zyme revealed that in the course of inactivation the band corresponding to the native enzyme disappeared while several new bands of lower molecular weight appeared. These data indicate that the polypeptide chain of lysozyme is cleaved during thermoinactivation, which was quantitatively assessed by measurement, with the use of gel scanning optical densitometry (*40*), of the intensity of the native band as a function of the time of thermoinactivation.
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24. Thermoinactivated lysozyme, reduced (*30*) and carboxymethylated (*31*), was completely hydrolyzed in concentrated HCl (*41*) and analyzed by high-performance liquid chromatography with the use of precolumn derivatization with phthaldialdehyde (*42*). None of the amino acid residues detectable by this method were destroyed during thermoinactivation. Acid hydrolysis breaks down tryptophan (*41*), which was, therefore, analyzed (*43*) in the inactivated lysozyme prior to acid hydrolysis; no differences in comparison with the native enzyme were observed. Also, since acid hydrolysis deamidates Asn and Gln (*41*), our method did not distinguish them from Asp and Glu, respectively.
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RESEARCH ARTICLE

Biochemical Modeling of an Autonomously Oscillatory Circadian Clock in *Euglena*

Ken Goto, Danielle L. Laval-Martin, Leland N. Edmunds, Jr.

There have been many efforts to discover the molecular mechanism (or mechanisms) for the circadian clocks underlying numerous physiological processes (*1*). Hypotheses have been proposed that stress the central role of transcription, protein synthesis and translation on 80*S* ribosomes, adenosine 3′,5′-monophosphate (cyclic AMP), and cellular membranes (*2–4*). There are difficulties inherent in distinguishing between the so-called "hands" of the clock (mechanism-irrelevant events) and the "gears" themselves (clock-relevant processes) which have impeded efforts to test these hypotheses. Phase-shift experiments have been used to ascertain whether or not a given process is an integral part of a circadian clock on the rationale that a transitory perturbation of either the state variables or the parameters that may be used to characterize an oscillation can cause a permanent phase shift in an overt rhythm (*5*). Unfortunately, the converse is not necessarily true; an observed phase shift may have occurred as a result of the effect of the drug or other perturbing agent on some site only secondarily affected by the drug rather than on its postulated primary target (*6*).

The rationale. A circadian oscillator can be expressed mathematically as a set of differential equations comprising both state variables and parameters. The state variables characterize the state of the oscillation, with each set of values defining each phase of the oscillation. The parameters are constants constraining the manner in which the state variables change and determining the dynamics of the oscillations; a different set of parameter values gives a different solution of the rate equations. Any transitory alteration or perturbation of either the state variables or of the parameters can cause a permanent phase shift in an overt rhythm but has no permanent effect on its period; in contrast, permanent changes in the parameter values can alter the steady-state period of the oscillation [see (*5*)].

We have now operationally designated any element as a "gear" (G) of a circadian clock if it can be expressed as a state variable or a parameter; if not, it is a "nongear" (~G). Together, the set of gears would constitute a closed control loop, or oscillator. Because an unperturbed ~G in its normal or physiological oscillatory range would not be expected to regulate the operation of the G's themselves, its artificial perturbation within this range (*7*) should not perturb circadian timekeeping (no steady-state phase shift in an overt rhythm should be observed). Consequently, if an experimental alteration in the level of a target within its normal range perturbs the clock and generates steady-state phase shifts, then that target is most likely a G (criterion A). It is conceivable, however, that the activation and resulting increase in the level of a ~G might perturb timekeeping, whereas its inhibition would not (or vice versa). For example, although the inhibition of protein synthesis can perturb the clock in several organisms (*8, 9*), its activation in both *Neurospora*

Ken Goto was a postdoctoral research associate in the Department of Anatomical Sciences, School of Medicine, State University of New York at Stony Brook, New York 11794, and is currently at the Institute of Applied Biochemistry, Yagi Memorial Park, Mitake, Gifu 505-01, Japan. Danielle L. Laval-Martin is a research associate professor in the Department of Anatomical Sciences, State University of New York at Stony Brook and Maître de Conférences de Physiologie Végétale, Laboratoire des Membranes Biologiques, Université Paris VII, France. Leland N. Edmunds, Jr., to whom correspondence should be addressed, is a professor in the Department of Anatomical Sciences, State University of New York at Stony Brook, Stony Brook 11794.

