Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10862–10866, November 1992
Neurobiology

Stopping the circadian pacemaker with inhibitors of protein synthesis

(rhythm/*Bulla*/mollusc)

SAT BIR S. KHALSA, DAVID WHITMORE, AND GENE D. BLOCK*

National Science Foundation Center for Biological Timing, Department of Biology, University of Virginia, Charlottesville, VA 22901

Communicated by Colin S. Pittendrigh, June 15, 1992

ABSTRACT **The requirement for protein synthesis in the mechanism of a circadian pacemaker was investigated by using inhibitors of protein synthesis. Continuous treatment of the ocular circadian pacemaker of the mollusc *Bulla gouldiana* with anisomycin or cycloheximide substantially lengthened (up to 39 and 52 hr, respectively) the free-running period of the rhythm. To determine whether high concentrations of inhibitor could stop the pacemaker, long pulse treatments of various durations (up to 44 hr) were applied and the subsequent phase of the rhythm was assayed. The observed phases of the rhythm after the treatments were a function of the time of the end of the treatment pulse, but only for treatments which spanned subjective dawn. The results provide evidence that protein synthesis is required in a phase-dependent manner for motion of the circadian pacemaker to continue.**

While there is considerable experimental interest in biological timing, little is known about the cellular and subcellular events which are responsible for generating circadian periodicities. Given the ubiquitous role of proteins and enzymes in intracellular processes and systems, it is reasonable to hypothesize that intracellular clocks are built by using proteins—that the biochemical processes responsible for generating circadian timing intimately involve transcription and/or translation and/or proteins themselves (1).

This hypothesis has received support from numerous and diverse studies. For example, mutations have been examined which show deficits or alterations in the physiology of the circadian pacemaker (reviewed in refs. 1–3). Furthermore, there are studies demonstrating proteins which oscillate in a circadian cycle representing putative "clock proteins" (4–6) (reviewed in refs. 7 and 8). Induction of proteins after phase-shifting perturbations of the circadian pacemaker has also been reported (9–13), and the role of protein synthesis as a process in phase shifting has been discussed (14). Other studies with transcription inhibitors have suggested a role for RNA synthesis in the pacemaker mechanism (4, 15–20), and models for a pacemaker mechanism incorporating transcription have been described and discussed (8, 21, 22).

Perhaps the largest body of evidence implicating proteins in the circadian pacemaker mechanism is from studies employing translation inhibitors (reviewed in refs. 7, 8, and 23–26). Continuous treatment of circadian pacemakers with translation inhibitors lengthens the period of the rhythms (27–30), whereas pulse treatments generate phase-dependent phase shifts of the circadian pacemaker (27, 28, 31–49) in a variety of organisms. On the basis of this type of experiment it has been suggested that translation is a requirement for the circadian pacemaker (17, 27, 28, 30–32, 35, 36, 40, 43, 44, 47, 49, 50), or more specifically, that translation is a phase-dependent requirement for the circadian pacemaker (24, 28, 32, 35, 36, 40, 43, 44, 49). A prediction drawn from this suggestion is that inhibition of protein synthesis should be capable of stopping the motion of the circadian pacemaker (14, 32); without protein synthesis, the pacemaker should not complete a full cycle of circadian oscillation.

Using an experimental paradigm that allows us to test the prediction that the circadian pacemaker can be stopped, we have conducted experiments with protein synthesis inhibitors on a molluscan preparation. The eyes of the molluscs *Bulla gouldiana* and *Aplysia californica* contain populations of electrically coupled retinal cells; recordings of optic nerve impulse activity from isolated eyes reveal a precise and persistent circadian rhythm (reviewed in refs. 23, 25, and 51). We now report evidence for a phase-specific requirement for protein synthesis in maintaining pacemaker motion: disruption of protein synthesis during a critical phase "stops the clock," which resumes its motion only upon removal of the protein synthesis inhibitor.

MATERIALS AND METHODS

B. gouldiana were obtained from Marinus (Long Beach, CA) and maintained in a temperature-controlled seawater tank at 15°C. Animals were exposed to a light cycle of 12 hr of light and 12 hr of darkness (L:D 12:12) for at least 1 week prior to experimental set-up. Two hours before the onset of darkness, animals were immobilized with an injection of 10 ml of isotonic $MgCl_2$ and then placed on ice for dissection.

