Plant Physiol. (1979) 64, 1000–1004
0032-0889/79/64/1000/05/$00.50/0

Effects of Temperature Perturbations on Circadian Conidiation in *Neurospora*[1]

Received for publication April 2, 1979 and in revised form June 29, 1979

CYNTHIA D. FRANCIS AND MALCOLM L. SARGENT[2]
Departments of Botany, and Genetics and Development, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Studies on the circadian rhythm of conidiation in the *bd* strain of *Neurospora crassa* Shear and Dodge have shown that temperature step-up and step-down perturbations produce phase advances and delays, respectively. Pulse-up and pulse-down treatments lead to both phase advances and delays. The resulting phase shifts can be very large, and few to no transients are observed.

Small amplitude temperature cycles are capable of entraining the circadian rhythm, and holding *bd* at low temperatures appears to stop the circadian oscillator late in the subjective night (circadian time 2200). Aspects of the temperature responses that are somewhat unusual include the high sensivity, the lack of transients, and the phase at which the oscillator stops under low temperatures.

Although the effects of steady-state temperature on the period of the circadian rhythm of conidiation in *Neurospora crassa* have previously been established (21, 24), the effects of temperature perturbations on this system have not been studied. Such an investigation is desirable in view of the increasing evidence (27) that membranes are components of circadian oscillators, and the speculation that temperature-induced alterations of membrane lipid composition may be related to temperature conpensation of biological rhythms (16). In addition, there are reports (1, 7) that microwaves shift the phase of the biological clock. Knowing the sensitivity of the clock to temperature perturbations is critical for the interpretation of these presumed microwave effects.

Previous work concerning the effects of temperature perturbations on circadian rhythms has been reviewed by Sweeney and Hastings (28), Wilkins (31), and Bünning (4) with most of the detailed information coming from studies on *Drosophila pseudoobscura* and *Phaseolus*. Three primary generalizations from this earlier research follow. First, step-up treatments cause primarily phase-shift advances while step-down treatments cause primarily phase-shift delays. The circadian oscillator apparently shifts phase immediately after the temperature step (13), but the driven rhythms of some organisms exhibit major transients after such a perturbation (20). Second, pulse-up and pulse-down treatments cause both phase advances and delays, and the direction and amount of the phase shift are reasonably predictable from the step-up and step-down phase response curves (34). The phase response curves from temperature pulses are similar in shape to those obtained with light or dark pulses, with sensitivity to light and high temperature being greatest during the subjective night, and sensitivity to darkness and low temperature being greatest during the subjective day. Third, temperature steps or pulses can start the clock of some organisms in which it has apparently stopped (33) and, furthermore, temperature cycles close to 24 h and of small increment (0.9 C; ref. 10) can entrain circadian rhythms to the temperature cycle. There is also increasing evidence from several species (15, 29) that holding an organism at a low, steady-state temperature may stop the circadian oscillator.

The experiments described in this paper were designed to evaluate the above generalizations with respect to the *Neurospora* system. The effects of temperature steps (up and down), pulses (up and down), and cycles on circadian conidiation in *Neurospora* were measured, as well as the effects of long term low temperatures.

MATERIALS AND METHODS

Culture Procedures. The band (*bd*) strain (MLS 41-4; Fungal Genetics Stock Center No. 1859, Humboldt State University Foundation, Arcata, Calif.) of *N. crassa* Shear and Dodge was used throughout. Stock maintenance and medium composition have been described elsewhere (24). Conidia were inoculated onto 8 ml of glucose-arginine medium in 55-cm Pyrex growth tubes. The tubes were incubated for 20 h at room temperature in fluorescent light, then placed in a light-tight darkroom at 25.5 ± 0.5 C. The growth front was marked at this time and at 24-h intervals. Brief illumination was provided by ruby-red safelights (GE BBX, 40-w) which have no effect on the *bd* conidiation rhythm (24). Temperature perturbations were conducted in either a growth chamber (Percival I-30L; ± 0.3 C) or an incubator (Lab-Line/CS&E model 600; ± 0.5 C) housed in the darkroom.

