CIRCADIAN RHYTHM FROM THE EYE OF *APLYSIA*: TEMPERATURE COMPENSATION OF THE EFFECTS OF PROTEIN SYNTHESIS INHIBITORS

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SUMMARY

1. The circadian rhythm of compound action potentials (CAP) frequency recorded from the isolated eye of *Aplysia* in culture medium and darkness was subjected to step and pulse treatments with anisomycin, a protein synthesis inhibitor.

2. The step application of anisomycin and its continued presence in the culture medium lengthened the period of the rhythm in a dose-dependent manner. At 10^{-8} M the period was increased from the normal 26·5 h to about 28 h and at 10^{-7} M the period was lengthened to 31 h or longer. At 10^{-6} M the rhythm was suppressed but the CAP activity continued without the cyclic variations in CAP frequency.

3. Six-hour pulses of anisomycin at 10^{-8} M caused phase-dependent phase-shifts of the rhythm. Maximum phase delays of 15 h were obtained at CT (circadian time) 2 and maximum phase advances of 4 h were obtained at CT 6. The phase response curves at 12, 15 and 17 °C were essentially identical.

4. Anisomycin appears to act rather selectively on the circadian clock mechanism. It does not alter the CAP amplitude and duration and it does not alter the bursting pacemaker mechanism of the optic nerve CAP or central neurones.

5. The results support the hypothesis that the synthesis of a protein or polypeptide on eucaryotic ribosomes is an essential part of the circadian clock timing mechanism. The sensitivity of the clock to anisomycin is the same at three different temperatures (12, 15 and 17 °C) within the physiological range of temperatures for *Aplysia*, as expected for a clock whose period length is temperature compensated (Q_{10} 1·02) over that same range.

6. At the critical phases of CT 1–4, anisomycin pulses often caused unusual perturbations of the rhythm. These effects are consistent with the hypothesis that the circadian rhythm is a multioscillator system.

INTRODUCTION

The idea that protein synthesis on the eucaryotic ribosome is a fundamental part of the cellular regulatory mechanism which constitutes the circadian clock has received strong support from recent studies. Protein synthesis inhibitors, such as anisomycin, puromycin and cycloheximide, caused shifts in the phase of the circadian rhythm of oxygen evolution in an alga, *Acetabularia* (Karakashian & Schweiger, 1967a), and in the circadian rhythm of neuronal firing in the eye of a gastropod, *Aplysia* (Jacklet, 1977; Rothman & Strumwasser, 1976). These inhibitors were applied in pulses of several hours and then removed, causing the phase of the rhythm to be advanced or delayed depending upon which phase of the rhythm the pulse was given. Also, the continuous presence of inhibitors, after being applied in a single step, increased the period length of the phototaxis rhythm of the flagellate, *Euglena* (Feldman, 1967) and the *Aplysia* eye rhythm (Jacklet, 1977). Thus the two diagnostic approaches to identifying fundamental processes of clocks, pulse and step experiments (Tyson *et al.* 1976), yield positive evidence of the necessity for protein synthesis in the circadian clock.

Having determined the importance of protein synthesis, a further inquiry is its specific function in the clock. Studies on *Acetabularia* (Karakashian & Schweiger, 1976b) showed that the shifts caused by pulses of cycloheximide had a temperature dependence different from the temperature dependence of the period length. The period was about 24·4 h at 25 °C and about 25 h at 20 °C, yet the phase-dependent sensitivity to cycloheximide obtained was 4–6 h longer in duration and shifted to a phase of the rhythm 8 h later at 20 °C than it was at 25 °C. The interpretation of this result was that protein synthesis at the eucaryotic ribosome does not itself generate the period of the rhythm in *Acetabularia* (Karakashian & Schweiger, 1976b) since the sensitivity to the inhibitor is so different at the two temperatures. The synthesis could be identified perhaps with processes associated with the clock rather than the timing mechanism itself.

In this report, experiments are described on the temperature dependence of the phase-shifting of the *Aplysia* eye rhythm by a protein synthesis inhibitor. The resultant phase response curves show that the sensitivity of the clock to the inhibitor is essentially identical at temperatures from 12 to 17 °C. As the period lengths of the rhythms are also the same at those physiological temperatures, both the sensitivity to the inhibitor and the period length are temperature compensated to the same extent.

METHODS

Aplysia californica (100–300 g) were obtained from Pacific Bio-Marine (Venice, CA) and kept in Instant Ocean aquaria exposed to light–dark cycles of 13–11 h maintained at 15–16 °C. Eyes with attached optic nerves were dissected from the animals 2–3 h after dawn and placed in 125 ml of culture medium, maintained thereafter at either 12, 15 or 17 °C in constant darkness. The optic nerve was drawn into a J-shaped tubing electrode (Jacklet, 1974, 1977) in the culture chamber allowing the compound action potentials (CAP) to be amplified and continuously recorded on a Grass polygraph. The frequency of CAP were counted automatically with an Ortec system and printed out each 20 min on a teletype. The culture medium contained 90% artificial sea water

(ASW) and 10% nutrient mixture which included amino acids, vitamins, dextrose, penicillin and streptomycin (Gibco) as used previously (Jacklet, 1974, 1977). Anisomycin (Pfizer) was dissolved in ASW and added to the culture medium at specific phases of the circadian rhythm in pulses of six hours or it was added in one step and left in continuously for several days. After the pulse treatments, the inhibitor solution was removed, the chamber was washed with 250 ml of ASW and fresh culture medium was added, all under dim red light. Exposure to the red light and changing the culture medium did not alter the rhythm.