For extracellular recordings, both eyes were removed from each animal and placed in separate dishes of artificial seawater (ASW, 20 ml per dish). The composition of ASW was 395 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 28 mM Na_2SO_4, 30 mM Hepes buffer, 100,000 units of penicillin per liter, and 100,000 μg of streptomycin per liter. The pH was adjusted to 7.8. The optic nerve from each eye was pulled up into an ASW-filled micropipette suction electrode mounted on a recording dish, which was then placed in a light-tight recording chamber and maintained at 15°C.

Optic nerve compound action potential activity was recorded on a Grass polygraph and also computer counted into 15-min bins. The phase reference point for circadian cycles of activity was determined by calculating the time of occurrence of the half-maximal spike frequency on the rising phase of each cycle. An acceptable cycle was defined as one which had at least one bin of eight events (a minimum frequency of 16 impulses per 0.5 hr), except for experiments measuring period lengthening with anisomycin, where a minimum bin of five events was considered acceptable.

Clock time or *Zeitgeber* time (ZT) based on the previous light cycle was used in the measurement of the phases of activity cycles and the timing of treatments. Therefore, phases and times reported in ZT in this study are slightly later than

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Abbreviations: CHX, cycloheximide; ASW, artificial seawater; ZT, *Zeitgeber* time.

*To whom reprint requests should be addressed.

true circadian time (CT), since the period of the *Bulla* ocular rhythm *in vitro* is on the average slightly less than 24 hr (51).

Two types of experiments were performed: (*i*) Continuous treatment experiments to evaluate changes in period. Eyes were set up *in vitro* in ASW at ZT 12 (subjective dusk). One hour later, at ZT 13, the experimental solution was applied and the preparations were then left undisturbed. (*ii*) Long-duration pulse treatments of inhibitor, in which up to five subsequent cycles of activity were recorded. Eyes were set up *in vitro* in ASW at ZT 12. Pulses were begun at ZT 13, ZT 18, or ZT 6 before or during the first cycle of activity, and pulse lengths ranged from 5 to 44 hr. At least two eyes were evaluated for each pulse length, and eyes from the same animal were never subjected to the same pulse length.

For each solution exchange, at least 95% of the volume of the dish was first removed and 20 ml of the exchanging solution (maintained at 15°C) was infused. For each pulse one complete exchange of the inhibitor solution was applied at the start of the pulse and five complete exchanges of ASW were applied at the end. Cycloheximide (CHX), anisomycin, and deacetylanisomycin were obtained from Sigma.

Protein synthesis inhibition was quantified by determining the [^{35}S]methionine incorporated into newly synthesized proteins, using a standard trichloroacetic acid precipitation protocol. Eight whole eyes were pulsed with 10 mM CHX as described above; the contralateral eyes served as controls. Eyes were transferred to an Eppendorf tube with 345.6 μl of ASW and 14.4 μl of [^{35}S]methionine (40 μCi/ml; 1 Ci = 37 GBq) and incubated in darkness at 15°C for 1 hr. Eyes were washed five times in 4°C ASW and homogenized in a grinding buffer of 75 μl of Tris·HCl, pH 7.4, and 30 μl of Anderson's buffer, which is 2% each of SDS, glycerol, Ampholine (LKB), 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and dithiothreitol. Samples were heated to 95°C for 5 min and frozen at −80°C.

To determine the amount of label incorporated, 10 μl of each sample was added to 300 μl of a bovine serum albumin solution (0.2 mg/ml) and 100 μl of 50% (wt/vol) trichloroacetic acid. This was mixed, placed on ice for 20 min, and centrifuged, and the supernatant was aspirated and the remaining pellet was washed three times in 5% trichloroacetic acid. The pellet was solubilized with scintillation fluid and the radioactivity was measured in a scintillation counter. The above procedure was repeated three times with additional 10-μl aliquots of every sample and the four resultant counts were averaged. The radioactivity of incubating solution was measured as a control to ensure that equal amounts of isotope were added to all eyes. Counts on four empty vessels served as background values. Inhibition of protein synthesis was calculated as 100% minus the percent of the average experimental counts relative to control counts.

RESULTS

Continuous application of both translation inhibitors CHX and anisomycin (52) lengthened the free-running period of the circadian rhythm in a concentration-dependent manner (Fig. 1). Where three or more cycles of activity were expressed, the period length was stable over the course of the treatment for both inhibitors. Period lengthening to 52 hr with 5 mM CHX and to 38 hr with 1 μM anisomycin was observed; the inhibition of spontaneous impulse activity at these high inhibitor concentrations precluded the observation of greater lengthening. The data in Fig. 1 are fit well with an exponential function, suggesting that the circadian pacemaker may be held "motionless" at concentrations above those which would drive the period to extreme values.

