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INHIBITORS OF PROTEIN SYNTHESIS ON 80s RIBOSOMES PHASE SHIFT THE *GONYAULAX* CLOCK

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SUMMARY

One-hour pulses of anisomycin (0·3 μM), streptimidone (30 μM) and cycloheximide (5 μM) caused strong phase-shifts (either advances or delays, of up to 12 h) in the circadian rhythm of the bioluminescence glow in the marine photosynthetic dinoflagellate, *Gonyaulax polyedra*. Similar pulses of emetine (0·1–100 μM) caused small (< 4 h) phase shifts. Drug pulses have quantitatively different effects when applied at different phases of the circadian cycle, thus giving rise to 'phase response curves' (PRC's). The results lend additional support to the generalization, based on results from several different organisms, that 80s ribosome protein synthesizing system is of key importance in the mechanism responsible for circadian rhythms.

INTRODUCTION

Although it is still not possible to specify unequivocally the key biochemical process(es) responsible for the cellular circadian clock, there is now much evidence to indicate that protein synthesis on 80s ribosomes (but not on 70s) is essential for the proper functioning of the timing mechanism. This is shown by the alterations in the phase and/or period of circadian rhythms induced by inhibitors specific for protein synthesis on 80s ribosomes. Such effects have been reported in *Euglena* (Feldman, 1967), *Acetabularia* (Karakashian & Schweiger, 1976*a*, *b*), *Aplysia* (Rothman & Strumwasser, 1976; Jacklet, 1977), *Neurospora* (Nakashima, Perlman & Feldman, 1981*a*) and *Gonyaulax* (Karakashian & Hastings, 1963; Walz & Sweeney, 1979; Dunlap, Taylor & Hastings, 1980; Rensing *et al.* 1980). Several different 80s inhibitors have been shown to be effective, and while it is possible that side effects (i.e. sites of action other than 80s ribosomes) could be responsible for their phase-shifting capabilities, the fact that the inhibitors have different structures and specific molecular mechanisms (Vasquez, 1979) argues against it. Furthermore, in a cycloheximide-resistant mutant of *Neurospora* (where the lesion is ribosomal), the phase of the circadian rhythm is unaffected by cycloheximide (Nakashima *et al.* 1981*b*).

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In our earlier work, pulses of cycloheximide were found to cause dose and duration-dependent phase shifts, which both advanced and delayed the glow rhythm of *Gonyaulax* (Dunlap *et al.* 1980). We here describe experiments using two other inhibitors of eukaryotic protein synthesis: anisomycin and streptomidine. These, along with cycloheximide, can result in 'strong' (type 0) phase shifting (Winfree, 1980). The data lend further support to the postulate that either some protein(s) periodically synthesized on 80s ribosomes, or the protein synthesizing system itself are essential parts of the clock mechanism (Schweiger & Schweiger, 1977).

MATERIALS AND METHODS

The cells used in these experiments (*Gonyaulax polyedra*, strain 70) were grown on light-dark cycles (LD 12:12; temperature, 19 ± 2 °C; light intensity, 150 micro-einsteins (μE) m^{-2} s^{-1}) in f/2 medium (Guillard & Ryther, 1962) as previously described (Dunlap *et al.* 1980). Cell number densities were determined using an electronic cell counter (Coulter Electronics, Hialeah, FL).

Ten ml aliquots of cells (in mid-log phase, about 5000 cells ml^{-1}) were transferred during the middle of the light period to glass scintillation vials and kept in LD for an additional 18 or 42 h before transfer (at end of the dark period) to the computerized glow measurement system. This automated system, controlled by an Apple II microcomputer (Apple Computer, Cupertino, CA), measures the glow of up to 60 vials of cells under constant conditions, with a determination for each vial every 15 or 20 m for up to at least 2 weeks (Dunlap *et al.* 1980; Taylor *et al.* 1981). With this system, glow peaks can be estimated with a precision of 10–30 m, depending on the strength and clarity of the signal.