In culture medium and complete darkness the period of the rhythm is about 26·5 h and on the first day in culture the CAP frequency increases to half its maximum rate of firing at subjective dawn (the time when dawn would have been observed by the eye had it not been dissected from the animal) which is indicated by convention as circadian time 0 (CT 0). On subsequent cycles of the rhythm the time when the frequency increased to half the maximum rate was used as a phase reference for CT 0 and the 26·5 h cycle was normalized to 24 circadian h for purposes of plotting the phase response curves. The phase of a 6 h pulse of anisomycin was designated in circadian hours at the midpoint of the pulse (i.e. a pulse designated as CT 3 was actually started at CT 0 and ended at CT 6). The period of the rhythm was measured in hours between successive half maximum rate increases or between successive centroids. Centroids were calculated for each cycle of CAP frequency changes from the maximum and back to 0. The time interval in which the median of the sum of frequencies occurred, from 20 min count intervals throughout the cycle, was the centroid (Benson & Jacklet, 1977a).

RESULTS

The endogenous electrical activity recorded from the optic nerve of the isolated eye in darkness consists of compound action potentials whose frequency waxes and wanes rhythmically with a circadian period. During subjective day the frequency is high and during subjective night the frequency is low. The amplitude of the CAP also fluctuates in phase with the frequency rhythm (Jacklet, 1974; Benson & Jacklet, 1977a). At high frequencies of firing the CAP tend to occur in bursts of 2–5 while at lower frequencies the CAP occur as singles. CAP at two recording speeds are shown in Fig. 1 just before, during, and just after an anisomycin pulse of 6 h. Anisomycin does not change the amplitude or duration of the CAP during or after the treatment beyond the variation observed in normal eyes. The frequency of CAP is usually altered to the extent that anisomycin stabilizes it at, or near, the frequency at which it was occurring when the inhibitor was applied. Thus, if it was applied during the rapid frequency increase at subjective dawn, the frequency would not continue to its expected peak but become stabilized at the frequency of firing when application of the anisomycin was made. Also when the inhibitor is removed there is no obvious excitation or inhibition of the CAP activity. This freedom from side effects on the traditional neuronal functions such as action potential conduction and synaptic transmission was observed previously for central neurones of *Aplysia* (Schwartz, Castellucci & Kandel, 1971) and Fig. 2 confirms that anisomycin has no detectable effect on the action potentials or slow wave potentials of central neurone R_{15}. The number of action potentials in a burst, the

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(A) Before

(B) During aniso

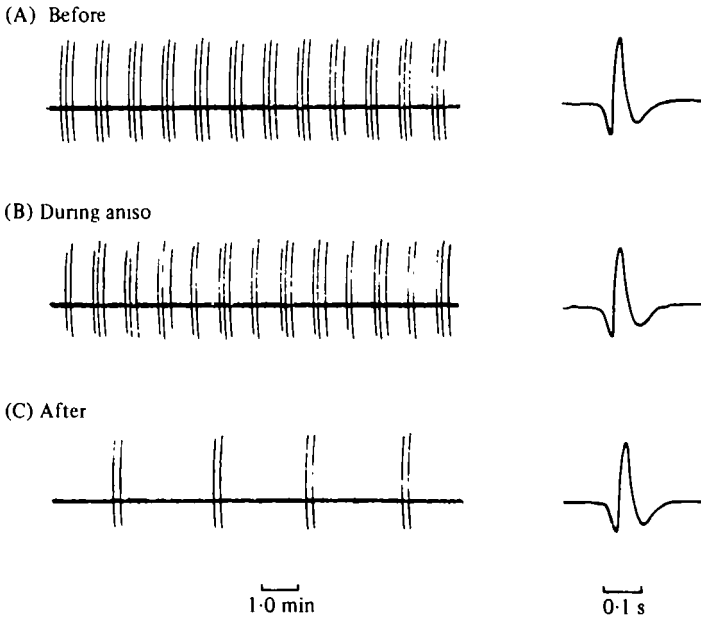(C) After

1·0 min   0·1 s

Fig. 1. Compound action potentials (CAP) recorded from the optic nerve of the isolated eye in culture medium. The CAP are shown at slow and fast recording speeds, 30 min before the application of 10^{-6} M anisomycin in A, 90 min after its introduction in B, and 30 min after it was removed in C. No unusual changes in CAP amplitude duration, or mode of firing occurred. The frequency was affected. It was stabilized near the firing rate at the time of anisomycin addition. CAP amplitude is approximately 100 μV, recorded on a Grass polygraph.