To test the hypothesis that the motion of the pacemaker can be stopped, we adopted an experimental paradigm in which the phase of the circadian rhythm subsequent to a treatment of various durations is used as an indication of

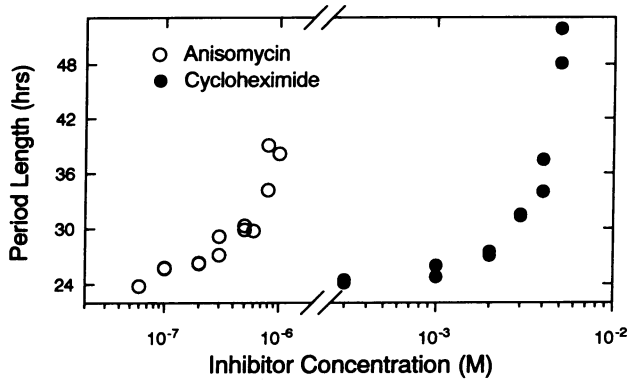

FIG. 1. Period length increases as a function of inhibitor concentration. Various concentrations of anisomycin (○) or CHX (●) were applied continuously. Period length was calculated as the average difference in time between successive cycles of activity for each eye. With increasing concentrations of the inhibitors the activity rhythms were progressively reduced in amplitude, with fewer cycles expressed.

pacemaker motion during the treatment (53, 54). Concentrations of CHX and anisomycin above those observed to cause the longest period lengthening were used as the treatment (10 mM and 2 μM, respectively). Fig. 2 summarizes the experimental protocol and shows examples of impulse activity records from three eyes subjected to different durations of CHX. In the summary plot in Fig. 2*D*, the shortest pulse of CHX (14 hr) fails to perturb the phase of the rhythm from that expected of an untreated control rhythm, near subjective dawn (ZT 0). However, the two longer pulses (23 and 32 hr) shift the phase of the rhythm with a magnitude which is a function of the end of the inhibitor pulse.

Summary plots of data from four complete experiments are shown in Fig. 3. For CHX pulses terminating before ZT 0 in Fig. 3*A*, the phase of the circadian rhythm appears unperturbed by the inhibitor treatment, since the phase reference points on subsequent cycles are near ZT 0, as expected for untreated eyes. This is also true in Fig. 3*B*, although only two pulses end before ZT 0 and show this effect. However, after longer-duration pulses that extend through ZT 0 in Fig. 3 *A* and *B* a distinctive relationship is established. The phase subsequent to the treatment is a strict function of the ending time of the treatment: *the phase of the subsequent rhythm is delayed by the number of hours that the inhibitor pulse extends past ZT 0*. In Fig. 3*C*, this relationship is confirmed; all of the phase reference points are determined by the ending time of the CHX treatment, since all pulses extend through ZT 0.

Long pulses of 2 μM anisomycin generated data similar to those for CHX. In Fig. 3*D* phase reference points after anisomycin pulses are a function of the ending time of the treatment, although the location of the phase reference points appears to be delayed relative to the same points obtained after CHX pulses (compare Fig. 3*C*). Pulses of the inactive analogue deacetylanisomycin (50) (2 μM) from ZT 18 to 12 over the first cycle did not inhibit spontaneous impulse activity during the treatment, nor did they generate significant phase shifts relative to untreated controls as measured on the fourth cycle (mean = −0.1 hr, SD = 1.0 hr, n = 4).

Protein synthesis inhibition was quantified at different time points over the course of a 17-hr pulse of 10 mM CHX delivered from ZT 18 to ZT 11, a treatment yielding a phase shift of about 12 hr (see Fig. 2*C*). Over the 1-hr period of ZT 20 to ZT 21 (beginning 2 hr after CHX application), protein synthesis inhibition was 85%. At the end of the inhibitor pulse, over the 1-hr period of ZT 10 to ZT 11 (16 hr after CHX), inhibition increased to 93%. Recovery from inhibition was measured over the 1-hr periods beginning 2, 4, 6, and 12