(*9*) and *Euglena* (*10*) does not; likewise, experimentally increasing (but not decreasing) the levels of cyclic AMP alters the period of the rhythm of conidiation in *Neurospora* (*11*). To more stringently differentiate between G and ~G, therefore, a further requirement for a target to be classified as a G (*12*) is that both the direct activation as well as direct inhibition of the target must perturb the clock (criterion B).

The causal relationships among circadian metabolic rhythms in the duckweed *Lemna gibba* G3 have recently been investigated (*13, 14*), leading to the suggestion that nicotinamide adenine dinucleotide (NAD^+), the mitochondrial Ca^{2+}-transport system, Ca^{2+}, calmodulin, NAD^+ kinase, and $NADP^+$ phosphatase might regulate their levels sequentially and thus constitute a self-sustaining, circadian oscillatory loop (*15*). We now extend our studies to the algal flagellate *Euglena*, a well-characterized unicellular circadian system (*16*) with an overt circadian rhythm of cell division (*17*), and test our hypothesis according to the given criteria. In principle, these criteria are applicable to any set of processes thought to constitute an autonomously oscillatory clock.

Oscillating elements in Euglena. The in vivo level of NAD^+, which was measured as described (*14*) in synchronously dividing and in very slowly dividing cultures of *E. gracilis* Klebs (Z strain) phototrophically batch-cultured at 25°C, oscillated with a circadian period (27 hours) when the cultures were exposed to a higher frequency cycle consisting of 3 hours of light followed by 3 hours of darkness (LD: 3, 3) (Fig. 1). Free-running circadian rhythms (not externally synchronized) have been shown to occur after transfer of the culture from continuous illumination to LD: 3, 3 (*17*). The ratio of the peak value [attained at circadian time 18 (CT 18)] to the trough value of the rhythm in NAD^+ level remained constant (~1.7) no matter whether the population showed only small factorial increases (stepsize, ss) in cell number (Fig. 1; ss = 1.14) or larger ones.

There is a circadian rhythm in the activity of NAD^+ kinase (peak at CT 0) (*18*) in extracts of *Euglena* (*18*) with a phase relationship such that it could induce the rhythm in the in vivo level of NAD^+ (Fig. 1) [see (*14*)]. As neither the ratio of NADH to (NAD^+ + NADH) nor that of NADPH to ($NADP^+$ + NADPH) oscillates when the circadian clock is operating in *Euglena* (*16*), our findings suggest that the circadian oscillation in the in vivo level of NAD^+ could be ascribable, as for *Lemna* (*14*), to that of the conversion between the reduced forms NADH and NADPH, but not to that of reduction-oxidation between NAD^+ and NADH.

Are NAD^+, NAD^+ kinase, and $NADP^+$ phosphatase "gears"? In order to determine whether or not NAD^+ constitutes a true clock "gear," small (25 ml) seed cultures displaying a free-running circadian rhythm of cell division were treated (for 2 hours) at various CT's with 0.5 m*M* NAD^+, 10 m*M* *p*-nitrophenylphosphate (*p*NPP, a competitive inhibitor of $NADP^+$ phosphatase), or 0.2 m*M* $NADP^+$ and then resuspended in 4 liters of fresh medium (effectively terminating the treatment) for subsequent automated monitoring of the cell division rhythm. Addition of these compounds would be expected to directly elevate their own in vivo levels and to secondarily increase or decrease the rate of the reactions catalyzed by NAD^+ kinase and $NADP^+$ phosphatase. Pulses of each of these compounds were able to generate steady-state phase shifts of the rhythm of cell division whose sign and magnitude were dependent on the CT at which the pulse was applied (Fig. 2). These phase shifts could not have been caused primarily by the perturbation of the cell division cycle (CDC) itself, inasmuch as the CDC has been shown to be driven, or phased, by the circadian clock. If the CDC were either transitorily delayed or advanced immediately after a drug pulse,