R15
10 min
before

Anisomycin
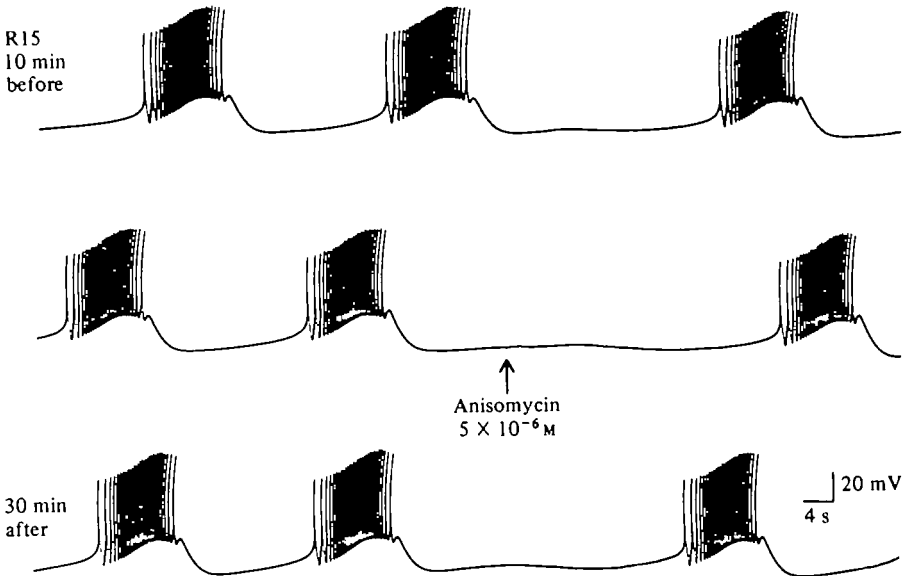5×10^{-6} M

20 mV
4 s

30 min
after

Fig. 2. Bursts of action potentials recorded from central neurone R_{15} 10 min before the addition of anisomycin (top line), at the time of application (middle line) and 30 min (bottom line) after the inhibitor was applied. There was no obvious change in the action potentials, bursts or slow membrane potentials. Spikes are attenuated in amplitude by the frequency response of the polygraph.
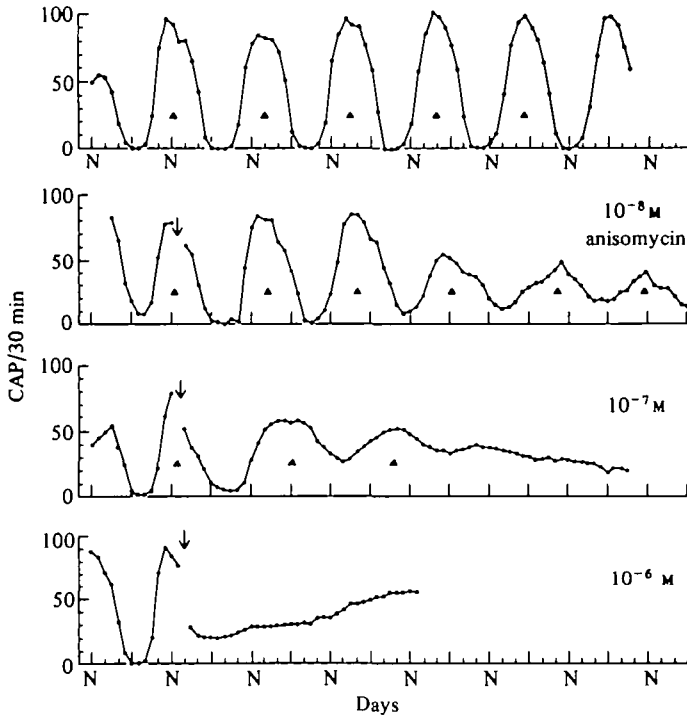
Fig. 3. CAP frequency rhythms during the continuous presence of anisomycin at 3 concentrations. The top graph is a control eye in darkness at 15 °C in organ culture medium. Black triangles mark the centroid points for each cycle and are used to measure the circadian period. The control periods are 26·5, 26·5, 26·8, 27 and 27 h. In graph 2, 10^{-8} M anisomycin was added at the arrow resulting in a lengthening of the periods to 27·5, 27·5, 28·5, 32 and 26·5 h. In graph 3, 10^{-7} M anisomycin lengthened the period to 35·0 and 31·0 h. In graph 4, 10^{-6} M anisomycin completely suppressed the rhythm but the CAP activity continued. Time scale in days marked at successive noons (N).

interval between bursts, and the amplitude of the post-burst hyperpolarization are not altered beyond the normal variation seen in these parameters. The lack of any direct effect on these parameters is rather remarkable since anisomycin has a profound effect on the circadian rhythm of CAP frequency in the eye as shown in the next section.

Step additions of anisomycin

Step experiments, in which the inhibitor was applied at one point and left in continuously for days thereafter, were performed at 15 °C in darkness. Fig. 3 shows the typical alterations of the rhythm produced by anisomycin at three different concentrations. The top graph is of a control eye. The period length averages 26·5 h, as previously found (Jacklet, 1974, 1977), with a slight increase to 27 h or longer by the end of a week in culture medium. Anisomycin at 10^{-8} M (second graph) caused an increase in the period length to about 28 h and eventually suppressed the range of the rhythm. Higher concentrations progressively increased the period of the rhythm to 31 h or longer, and suppressed the range until the rhythm was no longer detectable at 10^{-6} M anisomycin (bottom graph). However, the CAP activity was still quite strong. It continued at about the same average frequency (taken over several days) as in normal
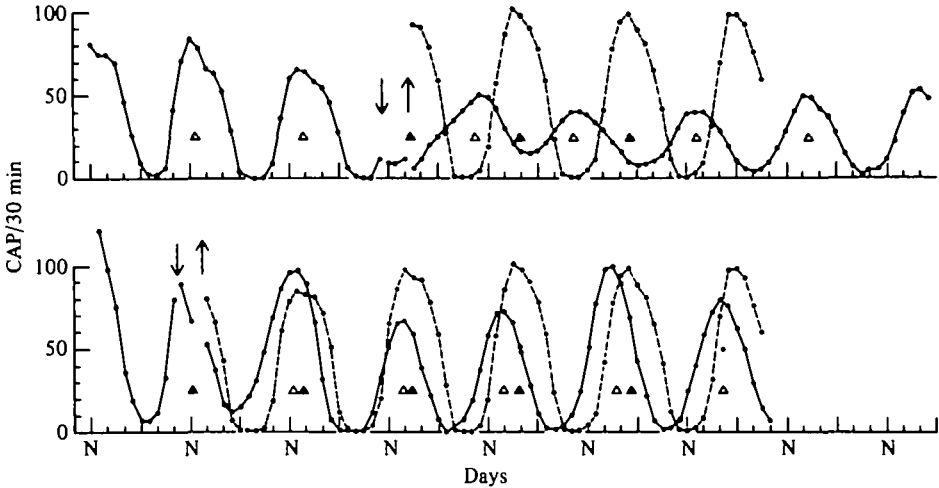
Fig. 4. Phase-shifts of the circadian rhythm at 15 °C produced by 6 hr pulses of anisomycin (10^{-4} M). Graphs show experimental eyes (——) compared to the control eyes (– – –). In the top graph the experimental eye activity was recorded for two complete cycles of the rhythm prior to the pulse, marked at the beginning (↓) and end (↑). Subsequently the experimental eye frequency is plotted with the control eye and the centroid points are marked (△, experimental; ▲, control). The pulse, centred at CT 3, caused a delay in the rhythm relative to the control of 15 h. The bottom graph shows a pulse centred at CT 5. It caused a phase advance of 3 h.

