# *LENGTHENING THE PERIOD OF A BIOLOGICAL CLOCK IN EUGLENA BY CYCLOHEXIMIDE, AN INHIBITOR OF PROTEIN SYNTHESIS**

By Jerry F. Feldman†

DEPARTMENT OF BIOLOGY, PRINCETON UNIVERSITY, PRINCETON, NEW JERSEY

*Communicated by Colin S. Pittendrigh, February 13, 1967*

This paper reports a lengthening of the period of a circadian rhythm (a biological clock) in *Euglena* by cycloheximide, an inhibitor of protein synthesis. Circadian rhythms have now been reported in organisms representing all of the major taxonomic groups except bacteria and blue-green algae. In many cases these rhythms have been shown to be under the control of an endogenous internal timing mechanism, i.e., a biological clock—rhythmicity persists in continuous darkness and at constant temperature with a period which closely approximates the 24-hour period of the earth's rotation. In recent years these circadian rhythms have aroused increasing interest because of the realization that at all levels of biological organization many steady-state conditions actually represent oscillatory steady states.

Despite many attempts, little has been learned about the biochemical nature of the basic oscillator involved in circadian rhythms. Biochemical oscillations have been detected and measured, but in no case has it been shown that the oscillation under study is the clock itself or part of the clock and not simply a periodicity caused by the clock. The clock in several organisms seems to require aerobic metabolism,[1-3] but in general it shows remarkable insensitivity to most metabolic inhibitors.[4] Although heavy water[5, 6] and ethanol[6,7] do affect the clock, these experiments give little information about what processes in the cell are being altered.

There have been several reports that actinomycin D, an inhibitor of DNA-dependent RNA synthesis, causes a loss of rhythmicity.[8, 9] In addition Strumwasser[10] has shown that a pulse of this drug induces a phase shift in the succeeding peak of spike output rate of an isolated ganglion, but technical limitations prevented the measurement of more than one cycle after the pulse. Therefore, since there were no results showing a *steady-state* change in the phase or period of the oscillation stable for at least several cycles, a direct effect on the clock by inhibitors of macromolecular synthesis has not previously been demonstrated.

*Methods.—Cell culture: Euglena gracilis*, strain *Z*, obtained from Dr. S. Hutner, was grown in an inorganic liquid medium modified only slightly from that of Cramer and Myers.[11] Primary liquid cultures were inoculated with cells from an agar slant and were maintained in a light-dark cycle of 12 hours of light and 12 of dark (referred to hereafter as LD 12:12) in constant-temperature cabinets at 25°C and allowed to grow to late logarithmic phase. Secondary liquid cultures were started from primary cultures and maintained under identical conditions.

For rhythm studies a secondary culture in late logarithmic phase was centrifuged and the pellet was resuspended in fresh inorganic medium at a density of about $1 \times 10^6$ cells/ml. This culture was returned to the same growth cabinet for at least two days before use in an experiment. Previous experiments[5, 12] have shown that centrifugation has no effect on the clock.

*Measurement of phototaxis:* The clock was assayed by measuring the circadian rhythm of phototactic response of a culture of cells. The equipment and procedure