Temperature Monitoring. Temperature was recorded with a copper-constantan thermocouple that was inserted into the agar of one of the growth tubes and connected to an amplifier-recorder (Heath-Schlumberger EU-200-01 plus EU-205-11). The system was calibrated against a thermometer (Parr Certified Calorimetric Thermometer model 1601; ± 0.01 C) maintained at various temperatures in an equilibrated water bath. At 1 mv full scale, the average deflection was 1.04 cm/C. After stabilization and calibration, temperature recording was generally accurate to within ± 0.1 C, although transient electrical noise occasionally reduced the accuracy to ± 0.5 C. After temperature changes of 5 and 10 C, the agar temperature equilibrated within about 10 and 20 min, respectively.

Light-dark Cycles. In experiments requiring light, the growth tubes were incubated in the growth chamber (I-30L) described above. Two fluorescent bulbs (cool-white F20T12-CW, Sylvania) provided an intensity of about 3,000 lux. Temperature was maintained at 25.5 ± 0.7 C.

Perturbation Experiments. Temperature perturbations were started on the 5th day of incubation in the dark at 25.5 C. Sets of three growth tubes were placed at higher or lower temperatures at appropriate intervals, and either maintained at those temperatures or returned to 25.5 C at suitable intervals. Incubation was continued for 4 to 5 days after the perturbation, so that the phase of the

[1] This work was supported by National Institute of General Medical Sciences Grant GM 16541.
[2] Author to whom reprint requests should be addressed.

rhythm on the 8th to 9th days could be used to measure any phase shift that might have been induced.

Data Analysis. At the end of an experiment a piece of tape (Scotch Brand Magic Transparent) was affixed to the bottom of each growth tube, and a permanent record was obtained by marking the position of the 24-h marks, the center of each conidial band, and the band shapes on the tape. Because growth of the *bd* strain along an agar surface is essentially linear with time (24), growth distance can be related to real time and to the circadian time of *Neurospora* (CT[3]; the normal period, about 22 h, normalized to 24.0 h; the band center defined as 2200 CT).

The CT of a perturbation was calculated by determining the CT at the previous 24-h mark, and adding to it the number of hours (normalized to CT) from that mark to the growth front at the start of the perturbation. Phase shifts (in hours normalized to CT) were determined by comparing the position of the ninth band center of an experimental tube with the averaged position of the equivalent band centers of the control tubes (a minimum of five tubes for each experiment).

Since there are major changes in the growth rate and minor changes in period at different temperatures (24), appropriate corrections were made when analyzing the experimental tubes. The greatest variability occurred near the breakpoints in the phase response curves where both the average phase shifts and the CT positions of the breakpoint were within 3 h of each other in similar experiments. Unless otherwise noted each data point in the figures represents one growth tube.

RESULTS

Redefinition of *Neurospora* CT. In the original report of circadian conidiation in the *bd* strain of *N. crassa* (24), CT was defined such that the middle of a conidial band occurred at 0800. To conform with the standard usage that has developed since then (19, 25), we are redefining CT in the *bd* strain to reflect the phase relationships that occur when the beginning of the dark period (subjective night) is taken as CT 1200. When *bd* was incubated in a 12:12 light:dark cycle at 25.5 C (Fig. 1A), the band center occurred about 10 h after the light to dark transition, *i.e.* at 2200 CT by the new definition. Under these entraining conditions the growth rate was 39 mm/day, and the period was 24 h.

Temperature Steps. When 5 C step-up treatments (25.5–30.5 C) were imposed on separate sets of cultures at 2-h intervals, the results shown in Figure 2 were obtained. This phase response curve shows predominantly phase advances, with a few small phase delays of questionable significance, and the phase advances could be very large, *i.e.* up to 15 h. These phase shifts were determined on the basis of the position of the ninth conidial band; however, when the phase shifts were plotted for successive bands, significant transients were not apparent (data not shown).