eyes but the circadian variation in frequency was absent. Anisomycin seems to act on the rhythm rather selectively and leaves untouched the short-term pacemaker activity.

Pulse additions of anisomycin

Six hour pulses of anisomycin produced phase-dependent phase advances or delays in the rhythm. Fig. 4 shows examples of a delay (top graph) and an advance (bottom graph). The experimental eye was usually allowed to run for two circadian cycles before the inhibitor was pulsed in order to measure the normal phase and period of the rhythm. The changes in phase and period caused by the experimental manipulations could then be compared to an expected phase and period based on the performance of control eyes run separately and the performance of the experimental eye prior to treatment. Fig. 4 shows that the phase of the experimental rhythm was delayed 15 h by a pulse begun at CT o and ended at CT 6, where CT o is taken as the point of half maximum frequency during the increase in frequency. The phase delay was determined by comparing the delay in hours of the first experimental centroid (open triangle) relative to the control centroid (solid triangle). The period length of the experimental rhythm showed transient alterations after the pulse. It was 24 h, then 29 h, before returning to the normal 27 h. This caused the measured delays to vary also before the stable delay was reached by the third post-pulse cycle. When the pulse was applied at a slightly later phase in the cycle the rhythm was advanced in phase rather than delayed, as shown for a pulse started at CT 2 and ended at CT 8 (bottom graph Fig. 4). Here the rhythm exhibited transient alterations in period also in the first few cycles after the pulse as seen by comparing the difference between control and experimental centroids for each post-pulse cycle. Again the relative advance was
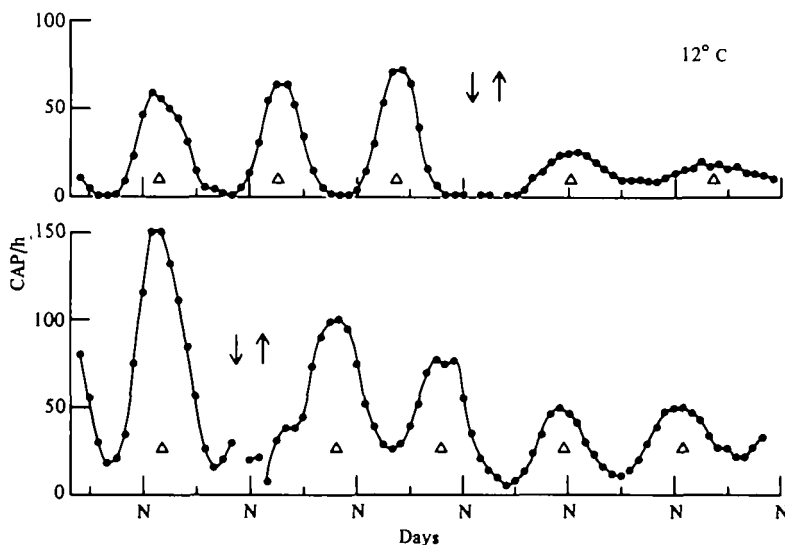
Fig. 5. CAP frequency and phase shifts of the circadian rhythm as 12 °C produced by 6 h pulses of anisomycin (10^{-4} M). The top graph shows a rhythm, with periods of 26·5 h, pulsed after the third cycle. The amplitude was reduced and the period between the last two centroids was 32 h. The bottom graph shows a rhythm with the pulse given earlier. The rhythm recovered and the post-pulse periods are 24, 28 and 27 h.

stable by the third cycle after the pulse. The advances in phase never equalled the magnitude of the largest delays obtained. The amount of advance or delay obtained for pulses given at each phase of the cycle for culture temperatures of 15 °C was recently published (Jacklet, 1977) showing maximum delays of 15 h and maximum advances of 5 h.

In order to test the temperature dependence of the anisomycin effect on the rhythm, experiments were also conducted at 12 and 17 °C. Reducing the temperature below 12°C diminished the amplitude of the CAP frequency peaks substantially and reduced the vigour of the rhythmic changes. In contrast, increasing the temperature above 17 °C enhanced the CAP frequency maximum and increased the vigour of the frequency changes.

Examples of rhythms at 12 °C are shown in Fig. 5. The CAP frequency was noticeably reduced but the period of the rhythm was less affected. The first two cycles in the top graph have periods (hours between centroids) of 26·5 h. The pulse, given at CT 21 caused an apparent 12 h delay in the rhythm but the amplitude and period of the rhythm did not recover to normal. This result of incomplete recovery from a pulse was typical of rhythms run at lower temperatures when the pulse was given after the eye had been in culture for 4 days or longer. If the pulse was given on earlier days (lower graph, Fig. 5) the rhythm did recover to normal periodicity. This pulse given at CT 21 caused a 12 h delay in phase and subsequently the period returned to the normal 27 h periodicity.