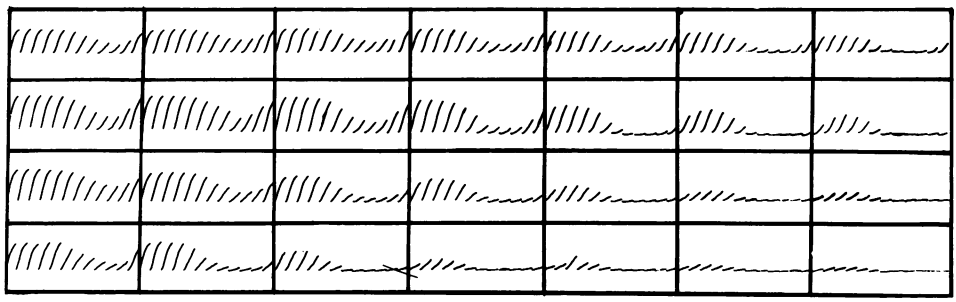


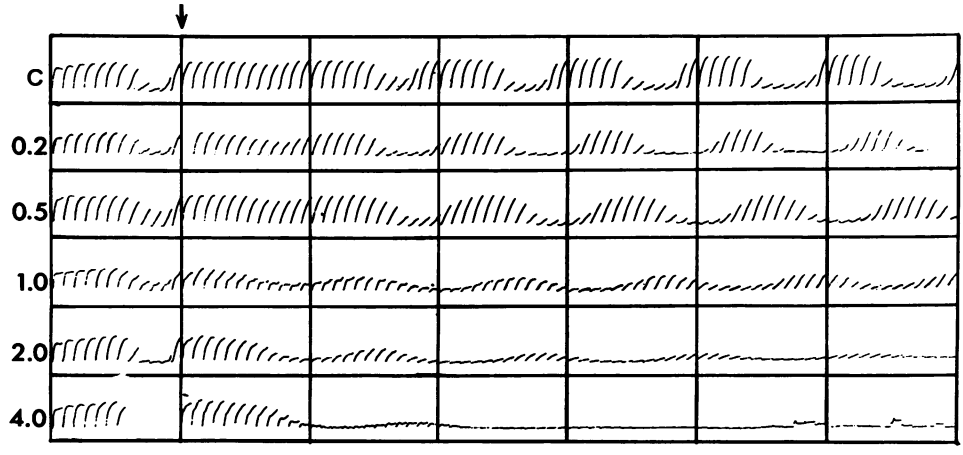
FIG. 1.—The free-running circadian rhythm of phototactic response of *Euglena gracilis*. Photograph of original records of four replicate cultures which had been in LD 12:12 (lights on at 1000 EST) and then transferred to continuous dark (DD). Only the cycles in DD are shown. Vertical lines, 24 hr apart, indicate 1000 EST. Constant temperature for all experiments is 25°C.

are similar to that previously described.[13, 14] Ten identical test chambers were used and recorded simultaneously. Ten ml of culture are contained in a 30-ml plastic Falcon tissue culture flask, which is placed on a platform surrounded by a water jacket inside a light-tight cabinet. Each cabinet contains two 4-watt fluorescent lamps (referred to as "day lamps") located directly above the culture which provide illumination for a day-night cycle (intensity = 100 foot-candles). Water at constant temperature is circulated through the water jacket. Below the culture is a "test lamp" for assaying the phototactic response, a 100-watt air-cooled projection lamp whose beam, after passing through heat-absorbing glass, is focused by a double convex lens and right-angle prism at the level of the culture. The diameter of the test beam as it enters the culture is 4 mm and the cross-sectional area of the beam represents about 5 per cent of the surface area of the culture. After passing through the culture, the beam from the test lamp falls on a photocell (Hoffman Electronics, model 2A). An adjustable fraction of the voltage output of the photocell is recorded on a Rustrak strip chart recorder, model 88. The test lamp thus serves two purposes: it acts as an attracting stimulus which elicits the phototactic response, and the transmitted light serves as a measure of the number of cells responding to the test light. In operation the test lamp is turned on for 24 minutes once every two hours, and the recorder operates only when the test lamp is on; records thus consist of 24-minute responses at two-hour intervals. The day lamps are always turned off when the test lamp is on, to prevent interference with the phototactic response. The test lamp cycle does not entrain the rhythm.

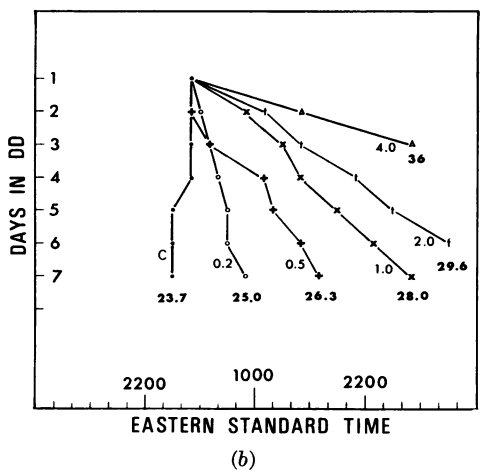
*Measurement of protein synthesis:* Protein synthesis was measured by adding 1 $\mu$c of $C^{14}$-phenylalanine to 10 ml of culture. Aliquots were precipitated with trichloroacetic acid, the precipitates collected on Whatman glass filters, type GF/A, and the filters counted in a Packard Tri-Carb scintillation counter.