Abstract. *Eukaryotic microorganisms, as well as higher animals and plants, display many autonomous physiological and biochemical rhythmicities having periods approximating 24 hours. In an attempt to determine the nature of the timing mechanisms that are responsible for these circadian periodicities, two primary operational assumptions were postulated. Both the perturbation of a putative element of a circadian clock within its normal oscillatory range and the direct activation as well as the inhibition of such an element should yield a phase shift of an overt rhythm generated by the underlying oscillator. Results of experiments conducted in the flagellate* Euglena *suggest that nicotinamide adenine dinucleotide (NAD^+), the mitochondrial Ca^{2+}-transport system, Ca^{2+}, calmodulin, NAD^+ kinase, and $NADP^+$ phosphatase represent clock "gears" that, in ensemble, might constitute a self-sustained circadian oscillating loop in this and other organisms.*

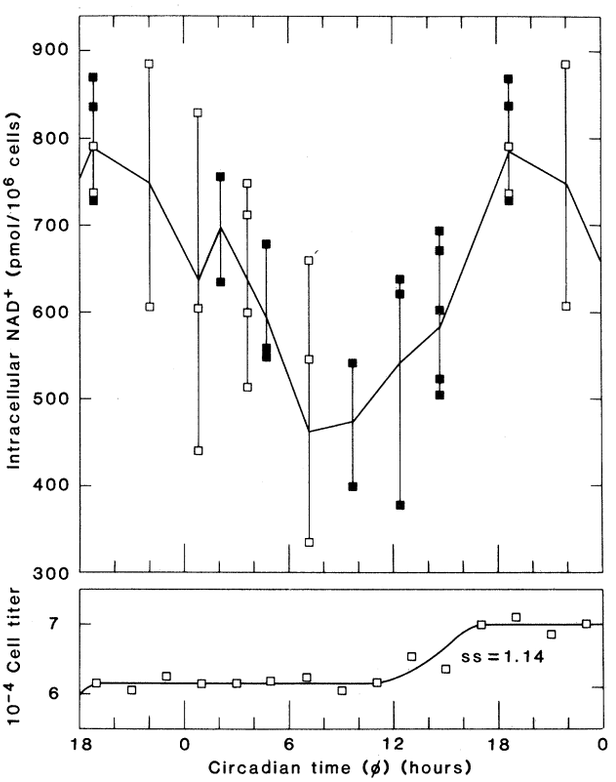


Fig. 1. (Upper panel) Circadian variations in the intracellular content of NAD^+ in very slowly dividing autotrophic cultures of *Euglena* maintained in LD: 3, 3 regimens. Two out-of-phase, free-running cultures (□ and ■) were sampled at different circadian times, and their NAD^+ contents were spectrophotometrically measured by an enzymatic assay (*14*) (two to five determinations for each time point). The curve connects the mean values of all data points obtained at a given CT (ϕ). CT (0) indicates the phase point of a free-running rhythm that has been normalized to 24 hours and occurs at the onset of light in a LD: 12, 12 reference cycle. (Lower panel) Representative growth curve for one of the two cultures. Cell titer is plotted (on a log scale) as a function of CT (ϕ). The very small factorial increase (ss = 1.14) indicates that only 14 percent of the free-running population divided during the cycle. The amount of intracellular NAD^+ increased by 70 percent during this fission interval.

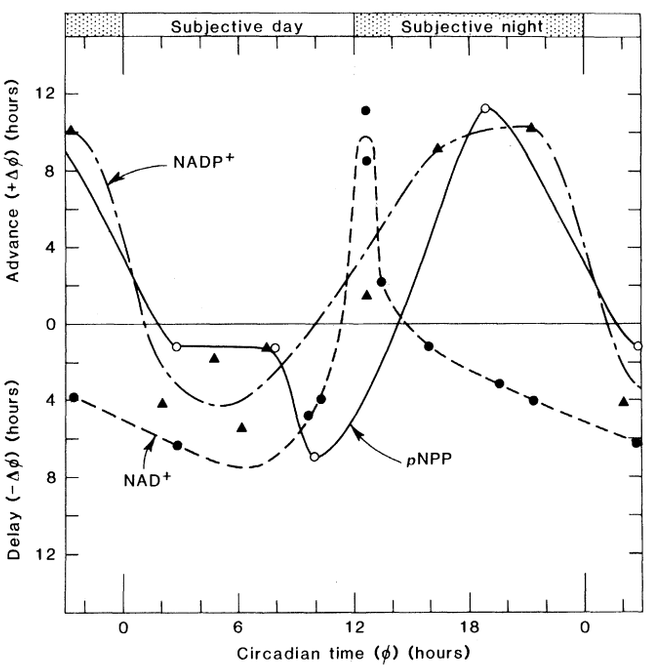
no permanent phase shift in the rhythm occurred. If the CDC were not shifted within the cycle during which a pulse was administered, it was shifted later after subsequent cycles.