The results from step-down experiments (30.5–25.5 C) as a function of CT (Fig. 3) demonstrate predominantly phase delays (up to 14 h) with a few small, perhaps insignificant, phase advances. Preliminary experiments indicate that the amount of phase shift is dependent upon the absolute temperature of the step as well as upon the temperature increment. The general shape of these other response curves is, however, similar to that presented here.

Temperature Pulses. The effects of 6-h pulse-up treatments (5 C; 25.5 to 30.5 to 25.5 C) given at 2-h intervals are seen in Figure 4. In contrast to the step experiments, both phase advances and delays of considerable magnitude were seen. As with the step-up treatments, the new steady-state phase relationships were reached within 1, or at most 2, days (data not shown).

The amount of phase shift is a function of the magnitude of the temperature pulse (Fig. 5). When *bd* was pulsed for 6 h with the

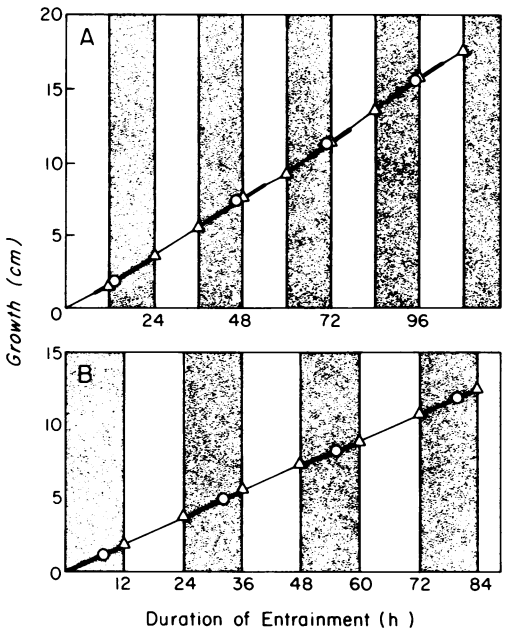

FIG. 1. Growth curves for entrainment of a *bd* culture by a 12:12 light-dark cycle (A) or by a 12:12 temperature cycle (B). The light intensity was 3,000 lux for A, and the temperatures for B were 25 and 20 C. Shaded regions indicate the dark (A) or cool (B) portions of the cycles. O: band center; dark bar: conidiation; Δ: position of growth front at lights-on and lights-off (A), or the start of the high and low portions of the temperature cycle (B).

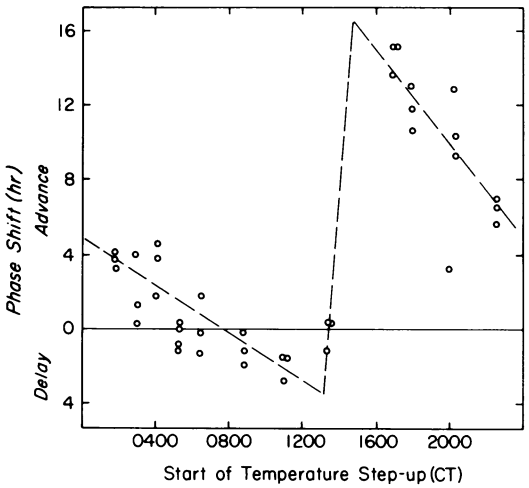

FIG. 2. Phase response curve for temperature step-up perturbations. Cultures were grown 5 days at 25.5 C and then transferred to 30.5 C for the remainder of the experiment.

temperature increment varying from 3 to 15 C (Fig. 5A), the amount of phase delay was reasonably proportional to the increment with a possibility of saturation at the larger increments. If a 5 C temperature increment were used while the duration of the pulse was varied from 1 to 10 h starting at 1100 CT, then maximum phase shifts were produced with a 3- to 4-h pulse, and longer pulses had little additional effect (data not shown).

A pulse-down response curve (Fig. 6) generated in response to 6-h pulses of a 5 C increment (25.5 to 20.5 to 25.5 C) also exhibited both phase advances and delays. Phase response curves for pulses of larger increment (10 or 15 C) were similar to Figure 6, but the phase shifts were larger, and there was less scatter in the data points. As with the previous treatments, no significant transients were observed.

