

pathogens which are often "silent" in cell culture systems or laboratory animals and can also persist at the molecular level as integrated inserts in chromosomal DNA (17, 18). The significance of the parvoviruses described in this report in the genesis of human connective tissue disease remains to be resolved.

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19. We thank J. O. Lampen and J. Casey for reading the manuscript, D. Hamerman for discussion, and H. Lechevalier for photographic assistance. M. Bloom kindly supplied parvovirus-specific antisera, and P. Mortimer provided human sera positive for B19 virus or antibody. We thank L. Anderson, R. Craig, T. Drogan, and R. Nuara for technical assistance and M. T. Fennell for typing the manuscript. Supported in part by funds from the Charles and Johanna Busch Foundation (Rutgers University), Kroc Foundation, and New York Arthritis Foundation (L.M.) and by NIH research grants AI-14359 (R.W.S.), AI-17262 (R.W.S.), and AM-15796 (C.A.S.).

1 November 1983; accepted 30 January 1984

Circadian Changes in Enzyme Concentration Account for Rhythm of Enzyme Activity in *Gonyaulax*

Abstract. A circadian rhythm in the activity of luciferase is partly responsible for rhythmic bioluminescence in the dinoflagellate alga *Gonyaulax polyedra*. The cyclic activity of this enzyme can be attributed to a corresponding rhythm in the concentration of immunologically reactive luciferase protein. Hence protein turnover (synthesis or degradation or both) is used by the endogenous clock to control the daily rhythm of bioluminescence.

In an effort to elucidate the biochemical basis of circadian rhythmicity (1), we studied the pathway by which the biological clock controls bioluminescence in the dinoflagellate alga *Gonyaulax polyedra*. In previous studies (2, 3) it was found that the in vitro activity of luciferase, the enzyme that controls the rate-limiting step in the bioluminescence reaction, is correlated with bioluminescence in vivo; both are rhythmic, even under constant environmental conditions. The 5- to 15-fold modulation in luciferase activity during the circadian cycle was shown to be due either to a covalent modification of the enzyme or to changes in the amount of enzyme (3, 4), and titration of enzyme activity with an inhibiting antibody to luciferase sug-

gested that day luciferase and night luciferase were immunologically indistinguishable (5). These studies implied that the enzyme is synthesized or degraded (or both) cyclically.

However, proteolysis of luciferase (4) or inhibition of luciferase activity by a heat-stable component in crude *Gonyaulax* extracts (6) during the incubation of luciferase and antibody could confound interpretation of the titration results. Moreover, the possibility that luciferase compartmentation might undergo rhythmic changes was never ruled out [and luciferase is at least partially compartmentalized (7)]. To evaluate these possibilities and to quantify luciferase, we directly measured luciferase protein with antibody to luciferase. We found that

luciferase is indeed modulated by the circadian clock; the magnitude of this change largely accounts for the rhythm of enzyme activity.

The polyclonal antibody we used inhibits luciferase activity in vitro (5). As shown in Figs. 1B and 2B, this antibody recognizes a single protein (molecular weight, 135,000) from crude extracts of *Gonyaulax*. The antibody also recognizes purified luciferase and the active proteolytic fragment of luciferase (molecular weight, 30,000 to 40,000), which is extractable under different conditions (4). We have little doubt, therefore, that the protein recognized by the antibody is luciferase.

Figure 1 depicts the daily cycle in activity and band density of luciferase from *Gonyaulax* cells maintained in an LD 12:12 cycle (12 hours of light followed by 12 hours of darkness). The amount of enzyme parallels the rhythm of enzyme activity from cells extracted every 3 hours during 51 hours in the cycle. To be sure that these rhythms are not merely driven by the LD cycle but are controlled by the endogenous clock, we performed the same experiment with cells maintained in constant dim light (LL). The results (Fig. 2) demonstrate that the circadian clock is indeed responsible for regulating turnover of this enzyme. Soluble protein extracts were used in the experiments represented in Figs. 1 and 2, but similar results were obtained with total protein extracts (which include organelles). Therefore, shuttling of luciferase between intracellular compartments is not responsible for the daily regulation of luciferase. The clock is controlling daily alterations in the total quantity of enzyme in the cell.