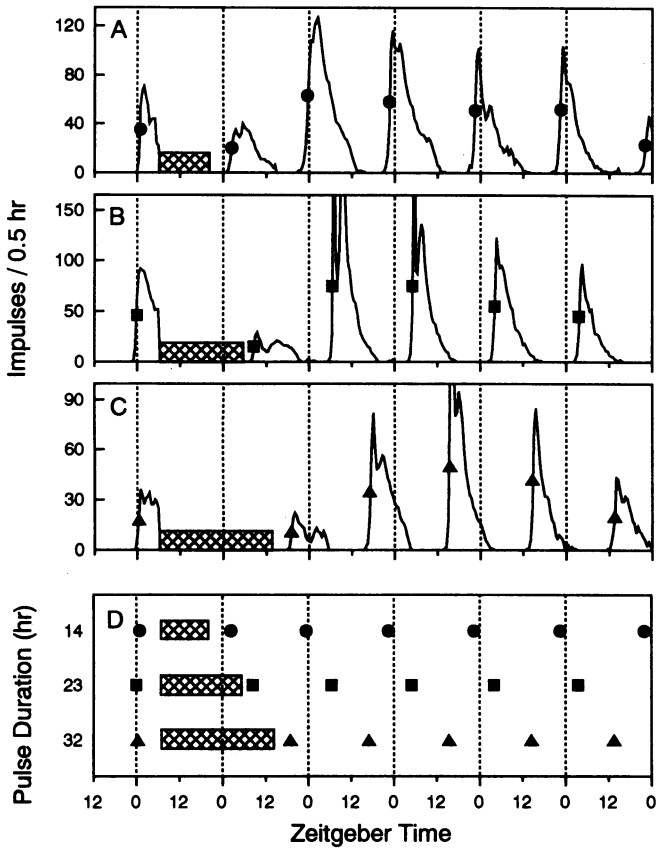

FIG. 2. Phase shifts in response to long inhibitor pulses and the experimental paradigm. In all plots the horizontal axis is clock time and begins at ZT 12 (subjective dusk relative to the previous light dark cycle), when the eyes are placed *in vitro* into constant darkness. (*A–C*) Records of the frequency of spontaneous impulse activity recorded from the optic nerves over time. In each record a pulse of 10 mM CHX begins at ZT 6 during the first cycle of activity as indicated by the cross-hatched bar on the time axis. The phase reference points are calculated as the time of the half-maximal impulse frequency at the beginning of a cycle of activity, and they are indicated on the rising phase of each cycle of activity. In *A*, the phase reference points fall close to subjective dawn (the vertical dotted line) on each cycle, as is typical of untreated control eyes, suggesting that the treatment in this instance had no effect on phase. The phase reference points and the pulse times for all three records are summarized in *D*, where pulse duration is plotted versus ZT, and the pattern of subsequent phase reference points with respect to the timing of the pulses is evident.