To determine the dependence of phase shift on the increment of a pulse-down treatment, appropriate data points were taken

[3] Abbreviation: CT: circadian time.

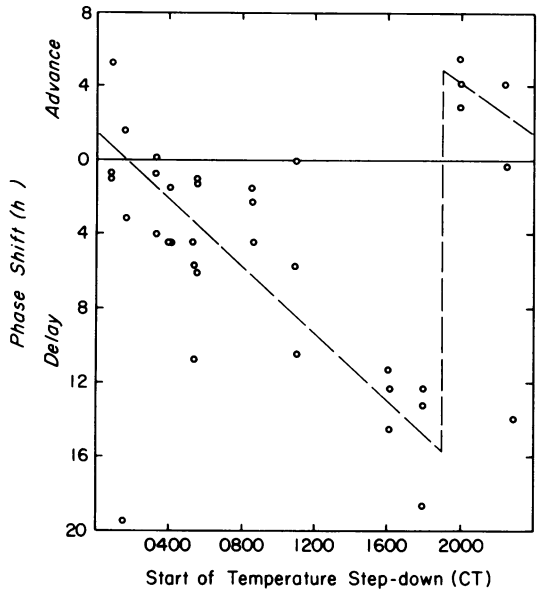

FIG. 3. Phase response curve for temperature step-down treatments. Cultures were grown 5 days at 30.5 C and then transferred to 25.5 C for the remainder of the experiment.

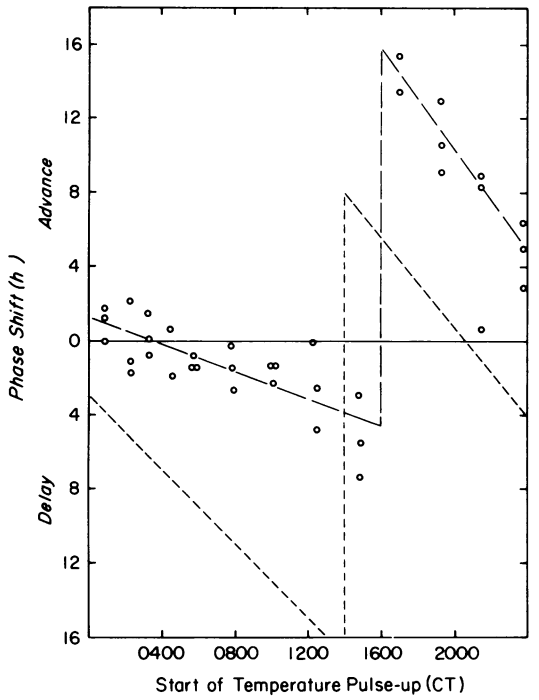

FIG. 4. Phase response curve for temperature pulse-up perturbations. Cultures were grown 5 days at 25.5 C, exposed to 30.5 C for 6 h, and then returned to 25.5 C for the duration of the experiment. Dotted line represents predicted values based on summation of step-up followed by step-down effects.

from the complete phase response curves described above. The results seen in Figure 5B indicate that for the two portions of the phase response curves studied, the amount of phase shift increases with increasing temperature increment. In the delay portion of the response curve, the increase in phase shift is rather minor with increments larger than 5 C, whereas in the advance portion saturation is reached only with larger increments. The amount of phase shift also increased with increasing pulse length (2–8 h). The response (data not shown) was quite linear, the amount of phase shift being less than the duration of the treatment.

Since in *Drosophila* the effects of pulse treatments can be predicted rather accurately in terms of a step-up followed by a step-down perturbation (34), we calculated expected phase shifts for 5 C pulses from 5 C step-up data (Fig. 2) and 5 C step-down data (Fig. 3). The calculations (dotted line in Fig. 4) show that the form of the pulse-up phase response curve is as predicted but the experimental curve is displaced vertically (4–6 h).

Entrainment to Temperature Cycles. When *bd* was put in a 12:12 temperature cycle (24.2:20.0 C) in constant darkness, it entrained and showed a conidiation period of 24 h (Fig. 1B). The band centers occurred about 8 h after the temperature decrease, in comparison to about 10 h after "dusk" in a light-dark cycle. A 25.5:23.5 C cycle (12:12) also entrained the rhythm, demonstrating that *bd* is sensitive to 2 C temperature increments.

