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PROTEIN SYNTHESIS REQUIREMENT OF THE *APLYSIA* CIRCADIAN CLOCK

TESTED BY ACTIVE AND INACTIVE DERIVATIVES OF THE INHIBITOR ANISOMYCIN

BY JON W. JACKLET

Department of Biological Sciences and Neurobiology Research Center, State University of New York at Albany, Albany, NY 12222, U.S.A.

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SUMMARY

1. The circadian rhythm of compound action potential frequency recorded from the isolated eye of *Aplysia* in culture medium and darkness was subjected to 6 h pulse treatments with either anisomycin, a protein synthesis inhibitor, or inactive derivatives of anisomycin.

2. Anisomycin caused phase-dependent phase shifts of the rhythm as expected from previous experiments, but none of the derivative molecules caused phase shifts or perturbed the rhythm.

3. Anisomycin inhibited eye-protein synthesis by 75% at the concentrations (10^{-6} M) used in the phase shifting experiments but none of the derivatives inhibited synthesis.

4. Only those molecules that actually inhibited protein synthesis caused phase shifts of the clock, although the inactive derivatives differed from anisomycin by only an acetyl group.

5. The results strengthen the conclusion that the inhibition of protein synthesis caused by anisomycin is important in perturbing the timing of the circadian clock and not some other characteristic effect of the inhibitor molecule. Together with the results from other systems, these findings imply that the daily synthesis of protein is a general requirement for circadian clocks.

INTRODUCTION

The proper timing of physiological events is crucial for an harmonious relationship between an organism and its environment and equally important for establishing temporal order among the many physiological processes that maintain the organism. One of the important physiological timers is the circadian clock (Pittendrigh, 1976), which is endogenous to the organism and may be reset by an appropriate 'time giving' environmental stimulus. Timing by the clock confers the definite advantage of allowing the organism to anticipate environmental change. It is not surprising that circadian clocks are a basic property of all eucaryotes.

The question of whether the proper functioning of the circadian clock is dependent upon protein synthesis and therefore can be perturbed by protein synthesis inhibitors

has been debated for more than a decade. A study (Feldman, 1967) showed the period length of the phototaxis rhythm of the flagellate *Euglena* was increased by the continuous application of cycloheximide. On the other hand, the effects of inhibitors on the clock of the dinoflagellate, *Gonyaulax*, were equivocal (Sweeney, 1969) and studies on the circadian rhythm of photosynthesis in the alga, *Acetabularia* (Sweeney, Tuffli & Rubin, 1967) suggested that the clock timing mechanism was independent of protein synthesis.

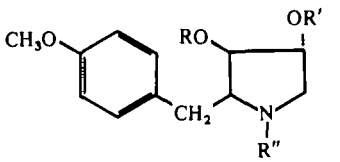
Support for the concept of required protein synthesis has come from recent experiments using protein synthesis inhibitors. In *Acetabularia*, a pulse of anisomycin or cycloheximide at low concentrations caused phase-dependent phase shifts of the O_2 evolution rhythm (Karakashian & Schweiger, 1976*a*, *b*). Phase-dependent phase shifts of the *Aplysia* eye rhythm were obtained with pulses of anisomycin (Jacklet, 1977) and similar responses occurred with puromycin (Rothman & Strumwasser, 1976). Even in *Gonyaulax* it is now known that inhibitors cause phase shifts of the rhythm (Dunlop *et al.* 1979).

Protein synthesis inhibitors are used to determine if new protein is required or how rapidly new protein must be synthesized for a function to continue. However, the inhibiting agent must be free of side effects on other cellular functions to conclude with certainty that the effects observed with the inhibitor are due specifically to inhibition of protein synthesis. This problem of specificity may be approached by using derivatives of inhibitor molecules that are slightly different in structure from the parent molecule and lacking in potency to inhibit protein synthesis.

A highly effective inhibitor of eurcaryotic protein synthesis is anisomycin (Grollman, 1967). It had proved to be remarkably free of side effects on neuronal functions, such as action potentials and synaptic potentials, when it was tested on the neuronal tissue of *Aplysia* (Schwartz, Castillucci & Kandel, 1971; Jacklet, 1977, 1979). To determine the specificity of the inhibitor effects on the circadian clock of the *Aplysia* eye derivatives of anisomycin were tested. Only those compounds that inhibited protein synthesis also caused phase shifts of the circadian clock.

