Proc. Natl. Acad. Sci. USA
Vol. 73, No. 9, pp. 3216–3219, September 1976
Cell Biology

Temperature dependence of cycloheximide-sensitive phase of circadian cycle in *Acetabularia mediterranea*

(biological clock/circadian rhythm/protein synthesis/80S ribosomes/photosynthesis rhythm)

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Communicated by Lawrence Bogorad, June 25, 1976

ABSTRACT **The biochemical nature of the circadian regulatory system that controls many cellular activities is still unclear. Recent results obtained from the application of protein synthesis inhibitors to individual *Acetabularia* cells expressing circadian rhythms of photosynthesis indicate that some protein(s) must be synthesized on 80S ribosomes during a discrete part of each cycle to insure correct time-keeping. A comparative study of the effects of brief cycloheximide treatments on cells investigated at different temperatures has revealed that the phase of cycloheximide sensitivity is 4–6 hr longer and occurs about 8 hr later in the cycle when cells are kept at 20° rather than 25°. Temperature is known to influence the function of the circadian regulatory system in *Acetabularia*, but the effect on frequency is small ($Q_{10} \cong 0.8$) due to the existence of a temperature-compensating feature. The large effects of temperature observed here thus favor the interpretation that protein synthesis on 80S ribosomes, while providing an essential component of the circadian timing mechanism, does not itself generate the period of the photosynthesis rhythm.**

A variety of cellular activities are regulated by a type of metabolic "clock" which measures time autonomously in intervals of about a day (= circadian) and which can be entrained to other frequencies by environmental cycles of light or temperature (1). The biochemical nature of this circadian regulatory system is still unclear, largely due to the difficulty of distinguishing between its component reactions and the processes which they control. Treatments that alter the period or the phase of a free-running circadian rhythm presumably act directly on the regulatory system, however, and this has been the rationale for the application of a variety of metabolic inhibitors to rhythmic systems (2) as well as for the detection of mutant clock systems (3–5).

There are conflicting reports about the effects of inhibitors of protein synthesis on circadian systems. Karakashian and Hastings (6) showed that chloramphenicol had no effect on the phase and period of the glow rhythm in *Gonyaulax polyedra*, while puromycin blocked the expression of the rhythm and induced small, but reproducible phase delays when it was given as an 8-hr chemical pulse. Sweeney *et al.* (7) concluded that protein synthesis was not involved in the circadian control of photosynthesis in *Acetabularia crenulata* after failing to find effects of prolonged exposures to chloramphenicol and puromycin on the period of its rhythm. Feldman (8), on the other hand, reported that long treatments with cycloheximide lengthened the period of the phototaxis rhythm in *Euglena gracilis*.

Following the development of a method for continuously monitoring the O_2 production of individual *Acetabularia* cells (9, 10), it was shown that long treatments with cycloheximide altered rhythmic expression in these cells (11) and that short treatments might be affecting phase. The improvement of procedures for describing and comparing the periods and phases of single cell rhythms (12) made it possible to more precisely evaluate the influence of inhibitors on the circadian system of this alga and thereby reexamine the question of whether protein synthesis is necessary for circadian regulation. It was shown recently that an 8-hr exposure to cycloheximide (0.1 μg/ml) or puromycin (50 μg/ml) delays the phase of the photosynthesis rhythm 6–14 hr, providing the drug is present during a discrete part of a cell's circadian cycle (13). Similar treatments with chloramphenicol (100 μg/ml) had no effect on phase. These results, obtained for cells kept at 25°, indicate that one or more proteins synthesized on 80S ribosomes participate in the mechanism for circadian control of photosynthesis in *Acetabularia*.

In the present contribution it will be shown that the phase of cycloheximide sensitivity is somewhat longer and occurs about 8 hr later in the circadian cycle, when cells are investigated at 20° rather than 25°. As will be discussed, this observation favors the interpretation that 80S protein synthesis, while necessary for the circadian regulation of photosynthesis, does not itself generate the period of the rhythm.

MATERIALS AND METHODS

Cells and Culture Conditions. Cells of *Acetabularia mediterranea* were grown at 20° under diurnal illumination in Erd–Schreiber medium, as has been previously described (14, 15). Only those cells that had increased their length from 1.5 to 2.0 cm in the week prior to an experiment were chosen for experimental study. Cells were routinely exposed to a standard light-dark cycle (light 0800–2000, 2500 lux; dark 2000–0800) at either 20° or 25° for 4 or 5 days before they were placed under continuous illumination (2500 lux). They were transferred to sterile 90% filtered seawater at the time they were put in the cell chambers of the oxygen-monitoring systems.