How large are these changes in the level of luciferase? The densitometry data in Figs. 1 and 2 suggest that the changes in enzyme level account for the change in activity, but this assay is valid only if the density is a linear function of protein concentration. Densitometry data corrected for linearity of the relation of density to protein (8) are compared in Table 1 for immunoblotted samples taken from the peak and trough of the oscillation. We also developed an enzyme-linked immunosorbent assay (ELISA) that allowed luciferase to be measured in micrograms per milligram of soluble protein (9). Table 1 shows that these methods of luciferase determination yield 6- to 16-fold night-to-day ratios of luciferase concentration corresponding with 9- to 34-fold activity ratios. The discrepancy between activity and concentration ratios might be due to inaccu-

racy of our methods or to some other factor [such as an activity inhibitor (6)] that could play an additional role in regulating luciferase activity.

Theoretically, periodic alterations in the rate of luciferase (i) biosynthesis, (ii) synthesis and degradation, or (iii) degradation alone could be responsible for the rhythm in enzyme concentration. The first two possibilities seem more likely a priori because the cases in which an enzyme concentration is known to be regulated solely by changes in degradative rate usually involve stabilization by cofactors, substrates, or ligands (10)—an unlikely mechanism for controlling luciferase. Recently, however, the amount of a protein that fluctuates rhythmically throughout the cell division cycle of invertebrate embryos was found to be regulated by periodic destruction and continuous synthesis (11). We do not yet have any direct evidence to resolve these three possibilities for luciferase; a pharmacological approach based on the use of protein synthesis inhibitors is precluded because these drugs cause large phase shifts of the oscillator controlling bioluminescence. Thus an apparently specific inhibition of luciferase synthesis could

be due merely to a phase shift of the biological clock (5).

Even though clock regulation of enzymes through synthesis or degradation or both may seem wasteful, two factors in *Gonyaulax* make protein turnover an inexpensive mode of control: (i) luciferase

accounts for only 0.006 to 1.50 percent of the total soluble protein pool (Table 1) and (ii) *Gonyaulax* is probably limited by nitrogen availability but is not energy-limited. These facts suggest that *Gonyaulax* may conserve nitrogen by degrading enzymes whose function is

Table 1. Day-night variations in luciferase concentration and activity, as determined by ELISA and by regression analysis of density data for immunoblotted samples. Luciferase concentrations are expressed as micrograms per milligram of total soluble protein. Day and night samples are the trough and peak samples, respectively, of the oscillation.

Experiment	Sample	Luciferase concentration*	Night:day ratio of luciferase		
			Concentration*	Concentration†	Activity
LD cycle					
1‡	Day	0.4	6.4	7.1	9.2
	Night	2.7			
2	Day	0.06	16.7		34.4
	Night	1.0			
Constant light (LL)					
1§	Day	2.0	7.7	5.7	10.8
	Night	15.3			
2	Day	0.8	9.9	7.1	17.8
	Night	7.8			
3	Day	1.3	7.4		11.0
	Night	9.6			

*Determined by ELISA (9).
blotted onto nitrocellulose (8).

†Determined by regression analysis of the density of various protein titers
‡Same experiment as represented in Fig. 1.
§Same experiment as represented in Fig. 2.