*Materials:* Cycloheximide (actidione) was a gift of the Upjohn Co., Kalamazoo, Michigan. 3-$C^{14}$-DL-phenylalanine (4.5 $\mu$c/$\mu$mole) was purchased from New England Nuclear Corp.

*Results.—DD free-run:* The reproducibility of the system is demonstrated in Figure 1, which shows the behavior of four replicate cultures which were recorded in LD 12:12 for two cycles and then transferred to continuous dark (DD) and al-




(a)




(b)

FIG. 2.—Period lengthening of the free-running rhythm by cycloheximide. (*a*) Photograph of original records of phototactic response of cultures to which cycloheximide was added at subjective dawn of the second day of DD (indicated by the arrow). The numbers at the left are the concentrations (in $\mu$g/ml) of drug added to each culture; $C$ = control, no drug added. Vertical lines, 24 hr apart, indicate 1000 EST. (*b*) The clock hours at which successive minima occurred are plotted for successive days for each of the cultures shown in (*a*). The numbers next to the data lines indicate the concentrations (in $\mu$g/ml) of drug added; $C$ = control. The numbers below the data lines indicate the period length (in hr). There is some slight variability (up to about an hour) in the exact amount of lengthening of the period induced by the highest concentrations of the drug (2 and 4 $\mu$g/ml).

lowed to "free-run." Rhythmicity persists in DD for at least seven cycles. Although the wave form and amplitude of the rhythm vary from culture to culture, the phase of the rhythm is quite reproducible and can be determined most easily by the minimum point of the oscillation; the period is then the time interval between successive minima. If there is a clearly defined maximum point, this may also be used as a phase indicator. In cultures where both minima and maxima can be clearly defined, the results are identical irrespective of which is used as a phase reference point. The period of the free-running rhythm shown here is about 23.7 hours, a value similar to that in previous reports.[12, 13]

*Lengthening of the period by cycloheximide:* Figures 2*a* and *b* show the lengthening of the free-running rhythm induced by cycloheximide. To each of five experimental cultures cycloheximide was added at different concentrations (0.2–4.0 $\mu$g/ml) at subjective dawn (the time when the lights would have been turned on if the preceding light-dark cycle had been continued) of the second day of DD. Each of the treated cultures had a steady-state period greater than that of the control, stable for at least five cycles (except in the case of the highest concentration). There is a

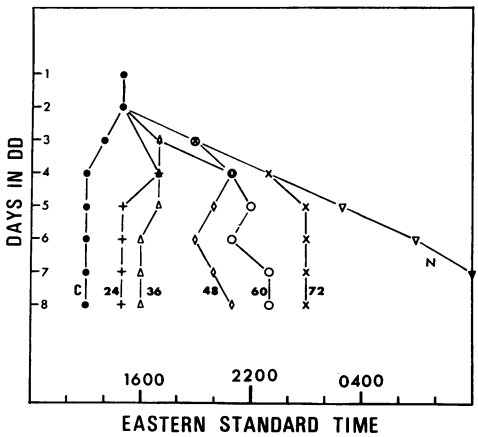



FIG. 3.—Reversibility of period lengthening induced by cycloheximide (2 μg/ml). The drug was added at subjective dawn of the second day of DD and washed out after varying lengths of time. The numbers next to the data lines indicate the length of the pulse (in hr); $C$ = control, no drug added; $N$ = culture from which the drug was never removed. The points represent the clock hours at which successive maxima occurred on successive days. Some overlapping symbols have been omitted for clarity.

correlation between the concentration of the drug and the lengthening of the period—the higher the concentration, the longer the period.

