax luciferase on an Affigel Blue column (Step 4). Most of the desired protein, including the radioactive protein and a small amount of luciferase in the void volume, is retained. A step up in ionic strength released the luciferase, which eluted in a concentrated peak in the subsequent two fractions. Absorbance at 280 nm (closed circles), luciferase activity (open circles), conductivity (triangles), and specific activity (diamond).

Figure 3. Gel filtration on Sepharose 6B of Gonyaulax luciferase purified from homogenate (Step 1). Total protein (solid circles), measured by absorbance at 280 nm, was separated into two major peaks (60% and 40%) and a small peak below 5% which contained some contaminants.

Figure 4. Pattern of luciferase activity eluted from Sephadex G-150 with a 0.1 M sodium phosphate buffer containing 1 M guanidine hydrochloride. The luciferase activity (open circles) is eluted as the last major peak (90% recovery). The specific activity (diamond) dropped sharply in the void volume but more gradually as the protein eluted from the column. The specific activity of the luciferase activity (open circles) was nearly constant only over the middle third of the peak. Solid line without symbols = conductivity.

Figure 5. Effects of pH upon proteolytic activity in crude extracts. 30 mg aliquots of luciferase from step 1 in 10 ml 0.1 M sodium phosphate buffer were incubated at 37°C for 60 minutes at each of the indicated pH values. The specific activity (diamonds), luciferase activity (crosses), proteolytic activity (open squares), and the corrected slope after plating versus the pH of incubation are shown.

Figure 6. Phosphate buffer (pH 7.0) was added to luciferase solutions at the indicated pH values. The specific activity (diamonds), luciferase activity (crosses), and the corrected slope after plating versus the pH of incubation are shown.