The period of a normal rhythm at 12, 15 and 17 °C was conserved quite well. Table 1 shows that the average period overall was about 26·4 h with the period at 17 °C slightly shorter. The greatest variation (S.D.) occurs in the first cycle even though the

Table 1. *Normal period length (τ) for the circadian rhythm at 3 temperatures*: 12, 15 *and* 17 °C.

The mean ($\overline{X}$), standard deviation (S) and number of observed cycles (n) are given for the first (τ_1), second (τ_2), etc., successive cycles in organ culture at each temperature. The average period for the first four cycles is very nearly the same at all the tempertaures, with a Q_{10} of 1·02 from 12–17 °C. Periods for τ_3 and τ_4 are from control eyes whereas periods for τ_1 and τ_2 are from control eyes and experimental eyes that were pulsed with anisomycin later.)

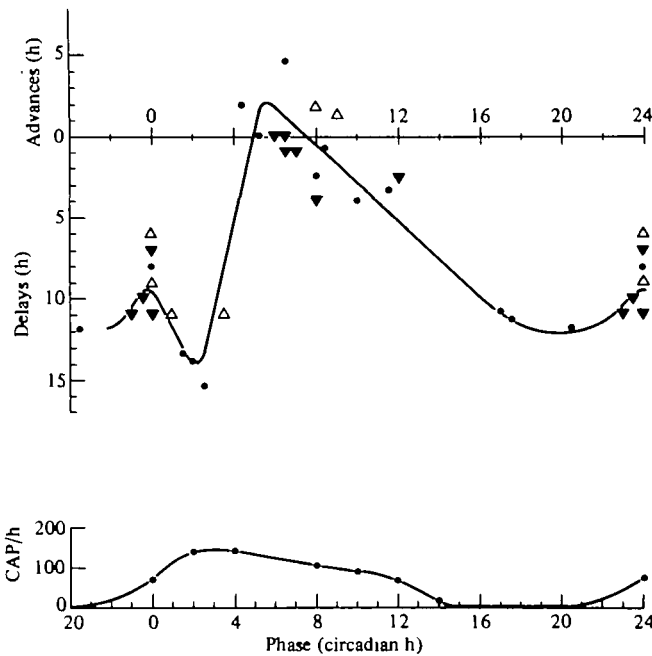|	12 °C			15 °C			17 °C		
	$\overline{X}$	S	n	$\overline{X}$	S	n	$\overline{X}$	S	n
τ_1	26·3	0·9	12	26·1	0·8	21	25·7	0·4	11
τ_2	26·3	0·3	6	26·8	0·6	9	26·1	0·1	4
τ_3	26·6	0·6	4	26·6	0·3	5	26·6	0·3	4
τ_4	27·0	0·7	2	26·7	0·3	3	26·7	0·3	3
Total	26·4	0·8	24	26·4	0·7	38	26·1	0·5	22

Fig. 6. Phase response curve for anisomycin (10^{-6} M) pulses (6 h) at 12 ° (▼), 15 ° (●) and 17 °C (△). The CAP frequency (CAP/h) rhythm is outlined in the lower graph as a phase reference for the response curve in the top graph. Phase is measured in hours of circadian time on the time axis with o equivalent to subjective dawn and the half maximum frequency increase of the rhythm. Hour 12 is equivalent to subjective dusk and the point of rapid decline in the CAP frequency. The advances or delays in phase of the rhythm are plotted at the midpoint of the 6 h treatment (3 h after addition), and the phase measurements are from the third post-pulse cycle. The solid line was drawn by eye to show the trend of shifts.

greatest number of observations (11) were made there. For the first cycle the periods were systematically longer, on the average, at lower temperatures. However, in later cycles (i.e. the third) the periods were almost identical; virtually perfect temperature compensation. The Q_{10} for the change in average period length, over the first four cycles and for 12–17 °C was 1·02, where $Q_{10} = [\text{rate } 17°/\text{rate } 12°]^{10/5}$ and rate is the frequency (or 1/period) at those temperatures.

Phase response curves for 12, 15 *and* 17 °C

The magnitude of the delay or advance in the rhythm caused by 6 h pulses of anisomycin (10^{-6} M) at different phases of the circadian cycle was systematically determined. Experiments were concentrated at those phases near the rapid reversal from delays to advances near CT 4. Fig. 6 shows the phase-response curve for the three temperatures. The average phase-shifts for pulses at 15 °C from Jacklet (1977) are replotted with advances upward and delays downward. Superimposed on this graph are points from 10 eyes (closed triangles) at 12 °C and points from six eyes (open triangles) at 17 °C. A total of 14 eyes at 12 °C and 10 eyes at 17 °C were pulsed. Some of these eyes either did not recover well from the pulse (i.e. top graph in Fig. 5) or the rhythm was so desynchronized that a reliable phase could not be measured (see bottom graph Fig. 7). In order to be included in the phase response curve the rhythm had to return to cycles with a normal period length after the pulse. The points on the phase response curve in Fig. 6 are phase measurements from the third cycle after the inhibitor pulse when the transient changes in period had subsided and a stable phase-shift was established. Fig. 6 shows that the different temperatures, 12, 15 and 17 °C, do not make any obvious difference in the phase-shifts produced at specific circadian times. At phases near CT 0 large delays are caused at all temperatures and the variability seen at 15 °C is also present at 12 and 17 °C. At CT 6–8 no advances were obtained at 12 °C but small advances were obtained at 17 and at 15 °C. At these two critical points of the phase response curve, maximum delays and advances, the responses were not different. Certainly none of the responses at a particular temperature were so far beyond the variation exhibited at other temperatures to indicate that they were different.