"Stopping" the Clock with Low Temperature. With 0.5-h ex-

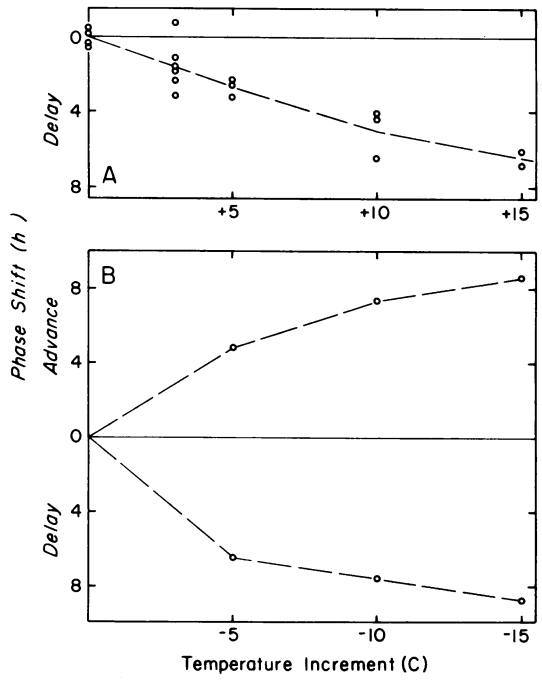

FIG. 5. Effect of temperature increment on the phase shift produced by a pulse-up or pulse-down of 6 h. A: cultures were grown 5 days at 25.5 C, exposed to the higher temperature at 1100 CT for 6 h, and then returned to 25.5C. B: points were taken from complete phase response curves for pulses to 20, 15, and 10 C. Phase shift advances were those from pulses started at 0800 CT, while the phase shift delays were from pulses started at 0300 CT.

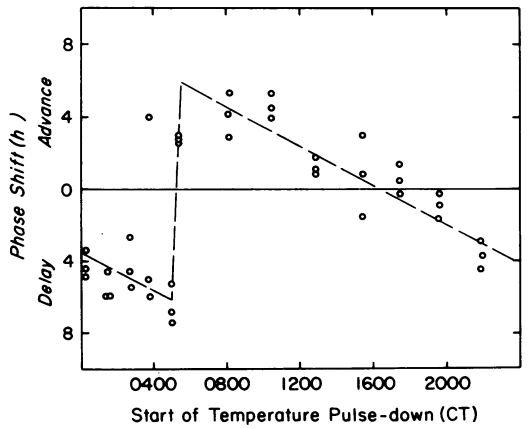

FIG. 6. Phase response curve for temperature pulse-down treatments. Cultures were grown 5 days at 25.5 C, exposed to 20.5 C for 6 h, and then returned to 25.5 C for the duration of the experiment.

posures to 4 C starting at 1100 CT the first and second band centers occurred about 10 and 32 h after the end of the pulse, respectively (Fig. 7), *i.e.* there was no phase shift. As the length of the pulse was increased, the band centers occurred at progressively shorter times after the end of the pulse indicating phase advances as seen in Figure 6. As the phase advances became larger and approached 10 h, the first conidial band formed closer and closer to the termination of the cold interval, became narrower, and finally was not evident at all. There was no additional phase-shifting effect with exposures longer than 6 to 8 h, and the "second" band center ("first" band center usually not visible) consistently appeared 22 to 24 h after the return to 25.5 C with the subsequent bands at circadian intervals. Similar results (data not shown) were obtained for pulses to 10.5 C. It is as if the clock "stops" at CT 2200 during long exposures to cold temperatures.

DISCUSSION

The results reported here demonstrate that the biological clock of *Neurospora* responds to temperature perturbations in much the same manner as the clocks of other species. For example, temperature step-up and step-down treatments provoke predominantly phase advances and phase delays, respectively, and the amount of phase shift is a function of the CT of the step. Temperature pulses, likewise, induce phase shifts with the direction and amount of the phase shift being dependent upon the temperature increment, pulse duration, and CT of the pulse.