METHODS

Aplysia californica (100–300 g) were obtained from Pacific Bio-Marine (Venice, CA) and kept in Instant Ocean aquariums exposed to light–dark cycles of 13–11 h maintained at 16–17 °C. Eyes with attached optic nerves were dissected from the animals 2–3 h after dawn and placed in 50 ml of culture medium, maintained thereafter at 17 °C in constant darkness. The optic nerve was drawn into a J-shaped tubing electrode (Jacklet, 1974) 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 or one of the derivatives (Pfizer) was dissolved in ASW and added to the culture medium at specific phases of the circadian rhythm in a pulse of six hours. After the



I: R = $COCH_3$;R',R'' = H (anisomycin)
II: R,R',R'' = H (deacetylanisomycin)
III: R,R'' = $COCH_3$; R' = H
V: R,R' = H; R'' = $COCH_3$
VII: R = $COCH_3$; R' = H; R'' = $(CH_3)_2$

Fig. 1. Structural formulas of anisomycin, 2-*p*-methoxyphenylmethyl-3-acetoxy-4-hydroxy-pyrrolidine, a protein synthesis inhibitor and several derivative (II, III, V, VII) not active in protein synthesis inhibition.

pulse treatment, the inhibitor solution was removed, the chamber was washed with 150 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. Another protein synthesis inhibitor, pactamycin, a gift from the Upjohn Company, was also tested. Pactamycin was first dissolved in acetone but otherwise handled the same as anisomycin.

Derivatives of anisomycin were obtained from Pfizer Inc, through the courtesy of Dr J. Beereboom. The parent compound, anisomycin, and four derivatives that are inactive in inhibiting protein synthesis (Grollman, 1967) were tested. The structural formulas are shown in Fig. 1 below.

The incorporation of tritiated leucine (New England Nuclear, NET-135H, specific activity 40–60 Ci/m-mol) into eye protein insoluble in trichloroacetic acid (TCA) was determined. Eyes from the same animal were compared. One was preincubated in ASW and buffer while the other was preincubated in ASW and buffer plus anisomycin or a derivative. After 15 min of preincubation eyes were placed in separate droplets containing labelled leucine. These droplets were prepared by drying down 20 μl of labelled leucine under vacuum and then dissolving it in 100 μl of ASW containing 30 mM Hepes buffer (pH 7·8). Eyes were incubated for 3 h in DD at 17 °C, then rinsed several times in ASW, rapidly frozen and homogenized. The homogenate was precipitated with 10% TCA, kept on ice 1 h, heated to 90 °C for 15 min, cooled and filtered on Gelman metricel membrane filters. Radioactivity was counted in a beckman LS-230 using 5 ml of Econofluor (NEN) scintillation fluid. Counting efficiency was 58%.

In culture medium and complete darkness at 17 °C the period of the rhythm is 26·1 ± 0·5 h (Jacklet, 1979) and on the first day in culture the CAP frequency increases to half its maximum rate of firing at subjective dawn (the solar 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 26 h cycle was normalized to 24 circadian hours. The phase of a 6 h pulse of inhibitor was designated in circadian hours at the mid-

point of the pulse (i.e. a pulse designated at CT 3 was actually started at CT 0 and ended at CT 6). The period of the rhythm was measured in hours between successive ½ maximum rate increases or between successive centroids (Benson & Jacklet, 1977*a*).