*Reversibility of the period lengthening:* An experiment demonstrating the reversibility of the period lengthening caused by cycloheximide is shown in Figure 3. Two μg/ml of cycloheximide were added to each of six experimental cultures; a control culture to which no drug was added is also shown. After a specified length of time (24–72 hours), each of five treated cultures was filtered with very gentle suction onto a sterile Millipore filter, type RA, washed with fresh inorganic medium, and resuspended in 10 ml of fresh inorganic medium. Recovery of cells from the filter was greater than 90 per cent. As long as the drug was present all of the cultures showed a period significantly longer than 24 hours (in this case, about 28 hours), but as soon as the drug was removed the cultures returned to their normal 24-hour period and remained out of phase with respect to the control.

*Phase-shifting of the rhythm by four-hour light pulses:* As has been shown previously,[14] the *Euglena* rhythm can be reset by light pulses. The amount of the phase shift depends on the phase of the cycle at which the pulse is given. Figure 4 is a "phase-response" curve for four-hour light pulses for *Euglena*. There is no phase shift if the pulse is given during the early subjective day; greater and greater delays result with progression from the late subjective day into the middle of the subjective night; and there is a short time during the late subjective night when slight advances occur. It is believed that this daily fluctuation in the sensitivity to light is a property of the underlying endogenous oscillation, i.e. of the clock itself.

*Phase-shifting by light pulses after cycloheximide addition:* With the use of this phase response curve, an experiment was performed to test whether the change in period of the overt oscillation of phototactic response truly reflected a shifting of the underlying (light-sensitive) clock. Cultures 1–5 (Table 1 and Fig. 5) received no drug; cultures 6–10 received 2 μg/ml. of cycloheximide beginning at subjective dawn on day 2 of DD. On day 4 of DD (two full days after addition of the drug), four-hour light pulses were given as follows: cultures 2, 3, 7, and 8—1000 to 1400 EST (the previous LD 12:12 cycle had lights on from 1000–2200 EST; thus, this pulse fell for controls 2 and 3 at their subjective dawn, referred to

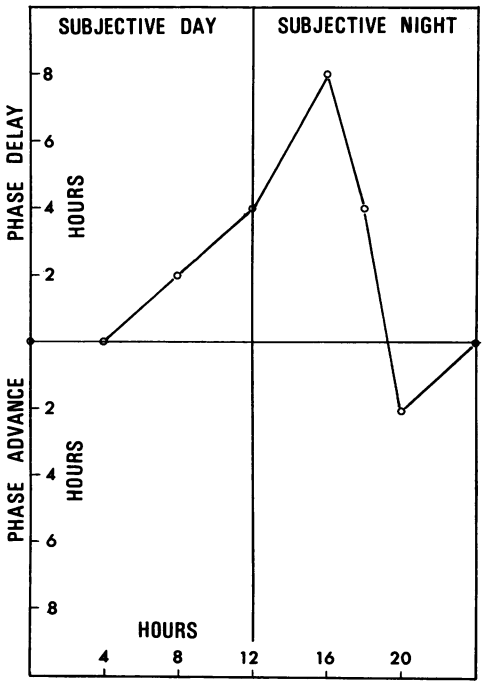



FIG. 4.—Phase-response curve for *Euglena* for four-hour light pulses given on the second day of DD. Points indicate the time of the beginning of the light pulse. Each point is based on the results of at least four independent experiments (except hour 18, for which there is only one experiment). Abscissa = circadian time. Circadian time 0 (subjective dawn) is the time when the lights would have come on on the first day of DD if the previous light-dark cycle had been continued. The first half of the circadian cycle is called the subjective day and the second half is called the subjective night.

as "circadian time" 0–4); cultures 4, 5, 9, and 10—0200 to 0600 EST (for controls 4 and 5, circadian time 16–20). Thus, all light pulses were given in duplicate to treated and untreated cultures. From Figure 4 it can be predicted that those controls (2 and 3) receiving the pulse in the early subjective day should show no phase shift and those controls (4 and 5) receiving the pulse in the middle of the night should show a large delay. Table 1 shows that this was the case.