Cycloheximide and puromycin were obtained from Boehringer–Mannheim. Streptimidone was provided by Dr Henry Dion of the Warner–Lambert Company, and anisomycin and its derivatives II, III, V, and VII (see Jacklet, 1980*b*) by Nathan Belcher of Pfizer. Phenylmethylsulphonylfluoride (PMSF) and leupeptin were obtained from Sigma. Since cycloheximide and anisomycin (and probably their derivatives) are degraded at alkaline pH (Cooney & Bradley, 1961; Tanner *et al.* 1954–5), stock solutions of these drugs were frozen in small aliquots and thawed just before addition to the cells. In order to terminate a drug pulse, the drug was removed by centrifuging cells for 3 m at low speed in a clinical centrifuge, aspirating the supernatant, and resuspending cells in fresh f/2 medium. Controls (to which no drug was added) included both those which were centrifuged and resuspended in fresh medium, and those not centrifuged at all. As previously reported (Hastings, 1960; Dunlap *et al.* 1980), there were some phase shifts due to centrifugation alone, but these were small (1 h or less) and not definitively systematic. Actually, in many such controls the first peak after centrifugation was delayed substantially (up to 4 h) in experiments carried out at all times of the circadian cycle, but these were largely transient shifts which decayed to 1 h or less by the fourth peak. For this reason, and because variability in the phase determination was increased after centrifugation, arguments can be made for utilizing uncentrifuged controls for calculations of phase shifts due to drugs. In some cases we have done this. In all cases we have estimated phase shi

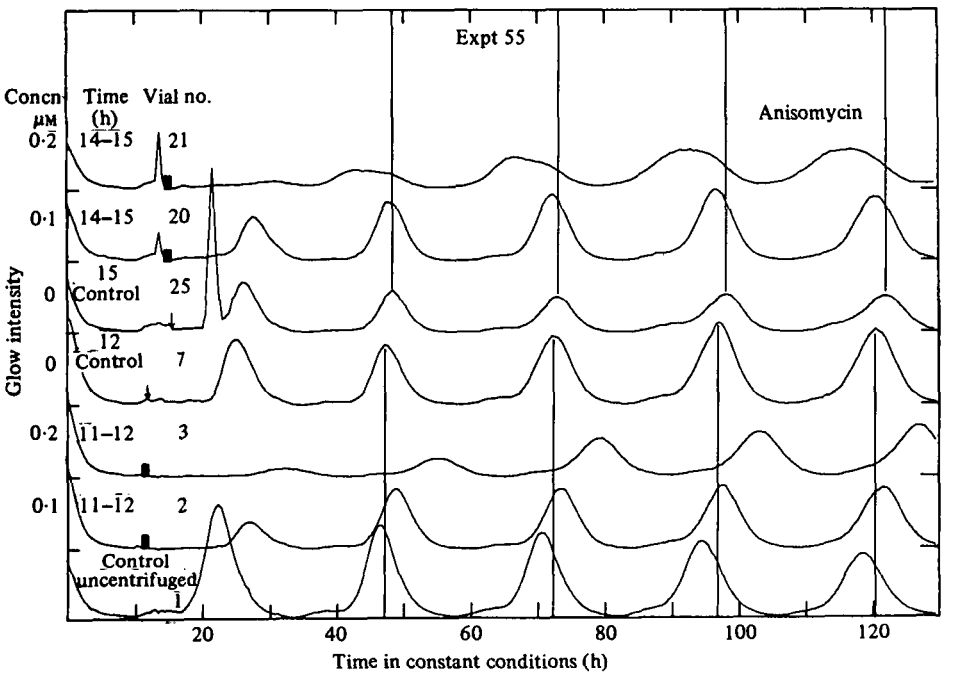

Fig. 1. Records of the spontaneous glow of bioluminescence in *Gonyaulax polyedra*, illustrating phase shifting of the circadian rhythm by pulses of anisomycin. Data points taken about every 20 m. Experiments are shown in which phase shifts in the rhythm were produced by 1 h pulses of 0·1 μM (vials 2 and 20) and 0·2 μM (vials 3 and 21) anisomycin. Vertical lines indicate positions of centrifuged control peaks (vials 7 and 25). Drug pulses given between h 11 and 12 (vials 2 and 3; bars on abscissa) after transfer of the cells to constant conditions resulted in phase delays as compared to the uncentrifuged control (vial 7); pulses given from h 14 to 15 resulted in phase advances compared to the centrifuged control (vial 25). As compared to a control that was not centrifuged (vial 1), the centrifuged controls show small phase shifts following larger transients at the outset. Light intensity, 35 μE m^{-2} s^{-1}, temperature 19 °C. (Experiment 55.)

by using peaks occurring after the third cycle subsequent to a drug pulse, thereby excluding transients, both those due to centrifugation and those due to the drug pulse itself (see text).