Altered rhythms due to pulses at CT 1–4

Pulses of anisomycin given at circadian times near the sharp transition from delays to advances at CT 1–4 (Fig. 6) caused unusually complicated phase-shifts. This behaviour was especially obvious at 17 °C as exhibited in Fig. 7. Graph 1 of the figure is a normal control rhythm with the rapid CAP frequency increases for each cycle marked with a dark vertical bar. The other graphs were aligned in time to coincide with this phase reference (dark vertical bar of the control). So the time axis is in circadian days (each equal to about 26·5 h). In graph 2, a pulse centred at CT 0 caused an instantaneous delay in phase and the first cycle after the pulse had a double peak. The second cycle however had recovered to the normal waveform with a single peak and a persistant delay of 11 h relative to the control. The rhythm subsequently recovered to the normal period and waveform but with the maintained phase delay. Pulses given at this phase of the rhythm yield 'clean' results; a large quick shift in phase with a few lingering transients but the period and waveform quickly recovered to normal. Pulses
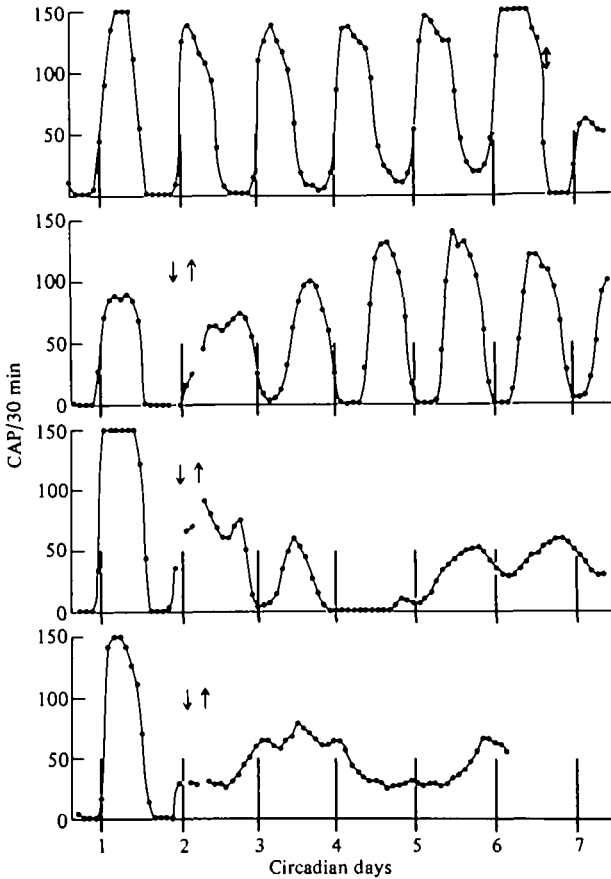
Fig. 7. CAP frequency rhythms at 17 °C for anisomycin (10^{-6} M) pulses given at circadian times 0–4. The normal control rhythm (top graph) is marked at each rapid CAP frequency onset by a solid vertical line which is carried over to the lower graphs to serve as a phase reference point. It is not an exact phase reference for all the graphs because of the normal variation in the period of the rhythms. In the second graph, a pulse of anisomycin given at CT 0 (midpoint of pulse) caused a delay in phase. In the third graph, the pulse given at CT 1 caused a delay in phase of the rhythm and unusual behaviour. The expected frequency onset on the second day after the pulse did not occur but it did on the third and subsequent cycles. In the fourth graph, the pulse given at CT 3·5 caused a delay but the frequency profile had three peaks. Time scale is circadian days, about 26·5 h. The first and sixth cycles on the top graph and the first cycle of the third graph are truncated at 150 CAP.

centred at circadian times from CT 5–24 gave such clean results. Pulses centred at CT 1–4 were more complicated. Graph 3 shows that a pulse centred at CT 1 caused the first cycle after the pulse to be double peaked and quite broad. The second cycle was reduced in amplitude and not delayed in phase as much as the rhythm in graph 2. The expected third cycle did not appear! The fourth and fifth cycles did appear and with a maintained delay in phase but the amplitude and waveform were not normal although the period was. The fourth graph shows a rhythm with a pulse centred at CT 4. A large delay is apparent and the cycle is very broad with three peaks, the first and third of which are a circadian period apart and the second peak coincides with the

peak timing in the graph above. Apparently, the rhythm has split with one component slightly advanced and the other component delayed so that they are about 180° out of phase. Subsequent cycles are complicated but the rhythm was apparently returning to normal at day 6 when it was prematurely terminated, unfortunately. Apparently, at the critical phases of CT 1–4, pulses act differentially on the constitutent components of the rhythm causing some to be shifted quickly to a new phase but allowing others to be affected less or shifted in the opposite direction. In subsequent cycles the disparate components appear to interact, causing the 'skipped beat' in graph 3 and the multi-peaked broad cycles in graph 4 of Fig. 7.

In all, 8 eyes were tested at phase 1–4. They all exhibited atypical post-pulse cycles as exemplified in Fig. 7. Two of the rhythms at 17 °C and 3 of the rhythms at 15 °C recovered sufficiently by the third post-pulse cycle so that a reliable phase delay could be measured. These delays are included in the phase response curve (Fig. 6).