If the overt rhythmicity reflected the phase position of the clock, then at the point when the signals were given (namely, two days after addition of the drug), the clock in the drug-treated cultures should lag the untreated ones by about eight–ten hours. It this were true, the light pulse to cultures 7 and 8 (1000–1400 EST) should effect a large delay and the pulse to 9 and 10 (0200–0600 EST) should

TABLE 1

PHASE SHIFTS INDUCED BY 4-HOUR LIGHT PULSES AFTER ADDITION OF CYCLOHEXIMIDE

| Culture | Time of Light Pulse: EST | Time of Light Pulse: Predicted circadian time | Predicted phase shift | Experimental phase shift |
|---|---|---|---|---|
| *Controls* (no cycloheximide) | | | | |
| 1 | — | — | — | — |
| 2 | 1000–1400 | 0–4 | 0 | 0 |
| 3 | 1000–1400 | 0–4 | 0 | 0 |
| 4 | 0200–0600 | 16–20 | 8-hr delay | 8-hr delay |
| 5 | 0200–0600 | 16–20 | 8-hr delay | 8-hr delay |
| *Experimentals* (2 μg/ml cycloheximide) | | | | |
| 6 | — | — | — | — |
| 7 | 1000–1400 | 16–20 | 8-hr delay | 8-hr delay |
| 8 | 1000–1400 | 16–20 | 8-hr delay | 8-hr delay |
| 9 | 0200–0600 | 6–10 | 0–2 hr delay | 0 |
| 10 | 0200–0600 | 6–10 | 0–2 hr delay | 0 |

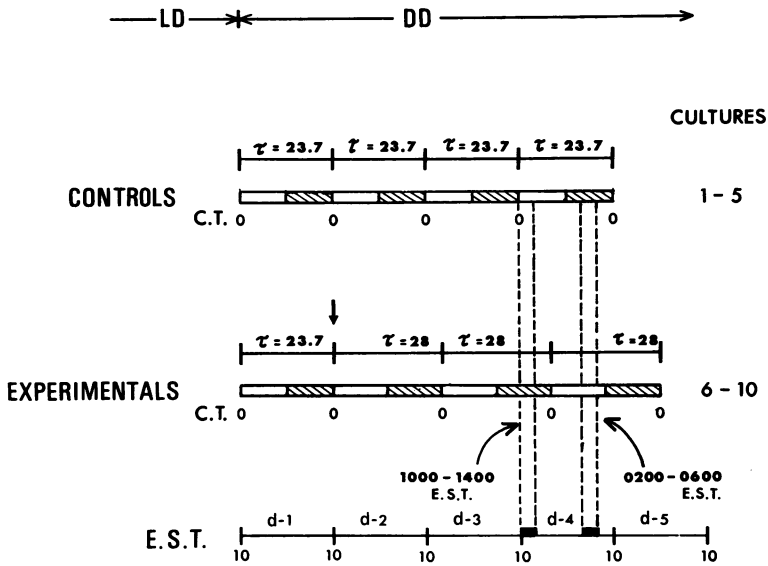



FIG. 5.—Resetting the rhythm with 4-hr light pulses after cycloheximide addition in DD. The bottom line indicates Eastern Standard Time (in the previous LD cycle, the lights were on from 1000 to 2200 EST). *d-1* is the first day of DD, *d-2*, the second, etc. The bar labeled "controls" indicates times of subjective day and night of the controls. Open bars = subjective day; hatched bars = subjective night. (Open and hatched bars do *not* represent a light-dark cycle; all cultures are in DD.) The bar labeled "Experimentals" indicates times of subjective day and night of the experimental cultures to which cycloheximide was added at subjective dawn of the second day of DD (indicated by the arrow). The solid blocks on the EST scale on day 4 (*d-4*) indicate the times at which each of the two 4-hr light pulses were given to separate cultures (each culture received only one of the two light pulses). Note that the pulse from 1000–1400 EST hits the control in the early subjective day and the experimentals in the middle of the subjective night, whereas the pulse from 0200 to 0600 EST hits the controls in the middle of the subjective night and the experimentals in the middle of the subjective day. $\tau$ = period length (in hours).

cause little or no phase shift. The data in Table 1 show that this, too, is the case and therefore are consistent with the hypothesis that the clock itself has been affected by the drug.