RESULTS

As judged by its ability to phase shift and, at certain times and doses, to alter the character of the circadian rhythm in *Gonyaulax*, anisomycin was the most potent of the drugs tested. Simple phase shifts (i.e. ones without apparent alteration in the subsequent shape of the typical rhythmic pattern) are illustrated by most of the experiments in Fig. 1. All of these shifts were produced by 1 h pulses of 0·1 or 0·2 μM anisomycin, starting at either h 14 (which resulted in apparent advances) or h 11 (which produced delays). These are experiments in which the distinction between advances and delays appears to be unambiguous by one of the usually adopted criteria (Winfree, 1980), namely treatment of the cells with the higher of the two inhibitor concentrations during the given time period resulted in a quantitatively greater phase

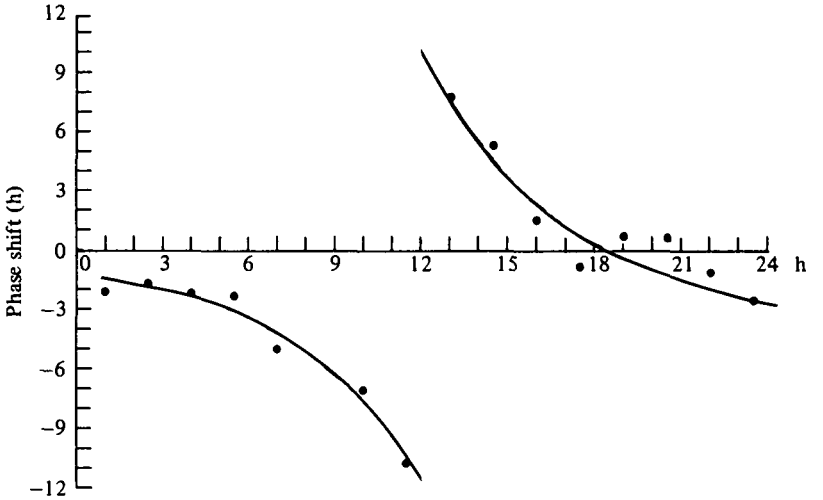

Fig. 2. A phase response curve for 1 h pulses of 0·3 μM anisomycin. Abscissa, beginning of time of pulses in hours since cells were transferred to constant conditions; ordinate, phase shift in hours; positive phase shift denotes advances. Temperature and light intensity as in Fig. 1. (Experiment 75.)

shift in the given direction. However, a distinction between phase delays and advances (based on either this or other criteria) was not always readily possible for shifts caused by anisomycin pulses (and also by pulses of some other inhibitors, see below), at various concentrations and/or at different times of day.

In several instances the rhythmic output exhibited an atypical wave-form after drug pulses (e.g. Fig. 1, vial 21). These often occurred in experiments in which the phase after the shift appeared to be located at or near the 'breakpoint' between delays and advances. Indeed, the occurrence of such peculiar wave-forms may be a good indicator of the position of the breakpoint.

Similar experiments carried out at several different times during the circadian cycle, but all with the same drug concentration, allow us to plot a phase-response curve (PRC) for anisomycin (Fig. 2). At the concentration (0·3 μM) and duration (1 h) used, the breakpoint between advances and delays appeared to occur at about h 12.

Fig. 3(*a*) illustrates experiments in which the concentration of the anisomycin pulses between h 8 and 10 was varied (0·01–5 μM). From these data, along with those in a similar experiment using pulses between h 12 and 14, the relationships between drug concentration and phase shifts (plotted as delays) for 2 h pulses is shown (Fig. 3*b*). The function does not exhibit clear saturation over the range for which data are available. Cells did not survive at the highest dose (5 μM; Fig. 3(*a*), vial 8), even though the cells were exposed to the drug for only 2 h. Extrapolation to zero phase shift falls at a dose of about 0·1 μM, which is ineffective in phase shifting.