RESULTS

Tests of phase shifting the rhythm

Previous studies of the anisomycin treatment of the circadian rhythm in CAP frequency recorded from the *Aplysia* eye have shown that the inhibitor has no detectable side-effects on the mechanism for CAP generation (Jacklet, 1977, 1979). Only the circadian clock control of the periodic firing was affected. Pulses of anisomycin phase shifted the rhythm to an extent dependent upon the phase of the rhythm at which the inhibitor was applied, which resulted in a characteristic phase-response curve for anisomycin (Jacklet, 1977, 1979). Derivatives were therefore tested for effects at a number of different phases of the rhythm. The results were then compared with a paired control eye treated with anisomycin or a paired control eye treated with normal culture medium. Fig. 2 contrasts the effects of anisomycin to those of deacetylanisomycin after each compound was delivered to one of a pair of eyes from the same animal. Before addition of the inhibitor (↓) the eyes were firing at a similar rate and the rhythms were precisely in phase. During the treatment (centered at CT 0) the CAP activity of the deacetylanisomycin treated eye continued as expected for an untreated eye and the eye treated with anisomycin performed as expected (Jacklet, 1977, 1979) for a rhythm treated at that phase; the firing frequency was stabilized. Following the pulse treatment the deacetlyanisomycin eye behaved like an untreated eye; the period of the rhythm was not changed and the CAP rate was normal. In contrast, the rhythm of the anisomycin eye was delayed in phase by 8 h but regained the normal period and amplitude a few cycles after the treatment as expected (Jacklet, 1977, 1979). These results show that rhythms treated with deacetlyanisomycin are virtually unperturbed compared to the large phase delay affected by anisomycin. In order to test the derivatives more precisely, the deacetylanisomycin treated eyes were compared to a paired control eye treated by changing the normal culture medium only. One result is shown in Fig. 3. No consistent alteration of the deacetylanisomycin treated eyes could be detected; the periods were normal, the phase and amplitude were not changed beyond that expected for a pair of normal eyes (Jacklet, 1974, 1979). Other experiments on pairs of eyes treated similiarly to those illustrated in Fig. 2 and 3 are listed in Table 1. Normal/deacetylanisomycin pairs were tested at two phase points (CT 23·5 and CT 0·5) at which maximum phase changes were obtained for anisomycin pulses. No phase shifting by deacetylanisomycin was observed and the period of the rhythm was normal before and after the pulse. Deacetylanisomycin/anisomycin pairs were tested at phases where large delays were expected for anisomycin (CT 0, CT 0·5 and CT 2) and a phase at which no phase shifting by anisomycin was expected (CT 4). In each case the deacetylanisomycin treated eyes were not phase shifted or otherwise perturbed. Three other eyes were treated with derivatives (CPD's III, V, and VII) similarly and compared to a normal control (Table 1). These derivatives also did not change the rhythm.

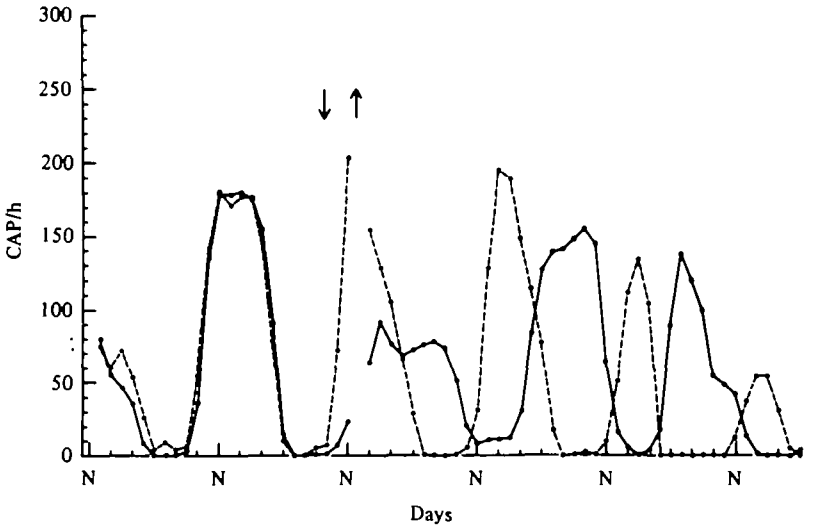


Fig. 2. Phase shifting by anisomycin (solid) but no effect by deacetylanisomycin (dashed) following a 6 h. 10^{-4} M pulse (arrows), centred at solar time 10 or CT 0, of the circadian rhythm of CAP activity. Time axis is marked in successive solar day noons (N) for pair of eyes from same animal at 17 °C in constant darkness.