*Inhibition of protein synthesis by cycloheximide:* Cycloheximide has been reported to be a potent inhibitor of protein synthesis in mammalian systems,[15] yeast,[16] and *Euglena*.[17] However, it was important to determine its effectiveness at the concentrations used in these experiments and under conditions identical to those under which the phototactic response was assayed. Therefore, $C^{14}$–phenylalanine was added to the 10-ml cultures in the recording apparatus with and without cycloheximide and the rate of protein synthesis determined as described above. Isotope pulses were given for several days after the addition of the drug. The results obtained on the second day after addition of the drug are shown in Figure 6. In the range of concentrations used cycloheximide did not inhibit phenylalanine incorporation 100 per cent, but with increasing concentration there was an increasing inhibition.

*Discussion.*—The results presented here show that cycloheximide causes an in-

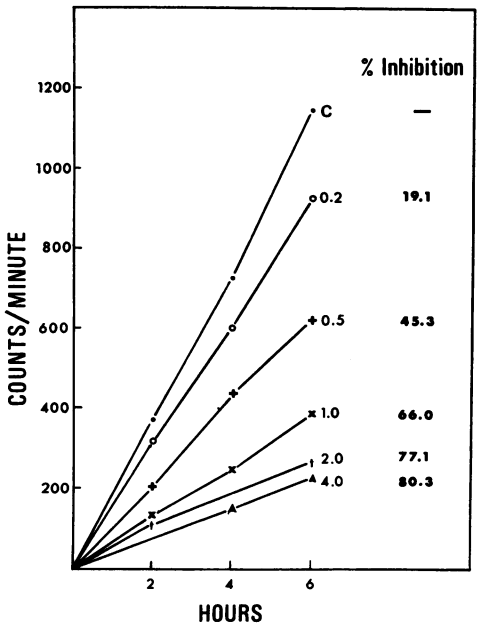



FIG. 6.—Inhibition of protein synthesis by cycloheximide. The numbers next to the data lines indicate the concentration (in $\mu$g/ml) of drug. The numbers at the right of the figure indicate the % inhibition of protein synthesis relative to the control (*C*). Hour 0 is the time of addition of $C^{14}$-phenylalanine and is 24 hr after addition of cycloheximide.

crease in the period length of the circadian rhythm of phototaxis in *Euglena*, which is a function of the concentration of the drug. Cycloheximide also inhibits protein synthesis under conditions identical to those used for assaying the phototaxis rhythm. The experiments in Figures 2 and 3 demonstrate a clear, reversible change in the period of the oscillation which is stable for at least five or six cycles. Such a steady-state lengthening of the period indicates that the clock itself has been "slowed down" by the drug.

The daily fluctuation in the sensitivity of the oscillation to be phase-shifted by light, represented by the phase-response curve in Figure 4, reflects the phase position of the clock. Therefore, the experiment summarized in Table 1 demonstrating that this "light-sensitive oscillation" is also shifted by cycloheximide is consistent with the inference that the clock has been slowed down.

It would be gratifying to be able to conclude that protein synthesis is an integral part of the biological clock of *Euglena*. However, such a conclusion is subject to several qualifications at this time. (1) It is not certain that the only primary effect of cycloheximide is to inhibit protein synthesis. Therefore, it will be necessary to test other inhibitors of protein synthesis to see if they, too, induce period changes in the rhythm. Unfortunately, permeability problems hinder this type of study in *Euglena*.[18] (2) Secondary metabolic effects, such as alteration of pools of metabolic intermediates and/or the levels of energy pools, might result from inhibition of protein synthesis. The observed effects on the clock might result from any of these or other secondary effects. Another type of experimental approach will be necessary to overcome this reservation.