The phase response curves from these experiments were type 0 (strong; 32), but it is possible that weaker perturbations would produce type 1 (weak) response curves. Indeed, response curves from a 5 C pulse-down treatment (Fig. 6) and treatments of smaller increment (not illustrated), seem to approach a type 1 response curve. *Drosophila* (6) and *Kalanchoë* (8) demonstrate both type 0 and type 1 steady-state response curves with strong and weak temperature perturbations, respectively.

In general, the shapes of the phase response curves for *Neurospora* correspond closely to those for other species, especially for those with recent, detailed response curves. More specifically, the *Neurospora* step-up response curve (Fig. 2) demonstrates highest sensitivity during the night, and resembles the response curves for *Phaseolus* (14) and *Drosophila* (34).

The *Neurospora* pulse-up response curve (Fig. 4) is nearly identical to response curves from *Kalanchoë* (8), *Phaseolus* (5, 14), *Solanum* (11), and corresponds well to the steady-state response curves from *Drosophila* (6, 34) and *Lycopersicum* (11). With the *Neurospora* pulse-down response curve (Fig. 6), maximum sensitivity occurs during the subjective day as with other species (31). There is good correspondence with the response curves from *Drosophila* (6, 34), *Phaseolus* (29), and *Leucophaea* (22). As was previously noted in *Kalanchoë* (8), the pulse-up response curve of *Neurospora* (Fig. 4) is nearly identical to the response curve for light-induced phase shifts (Fig. 4 of ref. 23; note change in definition of 2400 CT). The *Neurospora* pulse-down response curve is also remarkably similar to the two published response curves for dark pulses, those of *Acetabularia* and *Gonyaulax* (12), and is shifted one-half cycle along the CT axis in comparison with the pulse-up response curve as was predicted (30) on the basis of work with *Bryophyllum*.

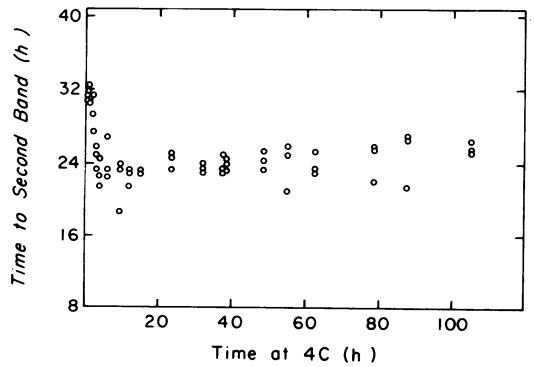

FIG. 7. Phase relationships of cultures held at 4 C for various intervals. Cultures were grown 5 days at 25.5 C, exposed to 4 C at 1100 CT for the intervals indicated, and then returned to 25.5 C.

Two other effects of temperature perturbations on the *Neurospora* rhythm clearly fit patterns found in other species (4, 10, 28, 31). First, temperature cycles of rather narrow increments are able to entrain the circadian rhythm (Fig. 1B), and second, pulses to low temperatures are able to stop or reset the clock (Fig. 7). For unknown reasons the *Neurospora* system seems to be different in that the clock appears to "stop" near 2200, rather than near 1200 as is ture for *Gonyaulax* (15), *Chlorella* (9), *Phaseolus* (29), and *Leucophaea* (22). Unfortunately, none of the above work has resolved the question as to whether the clock is arrested or actually running at the low temperature. It could be that the expression of the clock is suppressed, although the clock itself is still running. The clock might then be reset to the same phase, regardless of the CT, by the large temperature increment used to terminate the cold interval.

Two other aspects of the *Neurospora* response to temperature perturbations that do not simply support established generalizations include the lack of significant transients and the high sensitivity to temperature change. The circadian rhythms of many species, *e.g.* *Euglena* (2), *Phaseolus* (14), *Solanum* (11), *Lycopersicum* (11), and especially *Drosophila* (20), demonstrate large and complex transients after a temperature perturbation. Such behavior has been the basis for an influential model concerning circadian organization (20). *Neurospora*, however, is an example of a second group including *Bryophyllum* (31), *Kalanchoë* (8), *Uca* (26), and *Leucophaea* (22), in which transients are absent or very small. The significance of the difference between these two groups of species is not yet clear.