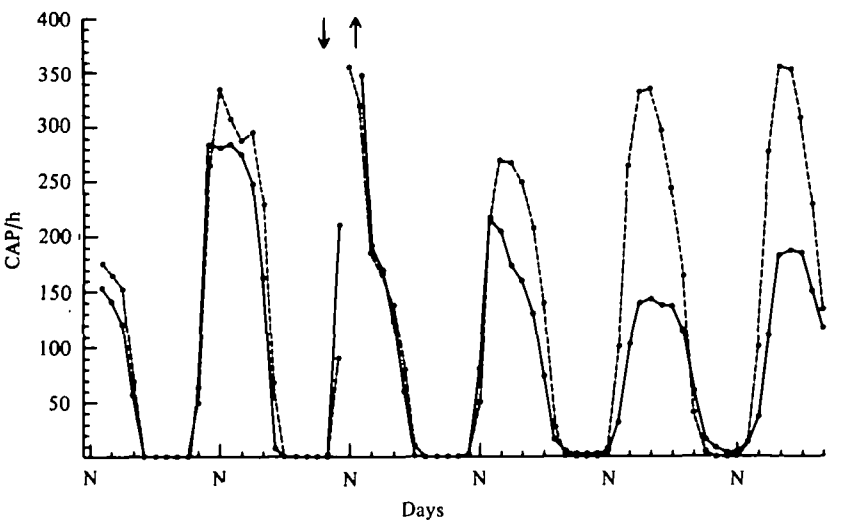


Fig. 3. Lack of phase shifting by either deacetylanisomycin (solid) or control solution (dashed) following a 6 h. 10^{-4} M pulse (arrows), centred at CT 0, of the circadian rhythm of CAP activity. Time axis in successive solar day noons (N) for pair of eyes from same animal. 17 °C in constant darkness.

Table 1. *Effects of inactive derivatives of anisomycin, Cpd II (deacetylanisomycin), Cpd III, V, and VII on the phase of the circadian rhythm*

(Period 1 was prior to the treatment, period 4 was after the treatment. Observed phase differences are measured at the second post-pulse cycle for the paired controls (six pairs) or unpaired control (CPD's III, V, VII). Expected phase differences are based on PRC to anisomycin (Jacklet, 1977, 1979) and expecting no effect by the derivative.)

Treatment (6h)		Period (h)		Phase difference (h)	
Type	Phase (CT) Midpoint	1	4	Observed	Expected if no effect
Normal	23·5	26·0	26·0		
Deacetyl.	23·5	26·0	26·5	0·5	0–1
Normal	0·5	26·0	27·0		
Deacetyl.	0·5	26·0	27·0	1	0–1
Deacetyl.	0·5	25·5	26·5		
Aniso.	0·5	25·5	26·5	−5	5–11
Deacetyl.	0	26·0	25·5		
Aniso.	0·5	26·0	27·0	−8	5–11
Deacetyl.	2·0	25·5	25·5		
Aniso.	2·0	25·5	26·5	−16	13–16
Deacetyl.	4·5	25·5	25·5		
Aniso.	4·0	25·5	25·0	0	0–1
Normal	0	25·5	25·5		
CPD III	0·5	25·5	25·5	0	0–1
CPD V	23·5	25·5	25·5	0	0–1
CPD VII	0	25·5	25·5	0	0–1