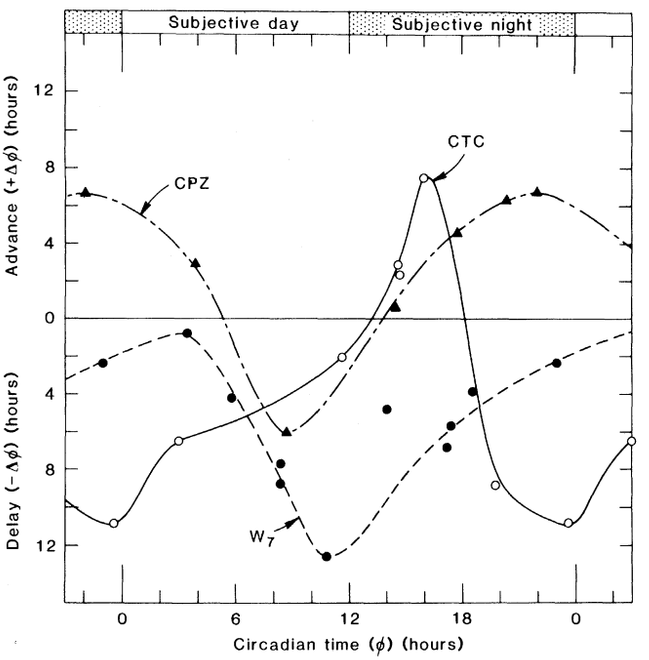
Furthermore, we have monitored the transitory change in the in vivo level of NAD^+, NADH, $NADP^+$, and NADPH at 30-minute intervals in a free-running culture of *Euglena* after treatment with NAD^+ (0.5 m*M*, 2.5 hours) given at CT 21.7, which caused a 4-hour phase shift (delay) in the overt circadian rhythm of cell division. Although the in vivo level of NAD^+ in an unperturbed, control culture decreased during this time span (Fig. 1), the level in the pulsed cells increased over the next hour to a value slightly lower than that of the maximum level attained by the oscillation in unperturbed cultures, and finally decreased. Similar variations were found in $NADP^+$ and NADPH contents in vivo, with all levels (as for NAD^+) falling within the normal range characteristic of unperturbed control cultures. The amount of NADH did not change significantly (*18*).

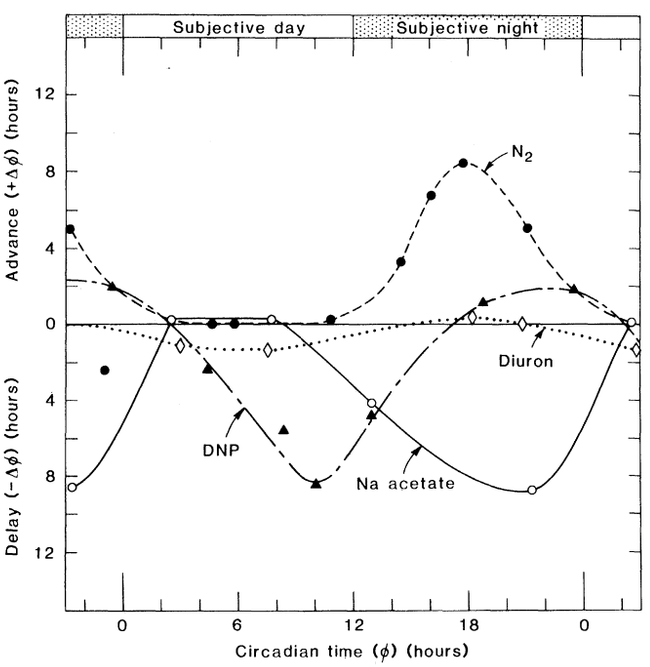
Taken together, these results satisfy criteria A and B, suggesting that NAD^+ (or NADPH or both), NAD^+ kinase, and $NADP^+$ phosphatase represent "gears" of the underlying circadian oscillator.