Fig. 4 shows the results of pulses of different durations ranging from 10 min to 10 h, all with 1 μM anisomycin. Even the 10 min exposure was effective and the function over this full range is plotted in Fig. 4(*b*). Extrapolation of the line calculated as the best fit to the line indicates that zero phase shift occurs with a pulse duration of 0·05 m. This indicates that washout is complete at this concentration and that t

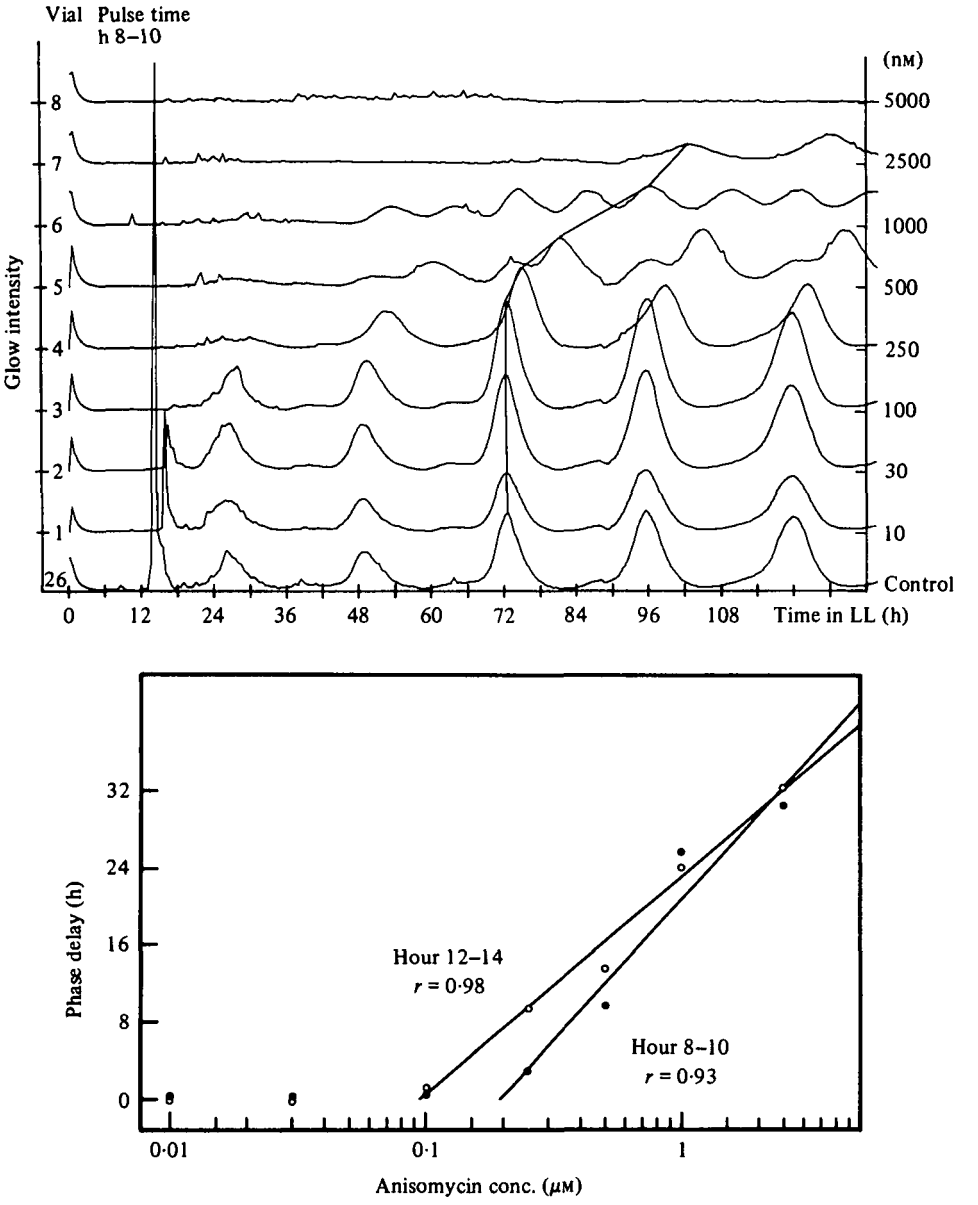

Fig. 3. (*a*). The effect of various doses of anisomycin (0·1–5 μM) given from h 8–10 (time since transfer of the cells to constant conditions) upon the phase of the glow rhythm. Abscissa, hours the cells have been in LL. Ordinate, glow intensity in arbitrary units. As a visual aid to following the phase shifts, a line has been drawn connecting what we believe to be the 3rd peak of all of the vials.