Oxygen Measurements. Details of the flow-through method for the continuous polarographic monitoring of the O_2 production of individual cells were as previously described (9, 10, 12). Absolute quantities of O_2 were not calculated, since only changes in the relative amounts of O_2 present in the seawater in individual systems were important for these studies. All O_2 measurements are therefore presented as millivolts which, for a given electrode-amplifier circuit, were proportional to the O_2 tension of the fluid flowing past the electrode.

Period Estimation and Phase Comparisons. Details of the experimental design for phase studies and conventions used for estimating period and calculating circadian time have been described (12, 13). The time of maximum photosynthetic O_2 production is used as a phase reference point and attributed to circadian hour 6. Therefore circadian time 0 is 6 circadian hours prior to the next maximum of O_2 evolution (12). In order to

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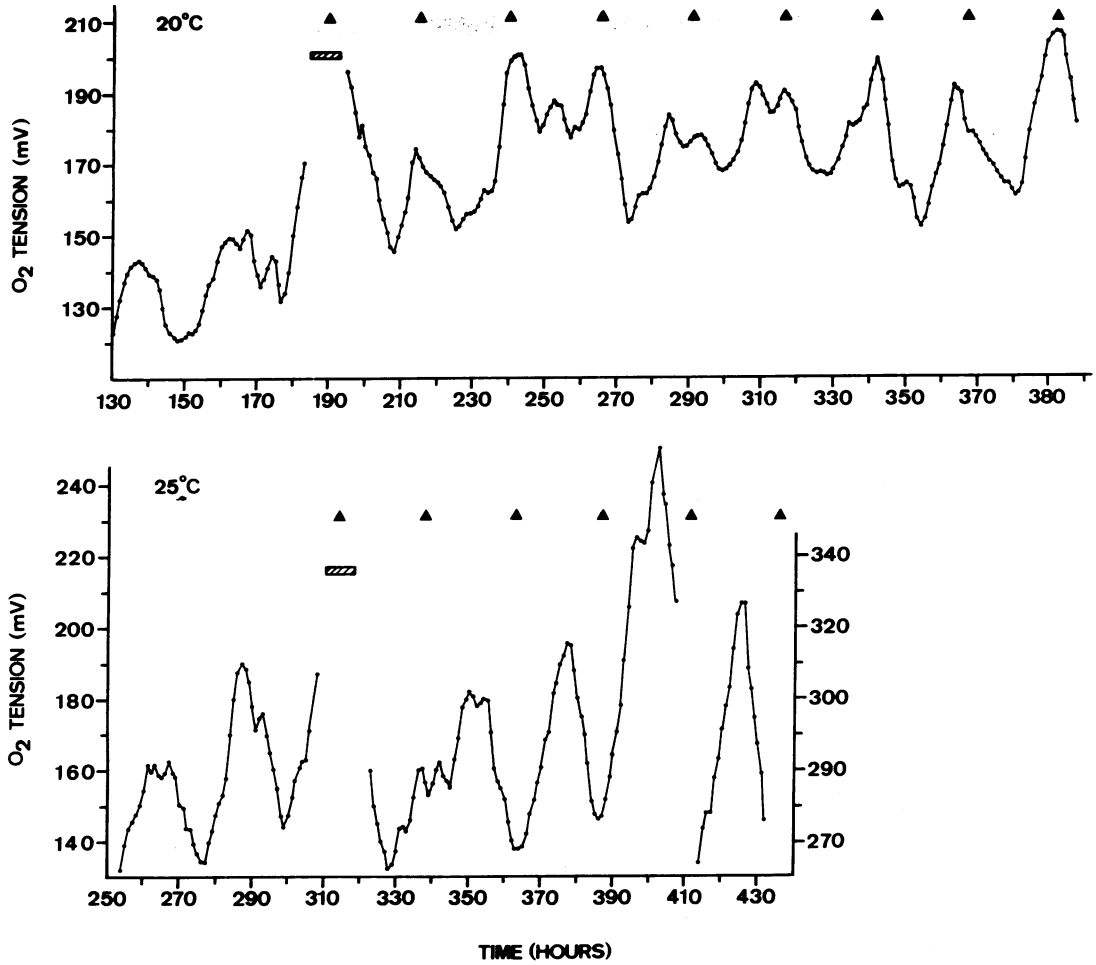

FIG. 1. The rhythm of O_2 production in individual nucleate cells exposed to 8 hr cycloheximide treatments at 20° and 25° during the early part of the circadian cycle. O_2 measurements were made polarographically at 60 min intervals in flow-through systems (0.4 ml/hr) containing cells that had been under continuous illumination (2500 lux) since hour 0. Values were smoothed by a moving average procedure before being plotted. The post-treatment phase (O_2 maximum) of each cell is shown in comparison to its original phase (▲). Cycloheximide (0.1 μg/ml) present between circadian hours 1.3 and 9.3 (bar) at 20° produced no effect on phase, whereas the presence of the drug between hours 2.6 and 10.7 at 25° induced a phase delay of approximately 14 circadian hours. At 20°, the cell's average periods before and after treatment were 25.4 and 24.8 hr, respectively. At 25°, the average periods before and after treatment were 24.4 and 24.3 hr.

eliminate the effect of intercellular period variability, all comparisons between cells are expressed in circadian hours as are the time and duration of drug treatments.