Are Ca^+ and Ca^+-calmodulin "gears"? The next question concerns the identity of the element that regulates NAD kinase and NADP phosphatase, already suggested to be "gears" in themselves. If these elements are G's, then the element regulating them should be a G also. Ca^{2+}-calmodulin activates NAD kinase in many plants (including green algae) (*19*) and in the sea urchin (*20*), and the circadian rhythms in the activities of NAD^+ kinase and $NADP^+$ phosphatase appear to be generated by a rhythm in the in vivo level of this complex in *Lemna* (*14*). Thus, Ca^{2+}-calmodulin would seem to be a likely candidate for this G in *Euglena*.

We attempted to *directly* cause a transitory decrease in $[Ca^{2+}]$ by means of 2-







Figs. 2 to 4. Phase response curves for the effect of pulses of different compounds on the free-running rhythm of cell division in photoautotrophic cultures of *Euglena* in LD: 3, 3. Steady-state phase shift ($\pm\Delta\phi$) is plotted as a function of the midpoint of the perturbation [CT (ϕ), normalized to 24 hours]. The number of data points for a given phase response curve was varied from five to ten, depending on the complexity of its waveform; assays were clustered in instances of apparent sharp discontinuities (for example, Fig. 2; NAD^+, CT 12) or to ensure that experiment-to-experiment variation was small (for example, Fig. 3; W_7, CTC). Fig. 2 (top left). Pulses of NAD^+ (0.5 m*M*, 2.3 hours), of $NADP^+$ (0.2 m*M*, 2 hours), and of *p*-nitrophenylphosphate (*p*NPP; 10 m*M*, 2 hours) applied during a light interval of the LD: 3, 3 cycle. Fig. 3 (top right). Pulses of W_7 (20 μM, 2.3 hours) and of chlorpromazine (CPZ; 50 μM, 2.3 hours) applied during a light interval of the LD: 3, 3 cycle, and of chlortetracycline (CTC; 200 μM, 3 hours) applied during a dark interval. Fig. 4 (bottom left). Pulses of nitrogen (N_2; 600 ml min^{-1}, 3 hours) applied during a dark interval of the LD: 3, 3 cycle; and of sodium acetate (10 m*M*, 2 hours), of dinitrophenol (DNP; 100 μM, 2 hours), and of diuron (10 μM, 2 hours) applied during a light interval.

to 3-hour pulses of chlortetracycline (*21*) (CTC, 200 μM), a membrane-permeable chelator of Ca^{2+}. Ca^{2+}-calmodulin was inhibited with similar short treatments with the calmodulin inhibitors W_7 (*22*) (20 μM) and chlorpromazine (*23*) (CPZ; 50 μM). These agents also produced pronounced phase shifts of the cell division rhythm (Fig. 3). Phase shifts in the overt rhythm were also obtained with the secondary increases in [Ca^{2+}], and the ensuing tertiary activation of Ca^{2+}-calmodulin, resulting from nitrogen and dinitrophenol (DNP) pulses (Fig. 4). These results suggest that both cytosolic Ca^{2+} and calmodulin constitute "gears" (*24*).

Is the mitochondrial Ca^{2+}-transport system a "gear"? There should be another G directly regulating [Ca^{2+}]. The main regulatory sites for many noncircadian systems are known to be the plasmalemma, the endoplasmic reticulum, and the mitochondria. In *Lemna*, the net uptake of Ca^{2+} across the plasmalemma does not oscillate, while that of K^+ does (*25*).