(*b*) Semilogarithmic plot of data from (*a*) and also additional data from pulses given from h 12–14, showing the dependence of the phase shifting effect on anisomycin concentration. Abscissa, anisomycin concentration (μM). Ordinate, phase delay in hours. The regression line for the h 12–14 points does not include the first two points at that time (0·01 and 0·3 μM) and the regression line for h 8–10 points does not include the first three points at that time (0·01, 0·3 and 0·1 μM).

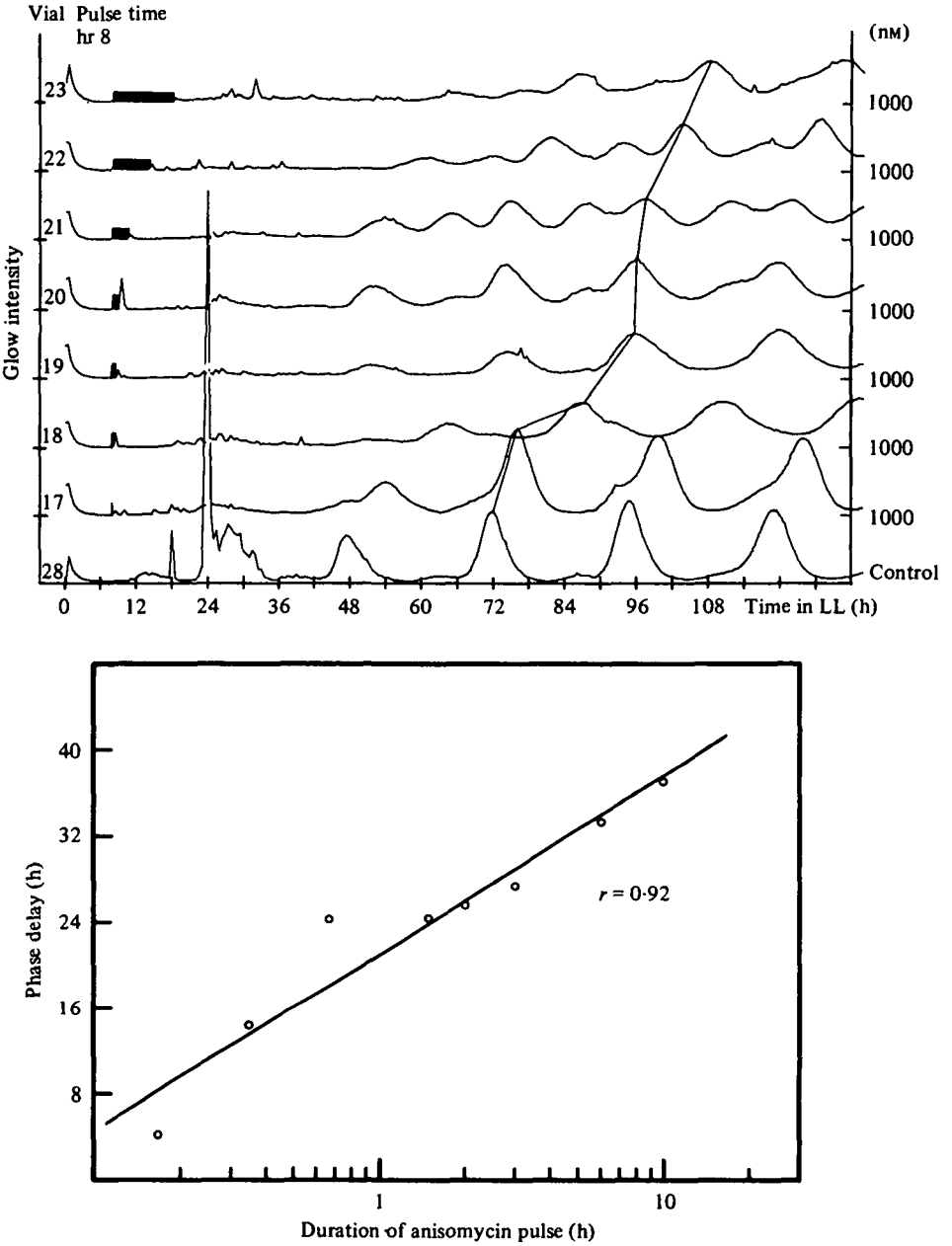

Fig. 4. (*a*) The effect of anisomycin pulses (1 μM) of varying duration (all starting at h 8) on the phase of the glow rhythm. Abscissa, hours the cells were in LL. Ordinate, bioluminescent glow in arbitrary units. A reference line is added to denote what we believe are corresponding peaks.

(*b*) A semilogarithmic replot of the data from (*a*). Abscissa, duration of anisomycin pulse (1 μM starting at h 8) in hours. Ordinate, phase delay in hours. Extrapolation of the regression line to 0 phase delay (not shown) predicts that 3 m pulses at this concentration should give 0 phase shift.

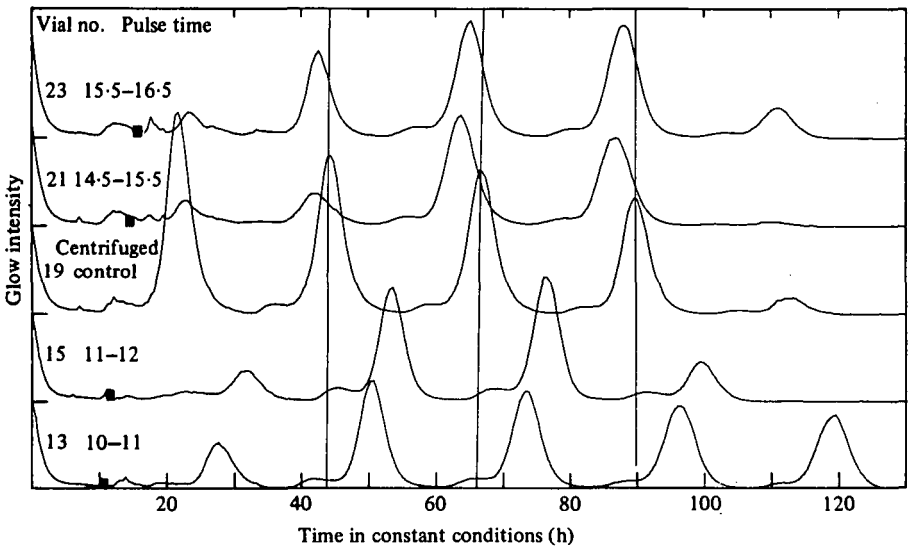