<div align="center">DISCUSSION</div>

The period length of the eye rhythm is well compensated between 12 and 17 °C. The Q_{10} of 1·02 for that range of temperatures is similar to the Q_{10} of 1·07 for the range 15–22·5 °C previously measured by Benson & Jacklet (1977a). The average period length at 12 °C found in this study was 26·6 h compared to the average of 28·5 h in Benson & Jacklet (1977a). Two things account for the difference. In the former study the rhythm completed one cycle at 15 °C before it was stepped down to 12·5 °C and periods from the second to the eighth cycle were averaged. In the present study temperatures were lowered to 12 °C as soon as the eye was isolated into organ culture and only the first four periods were averaged. Later periods increased in length at all temperatures (Benson & Jacklet, 1977a) so the inclusion of those periods increases the average length. When eyes were placed at the new temperature immediately after dissection they appeared to adapt to the new temperature to a greater extent than if they were stepped to the new temperature one day later.

Anisomycin, 2-*p*-methoxyphenylmethyl-3-acetoxy-4-hydroxypyrrolidine, is a potent inhibitor of protein synthesis on eucaryotic ribosomes. It acts on the transfer reaction following the formation of aminoacyl transfer RNA (Grollman, 1967). It has been tested previously on *Aplysia* central neurones, where it inhibited protein synthesis by 90 % at 1·8 × 10⁻⁶ M but did not interfere with the traditional functions of the neurones such as resting potentials, action potentials, pacemaker activity and synaptic transmission (Schwartz *et al.* 1971). They found that sparsomycin and pactamycin also strongly inhibited protein synthesis but high concentrations of puromycin and cycloheximide were needed to inhibit synthesis. Procaryotic protein synthesis inhibitors, such as streptomycin which is routinely added to the organ culture medium in eye rhythm experiments, did not inhibit protein synthesis. In the *Aplysia* eye (Jacklet, 1977) the incorporation of leucine into protein was inhibited by 90 % by anisomycin. It has no detectable effect on the neurophysiological properties of the eye since the CAP duration and amplitude are unchanged and the CAP frequency are normal except for the clock-controlled modulation of the frequency. The studies of Schwartz *et al.* (1971) on *Aplysia* central neurones and those on the eye provide agreement on the potency of anisomycin in inhibiting protein synthesis and its lack of effects on the

normal neuronal activities. It appears to affect the clock specifically. On the other hand protein synthesis is poorly inhibited by cycloheximide in *Aplysia* neurons (Schwartz *et al.* 1971), and this correlates well with its poor phase-shifting effect on the *Aplysia* eye rhythm, except at quite high 500–2000 μg/ml concentrations (Rothman & Strumwasser, 1976) when it has side effects on neuronal activity. It is puzzling that two inhibitors, cycloheximide and anisomycin, found to be specific for 80 S ribosomal protein synthesis inhibition in other systems have such different effectiveness in the *Aplysia* system.

The phase response curves for anisomycin pulses in the *Aplysia* eye rhythm are similar to the curve for puromycin pulses (Rothman & Strumwasser, 1976). Both have largest advances at CT 5–8 and largest delays at CT 20–24. The phases near CT 0 are not as thoroughly explored with puromycin as they are with anisomycin, so it is difficult to evaluate them in detail. Anisomycin produced larger delays than puromycin but the advances are about the same magnitude. The anisomycin response curves are unlike those for light pulses (Jacklet, 1974; Benson & Jacklet, 1977c) in which maximum delays occur at CT 14–16 and maximum advances occur at CT 20–24. The anisomycin phase response curve is very similar in general shape and position to curves obtained for dinitrophenol and sodium cyanide by Eskin & Corrent (1977) and the curve for low-temperature pulses (M. Deuser & J. W. Jacklet, unpublished) with the exception that the maximum phase-shifts are less than half the maximums obtained for anisomycin. Dinitrophenol, cyanide and low-temperature treatments are expected to inhibit energy production directly and many energy-dependent processes indirectly, including the transport of ions and the synthesis of proteins.

In studies on the alga *Acetabularia*, anisomycin (10^{-6} M) inhibited protein synthesis by 40% and caused delays in the phase of the rhythm of O_2 production in preliminary experiments (Karakashian & Schweiger, 1976a). Cycloheximide (10^{-6} M) is also quite effective in inhibiting protein synthesis and provoking phase-dependent phase delays in that rhythm (Karakashian & Schweiger, 1976a, b). Although it had been previously concluded that protein synthesis was not involved in the circadian control of photosynthesis in *Acetabularia* (Sweeney, Tuffli & Rubin, 1967), the development of a method for continuously monitoring the O_2 production of *Acetabularia* cells by Mergenhegen & Schweiger (1973) allowed the protein synthesis requirement for the clock to be discovered. The evidence now supports the idea that protein synthesis on eucaryotic ribosomes provides an essential component of the circadian timing mechanism in *Acetabularia* (Karakashian & Schweiger, 1976a, b). The daily synthesis of protein appears to be required for the clock to function but the daily synthesis of RNA is not required in whole or enucleate *Acelabutaria* for the rhythm to persist (Vanden Driessche, Bonotto & Brechet, 1970). This suggests that a long-lived m-RNA must be available for the required daily translation and protein synthesis.