To test the possibility that the mitochondrial Ca^{2+}-transport system might be a G, the phase-shifting effects of short (2- or 3-hour) pulses of nitrogen, dinitrophenol (DNP), and sodium acetate on the cell division rhythm of *Euglena* were examined (Fig. 4). Mitochondrial Ca^{2+}-transport is closely related to electron transport or to adenosine triphosphate (ATP) hydrolysis coupled to proton transport (*26*). Nitrogen or DNP would be expected to enhance the rate of net mitochondrial Ca^{2+} efflux by inhibiting energy-dependent Ca^{2+} influx, whereas sodium acetate enhances Ca^{2+} uptake via cotransport (*27*). As is evident from the phase response curves (PRC's) (Fig. 4), all the perturbing agents were effective in generating steady-state phase shifts (*28*). The PRC's for nitrogen and sodium acetate, agents having opposite effects on transport, are almost mirror images of each other. Diuron [DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea], an inhibitor of photosynthesis, should not have any significant effect on mitochondrial Ca^{2+} movement; it was shown to be ineffective for phase-shifting (Fig. 4) at the concentration used. Therefore, the mitochondrial Ca^{2+} transport system would appear to be a "gear" of the oscillator in *Euglena*.

Particularly relevant to this hypothesis is the observation that respiring mitochondria can maintain extramitochondrial (and thus presumably cytoplasmic) concentrations of free Ca^{2+} at a "set point." Depending on the kinetic parameters of the transport system (altered by certain effectors), this set point can change between 0.3 and $3.0 \times 10^{-6} M$ without changing the rate of respiration (*29*). Antibody to the glycoprotein thought to be involved in mitochondrial Ca^{2+} transport inhibited the Ca^{2+} efflux that was induced by the oxidation of NAD(P)H (*30*). Furthermore, NAD^+ lowered the affinity of the glycoprotein for Ca^{2+}, although NADPH and NADH did not. Thus, NAD^+ may be an activator of net mitochondrial Ca^{2+} efflux. It is conceivable that NAD^+ is a "gear" in the circadian oscillator that is coupled to another "gear," the mitochondrial Ca^{2+}-transport system.

A model for the circadian oscillator. Our findings in *Lemna* (*14, 15*) and *Euglena* have led to a working hypothesis for a closed loop that would constitute a self-sustaining circadian oscillator. This regulatory scheme (Fig. 5) together with the postulated primary actions of the drugs used in our experiments, includes the following three steps.

Step 1. NAD^+ would enhance the rate of net Ca^{2+} efflux from the mitochondria, resulting in a maximal concentration of cytosolic Ca^{2+} 6 hours (90°) later. Alternatively (step 1′), a photoreceptor (phytochrome, or perhaps a blue-light photoreceptor in *Euglena* and animal cells) stimulated by (red or blue) light would enhance either net Ca^{2+} efflux from the mitochondria or net Ca^{2+} influx across the plasmalemma into the cytoplasm.

Step 2. Ca^{2+} would immediately activate calmodulin by forming an activated Ca^{2+}-calmodulin complex in the cytoplasm (maximal level at 90°).

Step 3. This active form of Ca^{2+}-calmodulin would decrease the *rate* of net production of NAD^+ by both activating NAD^+ kinase and inhibiting $NADP^+$ phosphatase in the cytoplasm so that the rate would become maximal 12 hours later (at 270°) when Ca^{2+}-calmodulin reached its minimal level.

After six more hours (at 0°), the in vivo *level* of NAD^+ would reach its minimum. The regulatory sequence would then be closed.

Supporting evidence for step 1 has been discussed earlier. The evidence for step 1′ in other plants is that P_{fr} (*31*) enhances net Ca^{2+} efflux from isolated mitochondria (*32*) and net Ca^{2+} influx across the plasmalemma (*33*). In step 2, the rate of formation of an active form of Ca^{2+}-calmodulin would be limited by cytosolic Ca^{2+} but not by calmodulin