The reason for high sensitivity to temperature in *Neurospora* is not known, but it may be related to the fact that the growing tips are responsible for the expression of the monitored rhythmicity, and that the young, actively growing tips may be particularly sensitive to temperature. *Euglena* (2) seems to have similar temperature sensitivity, but the only other species that approach *Neurospora* in sensitivity, *e.g.* *Kalanchoë* (8) and *Leucophaea* (22), experience large phase shifts only with perturbations of greater increment or duration.

In general, the results presented in this paper support the conclusion (23, 24) that the basic circadian system in *Neurospora* is similar to other such systems in eukaryotic organisms. The results, unfortunately, are not unique or different enough to support or contradict the major models (4, 17, 18, 20) that have been proposed to account for the effects of temperature perturbations. Of significance, however, is the discovery that the phase of the *Neurospora* rhythm is extremely sensitive to changes in a common laboratory variable, since many investigators are adopting *Neurospora* as a test system for the study of circadian rhythms. The results, furthermore, provide a data base for experiments underway in this and other laboratories (3) to study the relationships, if any, between temperature, membrane composition and the operation of circadian oscillators.

Acknowledgments—We wish to thank Stuart Brody for helpful suggestions regarding the manuscript, and Thomas Lonergan and Robert Tuveson for advice and encouragement throughout the course of this work.

LITERATURE CITED

1. BARANSKI S, P CZERSKI 1976 Biological Effects of Microwaves. Dowden, Hutchinson & Ross, Inc, Stroudsburg
2. BRINKMANN K 1966 Temperatureinflüsse auf die circadiane Rhythmik von *Euglena gracilis* bei Mixotrophie und Autptrophie. Plant 70: 344–389
3. BRODY, S, SA MARTINS 1979 Circadian rhythms in *Neurospora crassa*: effects of unsaturated fatty acids. J Bacteriol 137: 912–915