Administration of Inhibitors. Seawater containing an inhibitor was rapidly flushed into the oxygen-monitoring system by accelerating flow 500-fold to about 4 ml/min. At least 10 ml of the inhibitor-containing medium was flushed through the system, i.e., an amount more than double the total volume of the system and accessory tubing. Pulses were ended by similarly flushing seawater through the system. Medium exchanges were thus completed within 3 min. O_2 measurements during and immediately after this procedure were greatly affected by these drastic changes in flow rate, but it caused no lasting effect on the phase and period of the rhythm (12).

Cycloheximide was obtained from Boehringer Mannheim GmbH and was put into solution just before use.

RESULTS AND DISCUSSION

During the course of the investigation of the effect of cycloheximide pulses on phase (13), it became apparent that the drug's effect was different when the cells were maintained and pulsed at 20° rather than at 25°. A cycloheximide pulse given between circadian hours 1.3 and 9.3 at 20° (Fig. 1, top) produced no change in phase, whereas a pulse at nearly the same time in the cycle, between circadian hours 2.6 and 10.7, was maximally effective for delaying phase at 25° (Fig. 1, bottom). Conversely, a pulse given between circadian hours 14.2 and 22.0 at 20° produced a phase delay of more than 11 hr (Fig. 2). At 25°, a pulse at this time had no apparent effect on phase.

The effects of cycloheximide pulses given during various parts of the circadian cycle at 20° and 25° may be summarized in the form of phase response curves, where each phase change observed is plotted according to the midpoint of the corresponding drug treatment (Fig. 2). Maximum sensitivity to cycloheximide at 20° clearly occurs later in the cycle and appears to be longer in duration than at 25°. The magnitude of the effect on phase tends to be greater at 20° than at 25°, too, but more data would be needed to establish that point with certainty. The flushing method of pulsing obscures the kinetics of phase change, but other pulsing methods have shown that cycloheximide-induced shifts are usually apparent within a day after treatment and follow an unusually long interval of reduced O_2 production (13). Accordingly, all phase changes induced by cycloheximide were arbitrarily classified as delays and measured as the average change during 5–6 cycles after treatment.

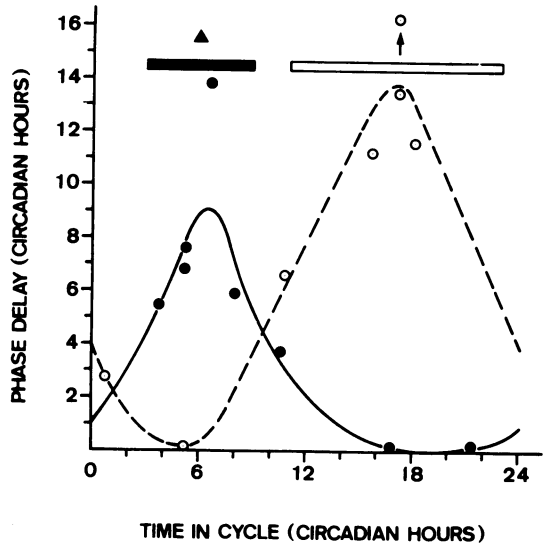

FIG. 2. Phase response profiles of cells maintained at 20° and 25° and exposed to single treatments with cycloheximide (0.1 μg/ml). The effect on phase of each treatment is plotted according to the midpoint of the cycloheximide pulse given. All phase changes were arbitrarily classified as delays and are expressed in circadian time. ●——●, phase response to cycloheximide treatments given at various times in the circadian cycle at 25°; O - - - - - O, phase response to cycloheximide treatments given at various times in the circadian cycle at 20°. For orientation in the circadian cycle, the time of the maximum of O_2 production (▲) is shown at hour 6. The putative sensitive phases identified by topological analysis are shown by the bars at the top of the figure. Arrow indicates a cycloheximide-induced phase delay in an enucleated cell.