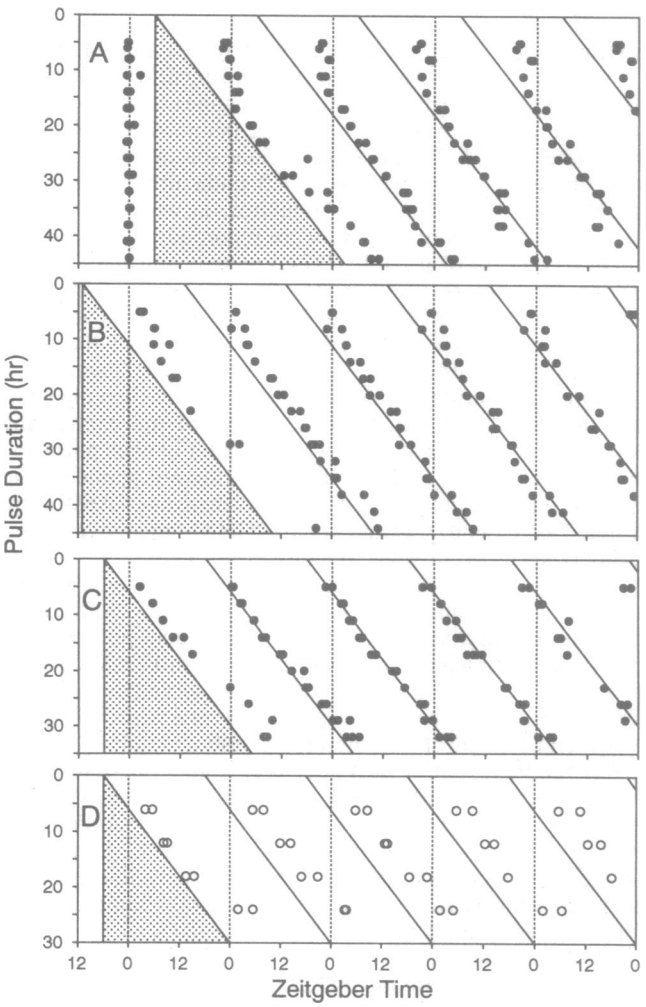

FIG. 3. Analysis of phase after inhibitor treatments of variable duration and starting time. Pulse duration is plotted versus ZT as described for Fig. 2, except that the pulse duration is now indicated by the shaded triangular region. In *A* 10 mM CHX pulses of various durations are applied starting at ZT 6, in *B* starting at ZT 13 (17 hr earlier), and in *C* starting at ZT 18. In *D* 2 μM anisomycin pulses of four different durations are applied starting at ZT 18. Subsequent projected dawns (ZT 0) are indicated by vertical dotted lines, and phase reference points appearing at this phase suggest that the treatment did not perturb the pacemaker. This is apparent in *A* for pulse durations of 14 hr and less and in *B* for durations of 8 hr or less; in both cases these pulse treatments do not extend past ZT 0. The diagonal lines represent 24-hr intervals following the ending time of the treatment. Phase reference points aligning with the projected ending time of the treatment suggest that the inhibitor is strictly determining the subsequent phase of the circadian rhythm. This appears to be the case for pulses extending through ZT 0 in *A* and *B* and for all pulses in *C* and *D*.

hr after CHX washout at ZT 11; protein synthesis was inhibited by 85%, 53%, 63%, and 46%, respectively.

DISCUSSION

Concentration-dependent lengthening of period in the presence of continuous inhibitor treatments can be taken as evidence that the rate of the inhibited process is important to the pacemaker. If the period lengthening is substantial and does not appear to saturate with increasing levels of the treatment, it is possible that the inhibited process may be a critical requirement for pacemaker motion. Although the observation that period is lengthened in the presence of protein synthesis inhibitors is not novel, the period lengthening data reported in this study are valuable in several respects. Surprisingly, only three previous studies have reported positive results with period lengthening to translation inhibitors: anisomycin lengthened the period to 31 hr or longer in *Aplysia* (28), and cycloheximide lengthened period up to 36 hr in *Euglena* (29) and up to 33 hr in *Chlamydomonas* (30). Possibly, expression of circadian rhythmicity is not observable with constant inhibitor treatment at higher concentrations where period lengthening occurs (16, 38, 55). Therefore, the period lengthening observed in *Bulla* adds support to the view that translation inhibitors do lengthen period, and it also demonstrates that CHX can lengthen period in a multicellular organism. Furthermore, the period lengths obtained in *Bulla* exceed those reported in previous studies, especially for CHX, suggesting that the molluscan ocular preparation is well suited to such inhibitor studies. Finally, the long periods observed, together with the exponential lengthening with concentration, strengthen the hypothesis that high concentrations stop the pacemaker.

The generation of phase shifts to inhibitor pulses is evidence that the process inhibited has access to the pacemaker

mechanism, and it may be evidence that the process is involved in the pacemaker mechanism. A large body of data has been generated by using translation inhibitors applied in pulses to generate phase shifts. Phase shifts and/or full phase response curves have been obtained for cycloheximide pulses in *Acetabularia* (35, 43), *Neurospora* (36, 40, 43), *Gonyaulax* (38, 42, 45–47), *Phaseolus* (39), *Aplysia* (31, 34), and hamster (33, 41) and for anisomycin pulses in *Gonyaulax* (37, 38, 42, 45, 47), *Aplysia* (27, 28, 32, 34), chick pineal (49), and hamster (33, 48). On the basis of these data, it has been suggested that translation is a requirement for the circadian pacemaker mechanism (24, 27, 28, 30–32, 35, 36, 40, 43, 44, 47, 49, 50), or more specifically, that translation is a phase-dependent requirement (24, 28, 32, 35, 36, 40, 43, 44, 49). Furthermore, models describing the circadian pacemaker mechanism which include translation have been extensively described and discussed (14, 23–26, 28, 39, 40, 43).