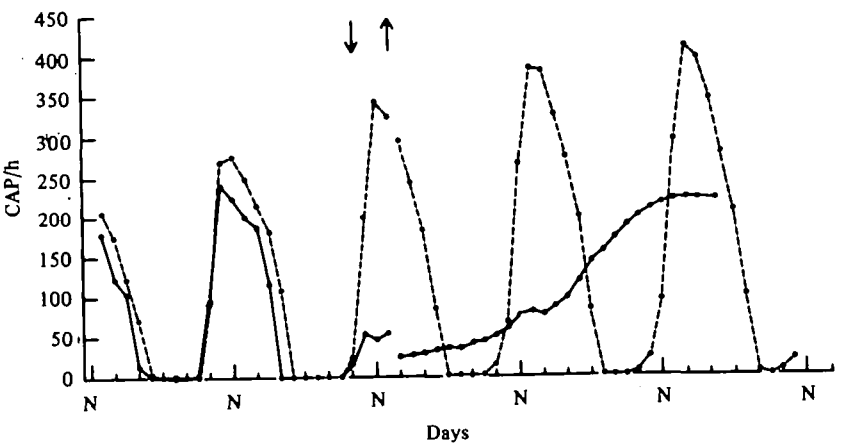


Fig. 4. Effect of pactamycin (solid) 2×10^{-6} M, following a 6 h pulse (arrows). The rhythm was suppressed and did not recover but the CAP activity continued. Time axis is marked in successive solar day noons (N) for pair of eyes at 17 °C in constant darkness.

Table 2. *Inhibition of tritiated leucine incorporation by anisomycin but not by derivatives at levels of* 10^{-6} M

Tissue	Treatment, (N)	cpm $\bar{X} \pm S$	Expt./control ratio
Eyes	Anisomysin (6)	9523 ± 5885	0·25
	Control (6)	40017 ± 22616	
	Deacetylanisomycin (6)	43454 ± 6536	0·93
	Control (6)	46716 ± 17411	
Buccal ganglia	Anisomycin (8)	21790 ± 15471	0·20
	Control (8)	107761 ± 50506	
	Deacetylanisomycin (6)	121677 ± 55229	1·33
	Control (6)	91662 ± 37348	
Pleural ganglia	Anisomycin (3)	16221 ± 6480	0·16
	Control (3)	100948 ± 13852	
	CPD III (9)	202512 ± 97974	1·14
	Control (9)	178181 ± 73349	

Another protein synthesis inhibitor, pactamycin, known to have a potent effect on protein synthesis in *Aplysia* central neurones (Schwartz, Castellucci, & Kandel, 1971) was also tested. It proved to be of only slight value in testing the rhythm since the rhythm did not recover from the treatment as shown in Fig. 4. The eye continued to be active after the treatment as evidenced by the continued CAP frequency but the rhythmic alterations in frequency that are the manifestations of clock control are absent. Such a result is hard to interpret. The clock may be stopped, uncoupled from the CAP generation mechanism or otherwise impaired.

Test of protein synthesis inhibition

Pairs of eyes from the same animal were compared for their ability to incorporate tritiated leucine into protein after one of the pair was treated with anisomycin or a derivative at 10^{-6} M concentrations. The results in Table 2 show that for six pairs of eyes the average leucine incorporation was 40017 cpm for normal controls and 9523 cpm for anisomycin treated eyes for a ratio of 0·25, or about 75% inhibition of protein synthesis by anisomycin. Deacetylanisomycin caused no significant inhibition of protein synthesis. Other neural tissue (buccal and pleural ganglia) were also tested and the results were similar; no inhibition of protein synthesis by deacetylanisomycin or CPD III as contrasted to 75–85% inhibition by anisomycin. The levels of incorporation of leucine for control tissues were 0·12 p-mol/h for eyes, 0·32 p-mol/h for buccal ganglia and 1·25 p-mol/h for pleural ganglia. Giant neurone R_2 of the abdominal ganglia of *Aplysia* incorporated 5 p-mol/h as a comparison (Schwartz *et al.* 1971). The variability of leucine incorporation was rather high even for paired eyes from the same animal. The reasons for it remain largely unexplained and a similar high variability was noted in studies of central neurones by Schwartz *et al.* 1971).

DISCUSSION

Protein synthesis is strongly inhibited (90%) by anisomycin at 10^{-6} M in HeLa cells, intact rabbit reticulocyte (Grollman, 1967) and in *Aplysia* central neurones (Schwartz, Castellucci & Kandel, 1971). The inhibition is reversible with synthesis

being quickly restored to 2/3 of the original value after 1 h of treatment (Grollman, 1967). Derivative of anisomycin tested by Grollman and used in the present study had no more than 1% of the inhibitory activity of the parent molecule even though the changes in structure of the derivative molecules involved the removal of only a single acetyl group in CPD II (deacetylansiomycin) or the addition of an acetyl group (CPD III). The finding that none of the inactive derivatives caused any phase shifting of the rhythm or produced any alterations of the normal periodicity considerably strengthens the argument (Jacklet, 1978) that it is indeed the inhibition of protein synthesis which causes perturbation of the clock by anisomycin.