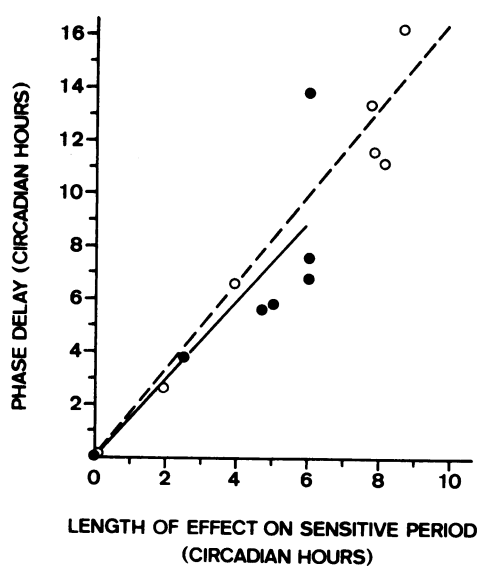

FIG. 3. Cumulative response to cycloheximide during the putative sensitive phases at 20° and 25°. Reproduced are two regression lines and the points on which they are based. Points are derived from a topological analysis undertaken to more precisely estimate the duration and phase of the sensitive portion of the cycle at each temperature (see *text*). ●——●, relationships between results obtained and a sensitive phase between circadian hours 3 and 9 at 25° (r = 0.865); O - - - - - O, relationship between results obtained and a sensitive phase between circadian hours 11 and 23 at 20° (r = 0.972). These two curves were selected because they had the largest regression coefficients among the many relationships tested. The relatively good fits to linearity suggest that the response to cycloheximide is cumulative during the sensitive phase at each temperature.

The complications of estimating phase changes against the background of high inter- and intracellular period variability have been discussed (12). An enucleated cell pulsed during the cycloheximide-sensitive part of its cycle (Fig. 2, arrow) displayed a phase delay equivalent to that observed for nucleate cells.

A more precise estimation of the duration and phase of the cycloheximide-sensitive portion of the cell's circadian cycle was sought by topological methods. If one postulates sensitive phases of 4, 6, 8, 10, 12, 14, and 16 hr duration and places each of them at successively later portions of the cycle until the entire cycle is scanned, one can estimate the amount of theoretical sensitive phase affected by the pulses for each case and compare it with the phase changes observed. This analysis yields a series of relationships which can be subjected to linear regression analysis (16). The range of correlation coefficients derived identifies an early part of the circadian cycle as cycloheximide-sensitive at 25°; best fits to linearity are obtained for 6 and 8 hr sensitive phases between circadian hours 3–9 (Fig. 3, r = 0.865) and 3–11 (r = 0.857), respectively.

Similar analyses of the data obtained at 20° corroborate the change in the sensitive phase at this temperature. The best fit to linearity (r = 0.972) is obtained for a 12 hr sensitive period between circadian hours 11 and 23 at 20° (Fig. 3). The relatively good fits to linearity at both temperatures suggest further that the effect of cycloheximide on phase is cumulative during the sensitive part of the cycle. Such a cumulative response might be expected if the protein(s) concerned participate quantitatively in circadian regulation.

Cycloheximide is believed to be highly selective in its action on translation on 80S ribosomes (17), but the dangers of drawing premature or inaccurate conclusions based on its presumed specificity have recently been emphasized (18, 19). Nevertheless, the demonstration that both puromycin and anisomycin pulses also shift the phase of the *Acetabularia* rhythm (13) supports the conclusion that some protein(s) must be synthesized on 80S ribosomes during each cycle to insure that a temporally correct sequence of events will take place. As discussed elsewhere (13), the protein(s) may be synthesized preferentially during part of the circadian cycle or they may be made continuously, turning-over or disappearing rapidly, and only be necessary for circadian regulation during a defined part of the cycle—to facilitate ion transport, for example.

Temperature is known to influence the function of the circadian regulatory system in *Acetabularia*, but the effect on frequency is small ($Q_{10} \cong 0.8$) due to the existence of a temperature-compensating feature (12). It is also known that the phase response profiles of cells subjected to dark pulses are essentially the same for cells kept at 20° and 25° (12). Therefore, the large effects of temperature on the phase of cycloheximide sensitivity would be unexpected, especially if it is postulated that protein synthesis on 80S ribosomes itself generates the period of the photosynthesis rhythm. Rather, it appears more likely that a 5° change in temperature markedly alters the availability of the cycloheximide-sensitive protein(s) necessary for circadian regulation. This could occur by its changing their rates of production and/or consumption in such a way that the inhibition of protein synthesis by cycloheximide has its impact on circadian control at different times depending on the temperature.

We wish to thank Dr. P. Dehm for many helpful discussions during this investigation and express our appreciation to Mr. D. Wolff, Mrs. M. Berthel, and Mrs. I. Waldecker for their excellent assistance with the collection and processing of the experimental data. This work was partially supported by the Deutsche Forschungsgemeinschaft.

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