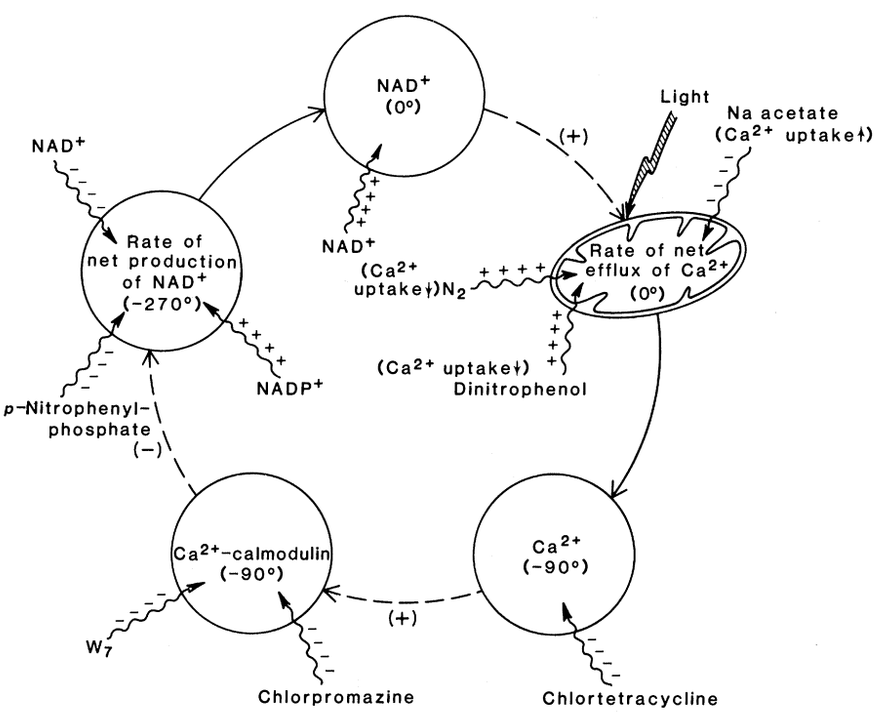


Fig. 5. Proposed control loop for the circadian oscillator in *Euglena* and *Lemna*. The pattern of regulation is indicated by both solid lines, which relate the reactions to their products, and dashed lines, which correspond to the sequence of steps. Each element oscillates with a circadian period (with peaks and troughs occurring 180° apart). The degrees in parentheses reflect the phases at which maximal values are attained [see (*14*)]. The maximal rate of a reaction precedes by 90° the maximal concentration of its product. The activation (+) or the inhibition (−) of each compound on the succeeding one is shown and can be considered to produce phase delays of 0° or 180°, respectively. The inhibitory effect of Ca^{2+}-calmodulin on the rate of net production of total NAD^+ derives from both its inhibitory effect on $NADP^+$ phosphatase activity and its activating effect on that of NAD^+ kinase. A number of different compounds and agents known to have either positive (activation: $\overset{+}{\sim\sim\rightarrow}$) or negative (inhibition: $\overset{-}{\sim\sim\rightarrow}$) effects on their targets are indicated.

(*34*). Our experiments showing that both the rise or fall in [Ca^{2+}] could perturb circadian timekeeping also support this notion. Step 3 has been corroborated in *Lemna gibba* (*14*), in other plants (*19*), and in the sea urchin (*20*).

This proposed feedback loop can autonomously oscillate because the "cross-couplings" are always of opposite sign (*35*). Thus, while the rise in NAD^+ causes increases in the rate of formation of the active Ca^{2+}-calmodulin complex by steps 1 and 2, the resulting rise in the level of active Ca^{2+}-calmodulin complex causes decreases in the rate of formation of NAD^+ by step 3. Step 1′ would entrain (synchronize) the system. In order for this oscillator to display limit cycle dynamics (*5*), additional requirements would have to be satisfied (*35*). The rise in NAD^+ must cause a decrease in the rate of net formation of NAD^+ (which is very likely). The rise in the level of active Ca^{2+}-calmodulin complex must cause an increase in its own rate of formation somewhere in the oscillatory region. This may be accomplished by the cooperative binding of Ca^{2+} to the four sites of each calmodulin molecule, which increases the binding affinity of calmodulin for Ca^{2+} (*34*). Finally, the strength of this "self-coupling" must be weaker than that of the cross-coupling.

Our model does not attempt to explain either the long period-length or the steady-state temperature compensation characteristic of circadian rhythmicity (*2*), primarily because of a lack of hard data. A limit cycle (*5*) could display the long (~24-hour) period given a particular set of parameter values or a network of interacting intracellular oscillators (*36*); alternatively, other time-consuming processes involving transcription, translation, or membrane-based ion transport may be important (*3, 4*). Similar explanations (*3, 4*) could be invoked as compensatory mechanisms (*37*) for the temperature-dependent steps (such as the NAD^+ kinase and $NADP^+$ phosphatase reactions) in our model. Finally, we have proposed a model—not for "the" clock—but for one oscillator in what is most probably a cellular "clockshop" (*38*).