4. BÜNNING E 1973 The Physiological Clock, Rev Ed 3. Springer-Verlag, Berlin, pp 71–88
5. BÜNNING E, M TAZAWA 1957 Uber den Temperatureinfluss auf die endogene Tagesrhythmik bei *Phaseolus*. Planta 50: 107–121
6. CHANDRASHEKARAN MK 1974 Phase shifts in the *Drosophila pseudoobscura* circadian rhythm evoked by temperature pulses of varying durations. J Interdiscipl Cycle Res 5: 371–380
7. CZERSKI, P, E PAPROCKA-SLONKA, A STOLARSKA 1974 Microwave irradiation and the circadian rhythm of bone marrow cell mitosis. J Microwave Power 9: 32–37
8. ENGLEMANN W, I EGER, A JOHNSSON, HG KARLSSON 1974 Effect of temperature pulses on the petal rhythm of *Kalanchoë*: an experimental and theoretical study. Int J Chronobiol 2: 347–358
9. HESS, M 1974 Der Einfluss niedriger Temperature auf die endogene Rhythmik der Reproduktionsfähigkeit von *Chlorella*. Z Pflanzenphysiol 71: 428–436
10. HOFFMAN K 1969 Zum Einfluss der Zeitgeberstärke auf die Phasenlage der synchronisierten circadianen Periodik. Z Vergl Physiol 62: 93–110
11. JUNKER G, W MAYER 1974 Die Bedeutung der Epidermis für licht- und temperaturinduzierte Phasenverschiebungen circadianer Laubblatt-bewegungen. Planta 121: 27–37
12. KARAKASHIAN MW, H-G SCHWEIGER 1976 Circadian properties of the rhythmic system in individual nucleated and enucleated cells of *Acetabularia mediterranea*. Exp Cell Res 97: 366–377
13. MAIER RW 1973 Phase-shifting of the circadian rhythm of eclosion in *Drosophila pseudoobscura* with temperature-pulses. J Interdiscipl Cycle Res 4: 125–135
14. MOSER I 1962 Phasenverschiebungen der endogenen Tagesrhythmik bei *Phaseolus* durch Temperatur- und Lichtintensitätsänderungen. Planta 58: 199–219
15. NJUS D, L MCMURRY, JW HASTINGS 1977 Conditionality of circadian rhythmicity: synergistic action of light and temperature. J Comp Physiol 117: 335–344
16. NJUS D, FM SULZMAN, JW HASTINGS 1974 Membrane model for the circadian clock. Nature 248: 116–120
17. PAVLIDIS T, W KAUZMANN 1969 Toward a quantitative biochemical model for circadian oscillators. Arch Biochem Biophys 132: 338–348
18. PAVLIDIS T, WF ZIMMERMAN, J OSBORN 1968 A mathematical model for the temperature effects on circadian rhythms. J Theoret Biol 18: 210–221
19. PITTENDRIGH CS 1974 Circadian oscillations in cells and the circadian organization of multicellular systems. *In* FO Schmitt, FG Worden, eds, The Neurosciences Third Study Program. MIT Press, Cambridge, pp 437–458
20. PITTENDRIGH CS, VG BRUCE, P KAUS 1958 On the significance of transients in daily rhythms. Proc Nat Acad Sci USA 44: 965–873
21. PITTENDRIGH CS, VG BRUCE, NS ROSENSWEIG, ML RUBIN 1959 A biological clock in *Neurospora*. Nature 184: 169–170
22. ROBERTS SK DE F 1962 Circadian activity rhythms in cockroaches II. Entrainment and phase shifting. J Cell Comp Physiol 59: 175–186
23. SARGENT ML, WR BRIGGS 1967 The effects of light on a circadian rhythm of conidiation in *Neurospora*. Plant Physiol 42: 1504–1510
24. SARGENT ML, WR BRIGGS, DO WOODWARD 1966 The circadian nature of a rhythm expressed by an invertaseless strain of *Neurospora crassa*. Plant Physiol 41: 1343–1349
25. SAUNDERS DS 1977 An Introduction to Biological Rhythms. Halsted Press, New York
26. STEPHENS G 1957 Influence of temperature fluctuations on the diurnal melanophore rhythm of the fiddler crab *Uca*. Physiol Zool 30: 55–69
27. SWEENEY BM 1976 Evidence that membranes are components of circadian oscillators. *In* JW Hastings, H-G Schweiger, eds, The Molecular Basis of Circadian Rhythms. Dahlem Konferenzen, Berlin, pp 261–281
28. SWEENEY BM, JW HASTINGS 1960 Effects of temperature on diurnal rhythms. Cold Spring Harbor Symp Quant Biol 25: 87–104
29. WAGNER R 1963 Der Einfluss niedriger Temperatur auf die Phasenlage der endogen-tagesperiodischen Blattbewegungen von *Phaseolus multiflorus*. Z Bot 51: 179–204
30. WILKINS MB 1960 The effects of light upon plant rhythms. Cold Spring Harbor Symp Quant Biol 25: 115–129
31. WILKINS MB 1965 The influence of temperature and temperature changes on biological clocks. *In* J Aschoff, ed, Circadian Clocks. North Holland, Amsterdam, pp 146–163
32. WINFREE AT 1973 The investigation of oscillatory processes by perturbation experiments. II. A singular state in the clock-oscillation of *Drosophila pseudoobscura*. *In* B Chance, EK Pye, AK Ghosh, and B Hess, eds, Biological and Biochemical Oscillators. Academic Press, New York, pp 479–501
33. ZIMMERMAN WF 1969 On the absence of circadian rhythmicity in *Drosophila pseudoobscura* pupae. Biol Bull 136: 494–500
34. ZIMMERMAN WF, CS PITTENDRIGH, T PAVLIDIS 1968 Temperature compensation of the circadian oscillation in *Drosophila pseudoobscura* and its entrainment by temperature cycles. J Insect Physiol 14: 669–684