The experimental paradigm in this study, a set of long pulse durations, is similar to traditional phase shifting experiments in that it uses a high inhibitor concentration in a restricted pulse. However, it extends the traditional phase-shifting paradigm to effectively demonstrate a nonsaturating "phase shift" to pulse duration. The subsequent phase of the pacemaker is therefore determined solely by pulse duration and the pacemaker does not continue its motion until the inhibitor pulse is terminated. Previously, Lotshaw and Jacklet (32), using 3-, 6-, and 9-hr pulses of anisomycin and puromycin in *Aplysia* starting at CT 21, observed an increase in phase shift which was linear with pulse duration and suggested that the pacemaker was being stopped. However, they did not extend pulse duration beyond 9 hr and throughout a circadian cycle, conditions that are necessary to demonstrate that the phase shift is truly not saturating with pulse duration.

The paradigm used in this study can also be thought of as an extension of the period-lengthening experiment. However, the period during the treatment is calculated retrospectively, and the concentration is chosen to yield effectively "infinite period" or a stopping of pacemaker motion. Previously, Feldman (29) used this concept in demonstrating the reversibility of CHX period lengthening. In this study a moderate period-lengthening concentration was applied for durations from 24 to 72 hr, and the subsequent phase after the pulse was consistent with the expected period lengthening occurring during the pulse.

The data in this study also address the issue of phase dependence of protein synthesis. Given that subsequent phase was not perturbed by inhibitor pulses until the pulse spanned subjective dawn (Fig. 3 *A* and *B*), and that the subsequent phase was determined solely by the termination time of the pulse for all CHX and anisomycin pulses which began as late as ZT 18 (Fig. 3 *C* and *D*), the critical phase must lie in a region within the late subjective night. The difference in starting times for different pulses in this study (3 hr apart) is too large to permit sufficient resolution for an exact determination of the time of the critical phase for protein synthesis. However, phase-shifting studies in *Acetabularia* (35), *Neurospora* (36), *Phaseolus* (39), *Aplysia* (27, 28, 31, 32, 34), chick pineal (49), and hamster (33, 41, 48) have also suggested that the critical phase is in the late subjective night since phase delays appear for pulses of inhibitor beginning in the mid to late subjective night. In *Aplysia*, critical protein synthesis occurs from CT 20 to CT 8 based on phase shifts to CHX pulses (34) and from CT 18 to CT 2 based on phase shifts to anisomycin and puromycin pulses (32).

The prediction that high concentrations of protein synthesis inhibitor should be capable of stopping the pacemaker at a unique phase derives naturally from the hypothesis suggested by numerous investigators, based on phase-shifting and period-lengthening data, that protein synthesis is a phase-dependent requirement for the circadian pacemaker. This study demonstrates this phase-dependent requirement and therefore serves as a confirmation of this widely held concept. Without protein synthesis at a critical phase of the circadian oscillation near subjective dawn, pacemaker motion is stopped. This confirmation is directly attributable to the application of the experimental paradigm used; however, it has been possible only because of the surprising tolerance of the molluscan eye preparation to the long durations of high inhibitor concentration applied.

To address the question as to whether the data with long CHX pulses may be a result of some action of CHX other than the inhibition of protein synthesis (33, 39–41, 44, 46), we have applied anisomycin, another translation inhibitor. Anisomycin was also capable of stopping the pacemaker for pulses beginning at ZT 18; however, the resultant phases following treatment were more delayed compared with the same pulse treatments of CHX (Fig. 3 *C* and *D*). The delays observed are possibly a result of the slow recovery time reported for the wash-out of anisomycin (32, 34). Eskin (34) found in a comparative study that CHX recovery time in *Aplysia* was much faster than that for anisomycin. Therefore, after long pulses of anisomycin, one would expect that the anisomycin remaining after wash-out would lengthen the period and result in a delayed subsequent phase of the rhythm as compared with the same duration pulse of CHX. The assumption that protein synthesis inhibition accounts for the observations is further supported by the result that the inactive analogue of anisomycin, deacetylanisomycin (38, 50), was ineffective at perturbing the phase of the rhythm. Finally, labeled methionine incorporation experiments in this study, which are a measure of protein synthesis inhibition, have indicated that CHX is effective at inhibiting protein synthesis over the time course of the long pulses used in this study. No appreciable degradation of inhibition was apparent over 17 hr of inhibitor application. Incorporation studies in *Aplysia* eye, using CHX and/or anisomycin, are also consistent with the effective inhibition of protein synthesis with these compounds (31, 32, 34, 50). The recovery of inhibition after 17 hr of CHX was slow, as compared with recovery reported in *Aplysia* eye after short pulses of CHX (34), since protein synthesis inhibition was still inhibited by 46% as long as 12 hr after the wash-out of the inhibitor. This observation may account for the low amplitude and distorted waveform often observed on the first cycle of compound action potential activity following long pulses (Fig. 2 *A–C*).