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12. Sometimes it will be impossible to attack a presumed target directly. Phase shifts generated by several drugs that only secondarily activate and inhibit a target, however, do not demonstrate necessarily that such a target is a G. Nevertheless, if a given target (B) is regulated by another target (A) and, in turn, regulates a third target (C), and if it is probable that A and C are gears, then it is likely that B is one also. In contrast, if both targets A and C are known to be hands (~G) of the oscillator, then target B is also a ~G. Only in the case where target A is shown to be a G and target C is proved to be a ~G can target B not be classified.
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28. Earlier work with inhibitors of mitochondrial respiration and oxidative phosphorylation has given conflicting results (*2*). Nitrogen pulses generated CT-dependent phase shifts in *Avena* [N. G. Ball and I. J. Dyke, *J. Exp. Bot.* **8**, 323 (1957) and in *Phaseolus* and *Kalanchoe* [E. Bünning, S. Kurras, V. Vielhaben, *Planta* **64**, 291 (1965)], but no significant effect in *Kalanchoe* [W. Steinheil, *Z. Pflanzenphysiol.* **62**, 204 (1970)]; likewise, dinitrophenol and cyanide caused phase shifts in *Kalanchoe* (Steinheil, *ibid.*), but little effect in *Oedogonium* [F. Bühnemann, *Biol. Zentralbl.* **74**, 691 (1955)] and *Gonyaulax* [J. W. Hastings, *Cold Spring Harbor Symp. Quant. Biol.* **25**, 131 (1960)]. These discrepancies might be due to the drugs' having been applied continuously, or pulsed only at one or two circadian times. Indeed, short pulses of dinitrophenol, cyanide, and azide shift phase in *Aplysia* [A. Eskin and G. Corrent, *J. Comp. Physiol.* **117**, 1 (1977)] and *Neurospora* [H. Nakashima, *Plant Physiol.* **76**, 612 (1984)].
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31. Entrainment and phase-shifting by light are mediated by the conversion of the red-absorbing form of phytochrome (P_r) to the far red–absorbing species P_{fr} in *Lemna* [Y. Oota and H. Nakashima, *Bot. Mag. (Tokyo)* (special issue), **1**, 177 (1978)] and many other higher plants [R. L. Satter and A. Galston, *Annu. Rev. Plant Physiol.* **32**, 83 (1981)], but one or more other photoreceptors would have to be responsible in *Euglena* and animal cells (which lack phytochrome).
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36. T. Pavlidis and W. Kauzmann have demonstrated [*Arch. Biochem. Biophys.* **132**, 338 (1969)] that a system dynamically equivalent to the glycolytic oscillator [with periods on the order of minutes; see (*5*)] theoretically could display a circadian period. Frequency reduction would be achieved by "enzyme starvation" through inhibitory coupling of a large number of individual oscillators [see also _____, *J. Theor. Biol.* **22**, 418 (1969)], by some type of inhibitory coupling in which the enzyme is used in a number of reactions (not necessary oscillator elements), or by "sink" and "deposition" effects within cell energy metabolism [see E. E. Sel'kov, *Eur. J. Biochem.* **59**, 151 (1975)].
37. The temperature-compensation mechanism may be independent of the basic timer itself, as has been suggested for *Neurospora* [D. L. Mattern, L. R. Forman, S. Brody, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 825 (1982)], perhaps reflecting a more recent, ancillary evolutionary adaptation.
38. In fact, it is possible that our circadian oscillator is identical with (or at least shares some common elements with) the escapement mechanism for an endogenous mitotic oscillator. The circadian oscillator may be modeled best as a low-amplitude limit cycle nested within the escapement mechanism. [J. J. Wille, Jr., in *Biochemistry and Physiology of Protozoa*, M. Levandowsky and S. Hutner, Eds. (Academic Press, New York, ed. 2, 1979), vol. 2, p. 142; L. N. Edmunds, Jr., and K. J. Adams, *Science* **211**, 1002 (1981)].

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