The level of protein synthesis inhibition observed in this study (75% at 10^{-6} M) by anisomycin are quite comparable to that observed previously (90% at 2×10^{-5} M, Jacklet, 1977) and to the levels found by Schwartz *et al.* (1971) (90% at $1{\cdot}8 \times 10^{-6}$ M and 95% at 18×10^{-6} M) for central neurones. The derivatives caused no detectable inhibition in the eye when tested at 10^{-6} M. This result is expected from the work of Grollman (1967) where a 1000× increase in concentration of the derivatives was needed to obtain the inhibition produced by anisomycin.

The incorporation of tritiated leucine into eye protein in this study was good but variable, with standard deviations about one-third of the mean of 43000 cpm for 12 control eyes. However, considerable variability was noted by others as well. The level of about 12000 cpm in SDS gels obtained by Rothman & Strumwasser (1976) was comparable to counts obtained in this study and their radioactive carbon counts averaged about 2900 cpm with a standard deviation of about one-quarter of the mean. In *Aplysia* central neurones variability was also seen with an average of 79 p-mol ± 24 (one-third of mean) for 39 abdominal ganglia studied by Schwartz *et al.* (1971). The source of this disconcerting variability remains obscure.

Other protein synthesis inhibitors such as puromycin and cycloheximide cause phase-dependent phase shifts of the eye circadian clock (Rothman & Strumwasser, 1976). The phase-response curve for puromycin from that study is very similar to the one for anisomycin (Jacklet, 1977, 1979) with the exception that the phase delays are limited to 6 h as compared with 15 h with anisomycin. The concentration of puromycin used to generate the phase response curve was rather high ($2{\cdot}3 \times 10^{-4}$ M) and some side-effects on the eye were evident. An explanation of the weak phase shifting effects of puromycin is that protein synthesis was also weakly inhibited. It required 20 μg/ml for 50% inhibition, according to Rothman & Strumwasser (1976), but in *Aplysia* central neurones Schwartz *et al.* (1971) found no inhibition at 80 μg/ml. Puromycin, then, would not be expected to cause phase shifts at low concentrations since protein synthesis is poorly inhibited. Cycloheximide is even less effective both as a phase shifting agent and as a protein synthesis inhibitor in *Aplysia*: Rothman & Strumwasser (1976) found 500 μg/ml of the chemical inhibited protein synthesis by 50% and caused phase delays at the same concentration while Schwartz *et al.* (1971) found no inhibition of synthesis at 60 μg/ml. Thus at levels where synthesis inhibition is 50% or so, phase shifts in the rhythm are seen, but where inhibition is slight, phase shifts are small or lacking.

Among the inhibitors, other than anisomycin, found to be effective at low concentrations on *Aplysia* central neurones were pactamycin and sparsomycin. Pactamycin

tested on the eye at 2×10^{-5} M was effective in altering the eye circadian rhythm without undue influence on the CAP or the short-term pacemaker activity. It altered the rhythm profoundly since it did not recover to its normal cyclic activity after the removal of the inhibitor. These results are not readily interpretable. It would be of considerable interest to know if the protein synthesis ability recovered. If it did not, the results would be consistent with the necessity of protein synthesis for proper clock timing.

The mode of action of anisomycin is to specifically block peptide bond formation and thus prevent the transfer reaction of protein synthesis. It acts on the same step as puromycin (Vasquez, 1974), but its action can be distinguished from that of puromycin since polyribosomes remain intact and nascent peptides remain attached during inhibition by anisomycin (Grollman, 1967). It binds specifically to the 60 S subunit of eucaryotic ribosomes (Vasquez, 1974) and may be acting on peptidyl transferase. Knowing the site of action of the inhibitor is useful for a first approximation of the necessary pathway for the circadian clock timing but it does not help much in the specific localization of the clock. Pertubations of the pathway leading to the timing mechanism may have effects on the clock that can not presently be unequivocally interpreted in terms of specifying the mechanism or exact site of the clock (Tyson *et al.*, 1976). That must await further penetrating inquiry.

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