Application of constant light has also been reported as a treatment which appears to stop pacemaker motion (53, 54). However, recent studies have raised the alternative interpretation that the underlying pacemaker actually continues its motion in constant light and that the transition from constant light to dark instantaneously "resets" the pacemaker to a specific phase (56–59). This interpretation is plausible for *Drosophila*, where the free-running period can be lengthened modestly (although exponentially with intensity), to about 26 hr, in constant light. Resetting is clearly the only interpretation for the effects of dim light pulses. The *Drosophila* pacemaker continues its motion during constant dim light with a circadian period of less than 25 hr, and yet a pulse of light at this intensity is capable of fully resetting the pacemaker (57). The resetting interpretation is not compelling for the *Bulla* eye, however, where long noncircadian periodicities are observed in the presence of lower concentrations of protein synthesis inhibitors. Applying the resetting interpretation in this case would suggest the implausible result that circadian periodicities are somehow present at high protein synthesis inhibitor concentrations, even though lower concentrations yield substantial period lengthening.

Although the action of CHX and anisomycin may be by their inhibition of protein synthesis, "whether the effects of inhibitors of protein synthesis on the circadian oscillator are

direct or indirect is not clear" (49). For example, protein synthesis inhibitors may act via an "input pathway" to the pacemaker (33, 34), or they may affect one or more processes in the cell which are more directly involved with the pacemaker mechanism (29, 30, 39). Another limitation of all studies based solely on application of protein synthesis inhibitors is that it is not possible to distinguish between protein synthesis as a central pacemaker variable and as a nonrhythmic supportive process (7, 8, 35, 40, 43). As aptly stated by Dunlap and Feldman (40):

> There are two general hypotheses. In one. . ., the periodic synthesis of one or more proteins is a required step in the cycle. Here, the process of synthesizing a protein or proteins is an indispensable part of the clock, and drug-induced resetting is due to interference with this process. . . . the kinetics of the clock reflect in some way the short-term rate of translation. A second type of model . . . suggests that proteins are needed to carry out an enzymatic reaction or transport process required for the clock, but synthesis *per se* of these proteins is not a step in the oscillation. Here, the short-term rate of protein synthesis is unimportant so long as a sufficient amount of the critical enzymatic activity has been made by the time it is needed. The critical proteins here are viewed as having a high turnover rate and, therefore, must be resynthesized each day during (but not necessarily only during) the time corresponding to the sensitive phase of the cycle.

Studies examining phase shifts to translation inhibitors in combination with temperature changes (42, 43, 45, 47), or to inhibitor-insensitive mutants (40), have provided evidence favoring the argument that translation is not an integral part of the clock mechanism but simply a requirement.

The experimental paradigm employed in this study, although not a novel one, is particularly suited for pacemaker analysis. In circumstances where the effects of a treatment abolish or obscure the overt circadian rhythm, the long-pulse paradigm can potentially serve as a useful technique for assessing the behavior of the underlying circadian pacemaker during the treatment. Previously, Pittendrigh (60) has demonstrated that the *Drosophila* circadian pacemaker appears to stop in a phase-dependent manner during exposure to oxygen-free nitrogen atmosphere. In *Bulla*, the circadian pacemaker appears to stop in a phase-dependent manner at low pH (61) and in the presence of transcription inhibitor (19). The results of period-changing and phase-shifting experiments can lead to the suggestion that a process plays an important role in the pacemaker system. The experimental paradigm used in the present study is a valuable adjunct to these techniques in providing direct evidence that a process not only influences free-running period or pacemaker phase but also is required for the pacemaker to complete a full cycle. Without the contributing process, the pacemaker stops, possibly in a phase-dependent manner, and motion resumes only when the process is restored.

We thank Dr. Barbara Bogart for extensive efforts on, and Dr. Arnold Eskin for assistance with, the trichloroacetic acid precipitation procedure. This work was supported by National Institutes of Health Grant NS15264 to G.D.B.

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