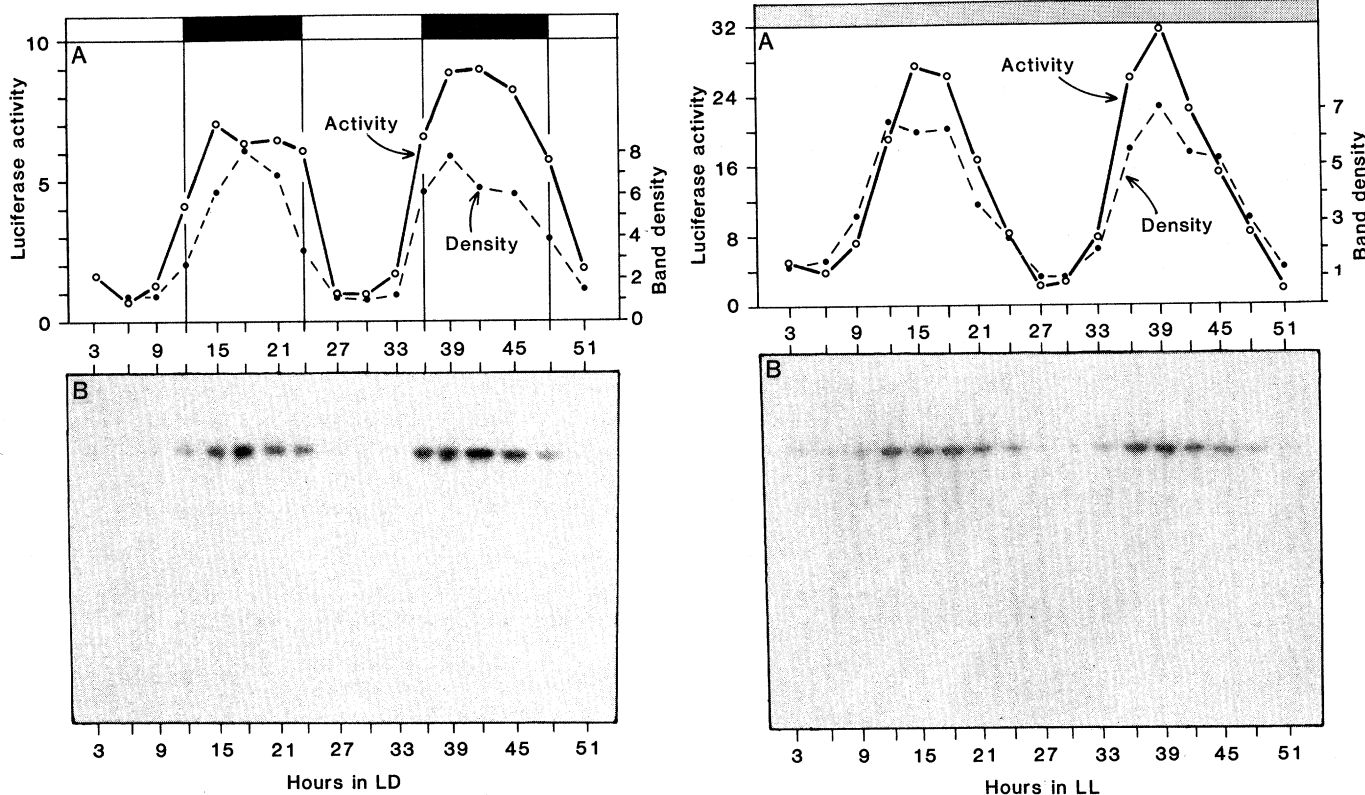


Fig. 1 (left). Rhythm of luciferase activity and amount in the LD cycle. *Gonyaulax* cultures at early stationary growth phase in LD 12:12 were harvested every 3 hours and extracted for protein (16). These extracts were assayed for luciferase activity (17) and prepared for sodium dodecyl sulfate electrophoresis, immunoblotting onto nitrocellulose, and autoradiography (18). (A) Activity of luciferase in light units per milligram of soluble protein (○) and densitometer scan of blot (●). (B) Immunoblot of total soluble *Gonyaulax* proteins reacted against antibody to luciferase (each lane contained an equal amount of protein extracted from *Gonyaulax* cells at the indicated phase in LD). Fig 2 (right). Rhythm of luciferase activity and amount in constant light (LL). *Gonyaulax* cultures were maintained in constant dim light (~ 1000 lux) throughout the experiment (zero time corresponds to the extrapolated dawn of the previously entraining LD 12:12 cycle).

temporarily unnecessary and recycling their amino acids while expending abundant photosynthetic energy (12).

However, the control of luciferase through its turnover may be one example of a more general strategy whereby the biological clock regulates enzymes. In modulating metabolic pathways, the clock regulates the activity of enzymes that are rate-limiting [such as *Gonyaulax* luciferase, pineal *N*-acetyltransferase, liver tyrosine aminotransferase, and β -hydroxy- β -methylglutaryl (HMG) coenzyme A (CoA) reductase]; rate-limiting enzymes are usually degraded rapidly, as with tyrosine aminotransferase and HMG CoA reductase (13). Therefore, since the daily clock regulates rate-limiting enzymes whose half-life is only a small fraction of the circadian period, it seems reasonable that it should exploit their characteristically rapid turnover. Indeed, our findings support the proposal that synthesis and degradation are involved in the control mechanism of pineal *N*-acetyltransferase (14) and hepatic HMG CoA reductase (15). Enzyme turnover may therefore be a common mode of circadian control over biochemical pathways.

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8. Relative amounts of luciferase were determined by electrophoresing and blotting various dilutions of day and night luciferase and comparing the ratio of titer to density of the luciferase band in the linear range by analogy with the messenger RNA "dot blot" method [B. A. White and F. C. Bancroft, *J. Biol. Chem.* **257**, 8569 (1982)].
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16. *Gonyaulax* cells (strain 70) were grown at 19° to 20°C (5). At given intervals 1 liter of cell culture

- (1×10^4 to 1.2×10^4 cells per milliliter) was harvested (without photoinhibition) by filtration (Whatman 541) and resuspended in 5 ml of extraction buffer (100 mM tris, 10 mM EDTA, and 5 mM 2-mercaptoethanol, pH 8.5 and 4°C). The cells were broken by passage through a Kirkland emulsifier (Brinkmann); cell debris was removed by a 10-minute centrifugation at 800g. This supernatant constitutes the total protein fraction (including organelles). A portion of this supernatant was then centrifuged for 20 minutes at 27,000g, giving the soluble protein fraction.
17. Luciferase activity was assayed (5) at pH 6.3 with a sufficient dilution of the extract for linear response (usually a 1:100 dilution).
 18. Soluble and total protein samples were subjected to electrophoresis through sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose paper overnight [H. Towbin, T.

Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979)]. This "blot" was reacted with antibody to luciferase, washed, and reacted with 125 I-labeled protein A (H. Towbin *et al.*, *ibid.*). After a final washing, the immunoblot was autoradiographed for 1 to 3 days with or without an intensifying screen (an intensifying screen was used for Figs. 1B and 2B, but the densitometer data in Figs. 1A and 2A are from the same blots autoradiographed without screens).

19. This report is dedicated to Colin S. Pittendrigh on the occasion of his 65th birthday. We thank L.-A. Yeh and L. Safranek for their assistance and advice and J. Dunlap for suggestions and antibody. Supported by NIH postdoctoral fellowship GM08288 (C.H.J.) and NIH grant GM19536 (J.W.H.).

3 November 1983; accepted 23 January 1984

Salt-Sensitive Hypertension: Contribution of Chloride

Abstract. *The effect of the anion associated with sodium loading on the development of hypertension in the Dahl salt-sensitive rat was determined. For 5 weeks rats were fed a diet containing normal or high concentrations of sodium chloride or high concentrations of sodium provided as a mixture of sodium bicarbonate, phosphate, and amino acids. After 1 week on these diets and until the end of the study the rats receiving high concentrations of sodium chloride had higher systolic blood pressures than the rats in the other two groups. There were no statistically significant group differences in plasma volume, arterial pH, or plasma concentrations of Na^+ , K^+ , Cl^- , Ca^{2+} , or creatinine, or in renomedullary prostaglandin E_2 production. Compared to the animals receiving normal concentrations of sodium chloride, those receiving high concentrations of sodium chloride or amino acids showed decreased plasma renin activity and plasma aldosterone concentrations. Thus, the anion ingested with sodium alters the development and severity of hypertension in the Dahl salt-sensitive rat.*

We previously proposed that inhibition of renin release by sodium chloride is specifically related to a renal tubular effect of chloride (1, 2). In 1850, Redtenbacher and Gesell reported that urinary chloride excretion decreased with febrile illness (3), and in 1904 Ambard and Beaujard reported that a salt-restricted diet resulted in lowering of urinary chloride excretion which was associated with a decline of blood pressure in hypertensive patients (4). The prominence of chloride rather than sodium in these early reports was related to the ease with which it could be measured. However, with the advent of techniques for measuring sodium, interest in salt-

dependent hypertension became focused on sodium. In the study described here, we evaluated the importance of chloride to hypertension in the Dahl salt-sensitive (Dahl S) rat—an experimental model of sodium chloride-induced hypertension.

Lewis K. Dahl bred Sprague-Dawley rats on the basis of their predisposition to develop hypertension on a high sodium chloride diet (5). We first compared the effects on blood pressure of adding 8 percent sodium chloride or equimolar sodium bicarbonate to the diets of 7-week-old Dahl S rats (6). Despite a massive positive sodium balance, blood pressure did not increase in the sodium

Table 1. Body weight, net electrolyte balance, and muscle electrolyte content in Dahl S rats on three different diets. Results shown are mean $\pm$ standard error of the mean.

Diet	Body weight (g)		Cumulative electrolyte balance (mEq/100 g body weight for 5 weeks)			Muscle electrolyte content ($\mu\text{Eq/g}$ dry weight)		
	Start of study	End of study	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
Normal NaCl (N = 8)	227.8 ± 7.7	394.3* ± 10.2	12.74* ± 0.44	31.38* ± 1.57	11.97* ± 0.61	84.4 ± 3.3	457.0 ± 24.6	90.9 ± 7.9
High NaCl (N = 8)	220.4 ± 6.2	355.6 ± 6.3	70.95* ± 2.33	23.16 ± 0.45	53.33* ± 1.95	86.7 ± 3.9	457.8 ± 13.9	90.7 ± 6.8
High NaAA (N = 8)	212.6 ± 5.4	358.1 ± 7.3	80.81 ± 2.92	20.97 ± 0.91	7.53 ± 0.53	99.1* ± 5.0	470.3 ± 13.5	89.9 ± 4.0

* $P < 0.05$ (or less) compared to the other two groups (Newman-Keuls test).