The phase response curves for protein synthesis inhibitor effects at 12, 15, and 17 °C in *Aplysia* are similar to those in *Acetabularia* (Karakashian & Schweiger, 1976b) at 20 °C, but very different from the curve at 25 °C. At 25 °C maximum phase delays occurred at CT 6, but at 20 °C maximum delays occurred 12 h later at CT 18 and minimum delays occurred at CT 6. In *Aplysia* the results were large delays at CT 18 and minimum delays (and advances) at CT 6 in agreement with the results on *Acetabularia* at 20 °C. Presumably 20–25 °C is within the physiological temperature

range for *Acetabularia* just as 12–17 °C is within the physiological temperature range for *Aplysia* and the periods of the rhythms are each compensated for those temperatures. The Q_{10} is 1·02 for the *Aplysia* rhythm and it may be about the same for rhythms of *Acetabularia* in the range 20–25 °C (Karakashian & Schweiger, 1966), but the rhythm is quite variable and the Q_{10} of 0·8 for 18–25 °C may be more typical (Karakashian & Schweiger, 1976c). The large difference in the phase of the sensitivity to cycloheximide at 20 and 25 °C was unexpected (Karakashian & Schweiger, 1976b) because the phase response curve of the rhythm to dark pulses was the same at 20 and 25 °C.

The *Aplysia* and *Acetabularia* results showed that they are very similar except for the disparate results at 25 °C in *Acetabularia*. This is encouraging, considering that the rhythm types are quite different. The *Acetabularia* rhythm is in the evolution of O_2 from photosynthesis conducted in constant light and the *Aplysia* rhythm is in the frequency of endogenous CAP activity recorded in darkness. If the disparate results at 25 °C could be explained, these results would argue for the necessity of a similar clock protein or polypeptide in both systems, and perhaps for circadian clocks generally. In *Acetabularia* the essential polypeptide is visualized as changing a membrane component of the circadian clock (Schweiger, 1977), and that function is quite compatible with its expected role in the *Aplysia* circadian clock.

The perturbations of the rhythm by anisomycin pulses are quite specific. It alters the phase of the rhythms but the waveform and period of the rhythm recovers to normal, so it is an excellent probe of the rhythm mechanism considering the criteria for pulse experiments (Tyson *et al.* 1976). The recovery of the normal periodicity after a pulse of anisomycin depended upon the phase of the rhythm at which the pulse was applied. Pulses given at CT 5–24 usually gave clean results in which the waveform was normal on the first post-pulse cycle but the period exhibited transient changes, being first shorter and then longer by 1–3 h before settling down to the normal periodicity by the third post-pulse cycle. Pulses given at CT 1–4 alter the waveform and period considerably as well as shifting the phase of the rhythm (Fig. 7). Parts or components of the rhythms seem to be phase-shifted to different extents by the pulse at these phases and subsequently these components, disparate in phase, interact in producing the waveform and period of the rhythm. This behaviour may be explained by assuming that the population of oscillators that make up the rhythm are slightly out of phase with one another. At the critical phase of CT 1–4 where phase shifts change abruptly from large delays to no effect, a pulse of anisomycin may fall on the delay phase of one group of oscillators and the no-effect phase of another group, producing a differential shifting of the groups. The actual waveform observed after a pulse is predictable, within limits, by knowledge of the phase at which the pulse was applied, suggesting a systematic fractionation of the rhythm by the pulse and a predictable interaction between the split components to shape the phase, period and waveform of the subsequent cycles. A single oscillator would not be expected to react this way. This result reinforces the conclusions from other studies (Jacklet & Geronimo, 1971; Benson & Jacklet, 1977b) that the *Aplysia* eye rhythm is the expression of a composite of cellular oscillators.

The effects of anisomycin on the circadian clock of the eye are consistent with the model oscillator proposed for the eye by Benson & Jacklet (1977c). This model has an

active, energy requiring, synthesis phase and a passive, relaxation, phase with the increase in CAP frequency occurring during the active phase and the decline in CAP frequency occurring during the passive phase. A chemical substance is synthesized during the active phase of the rhythm. It accumulates and when a certain level is reached, further synthesis is turned off. The sensitivity to pulses of anisomycin, as indicated by the amount of phase delay of the rhythm, is greatest during the active synthesis phase of the model. The action of the anisomycin would be to prevent the synthesis of the chemical substance (a necessary protein) and alter its rate of accumulation, causing a delay in phase. According to the model, the phase of sensitivity to low-temperature pulses, protein synthesis inhibitors, and inhibitors of energy production should be very similar since they act on the synthesis process. The results of Eskin & Corrent (1977) with dinitrophenol and cyanide, the results of Benson & Jacklet (1977a) on temperature, and the present results on anisomycin confirm this expectation. On the other hand, light affects the reference level for the chemical substance in the model and light should cause maximum advances at the same phase that large delays are caused by synthesis inhibitors. The results of light-pulse experiments (Jacklet, 1974; Benson & Jacklet, 1977c; Eskin, 1977) show this. The experimental results confirm the expectations from the model that there are at least two distinct types of phase response curves for the eye rhythm depending upon whether the active agent has its effect on the synthesis process, like anisomycin, or on the reference level, like light.

Anisomycin does not effect the basic bursting pacemaker mechanisms or neurophysiological properties of central neurones (Schwartz et al. 1971, and Fig. 3), and it does not have a direct effect on the CAP generation mechanisms of the eye either since the CAP activity continues in the presence of the inhibitor. Rather it does interfere with the modulation of the CAP frequency normally performed by the clock. If anisomycin exerts its phase-shifting effects by blocking protein synthesis, as expected, a protein or proteins synthesized on a daily basis must be engaged in a vital part of the clock timing mechanism because anisomycin pulses cause phase dependent phase-shifts of the clock and step experiments cause permanent changes in the period of the rhythm. These results are considered good evidence that the parameters or state variables of the clock are being altered rather than the hands of the clock (Tyson et al. 1976). However, whether the protein synthesis inhibitor is affecting the clock directly or indirectly through a chain of intermediates leading to the clock can not be unequivocally concluded until the actual clock variable shave been identified and the extent of the complexity of the clock is known (Tyson et al. 1976).

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