In connection with such possible secondary effects one must note that although there is a correlation of increasing drug concentration and increasing inhibition of protein synthesis with increasing period length, the inhibitor has a much greater effect on protein synthesis than on the period of the rhythm. In one sense this

absence of an equivalent effect of the inhibitor on protein synthesis and period length might suggest that the alteration of the clock may not be due directly to the inhibition of protein synthesis. However, it must also be remembered that temperature, which has large effects on growth rate and hence on the over-all rates of synthetic processes of the cell, has a very small effect on the period length of the rhythm, i.e., the period length is temperature-compensated. This is most clearly seen in those systems in which both the growth rate and the period of a circadian rhythm have been measured simultaneously at several temperatures.[19, 20] Since the temperature-compensation mechanism probably involves machinery to compensate for changes in the rates of some metabolic processes, the small lengthening of the period which accompanies a large inhibition of protein synthesis can be understood by assuming that part of the temperature-compensation mechanism includes a device to compensate for changes in the rates of protein synthesis. Our inability to prove at this time a *direct* involvement of protein synthesis in the operation of the clock, however, does not affect the previous conclusion that the light-sensitive driving oscillator has in fact been slowed down by cycloheximide.

*Summary.*—The period length of the circadian rhythm of phototactic response in *Euglena* is increased by cycloheximide, an inhibitor of protein synthesis. Furthermore, the effects of the drug are on the biological clock mechanism itself rather than on some parameter controlled by the clock or on some "coupling" mechanism between the clock and the parameter, as confirmed by experiments involving phase-shifting of the oscillation by light. The possible involvement of protein synthesis in the operation of the clock is discussed.

The author wishes to thank Drs. C. S. Pittendrigh, V. G. Bruce, A. B. Pardee, and G. K. Russell for their helpful suggestions and criticisms during the course of this work and in the preparation of this manuscript.

* This work was performed under contract Nonr 1858(28) between Princeton University and the Office of Naval Research.

† National Science Foundation graduate fellow.

[1] Ball, N. G., and I. J. Dyke, *J. Exptl. Botany*, **8**, 323 (1957).

[2] Pittendrigh, C. S., these PROCEEDINGS, **40**, 1018 (1954).

[3] Wilkins, M. B., *Planta*, **72**, 66 (1967).

[4] Hastings, J. W., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 25 (1960), p. 131.

[5] Bruce, V. G., and C. S. Pittendrigh, *J. Cellular Comp. Physiol.*, **56**, 25 (1960).

[6] Bünning, E., and J. Baltes, *Naturwissenschaften*, **50**, 622 (1963).

[7] Keller, S., *Z. Botan.*, **48**, 32 (1960).

[8] Karakashian, M., and J. W. Hastings, these PROCEEDINGS, **48**, 2130 (1962).

[9] Vanden Driessche, T., *Biochim. Biophys. Acta*, **126**, 456 (1966).

[10] Strumwasser, F., in *Circadian Clocks*, ed. J. Aschoff (Amsterdam: North-Holland Pub. Co., 1965), p. 442.

[11] Cramer, M., and J. Myers, *Arch. Mikrobiol.*, **17**, 384 (1952).

[12] Brinkmann, K., *Planta*, **70**, 344 (1966).

[13] Bruce, V. G., and C. S. Pittendrigh, these PROCEEDINGS, **42**, 676 (1956).

[14] Bruce, V. G., and C. S. Pittendrigh, *Am. Naturalist*, **92**, 295 (1958).

[15] Wettstein, F. O., H. Noll, and S. Penman, *Biochim. Biophys. Acta*, **87**, 525 (1964).

[16] Kerridge, D., *J. Gen. Microbiol.*, **19**, 497 (1958).

[17] Kirk, J. T. O., and R. L. Allen, *Biochem. Biophys. Res. Commun.*, **21**, 523 (1965).

[18] Hutner, S. H., and L. Provasoli, *Ann. Rev. Plant Physiol.*, **15**, 37 (1964).

[19] Sweeney, B. M., and J. W. Hastings, *J. Protozool.*, **5**, 217 (1958).

[20] Sargent, M. L., W. R. Briggs, and D. O. Woodward, *Plant Physiol.*, **41**, 1343 (1966).