Fig. 5. Experiments showing advance and delay phase shifts of the *Gonyaulax* glow rhythm produced by 1 h pulses of 30 μM streptimidone. Pulses that started 10 and 11 h after transfer of the cells to constant conditions (vials 13 and 15) resulted in phase delays, while those initiated at h 14·5 and 15·5 resulted in phase advances (vials 21 and 23). Control, not centrifuged. Temperature and light intensity as in Fig. 1. (Experiment 66.)

drug action in causing phase shifting is terminated at the time of its removal from the culture medium.

Both Figs. 3(*a*) and 4(*a*) show atypical wave-forms, notably ones with apparent double peaks. These occur at intermediate doses or periods of exposure, more typical oscillations appearing with higher doses and durations of exposure.

Streptimidone, a glutarimide antibiotic, is another inhibitor of protein synthesis on 80s ribosomes, which is similar in structure and mode of action to cycloheximide (Kohberger *et al.* 1960; Pestka, 1971; Vasquez, 1979). It was also very potent in its ability to cause both advances and delays without pronounced alteration in the character or pattern of the rhythm (Fig. 5). The PRC resulting from such experiments is shown in Fig. 6; although the breakpoint is at about the same time (h 12) as for anisomycin (Fig. 2), this may differ depending on the concentration and duration of the drug pulse (W. R. Taylor, R. Krasnow, J. C. Dunlap, H. Broda & J. W. Hastings, unpublished observations).

Experiments in which cells were exposed to 1 h pulses of 1 μM cycloheximide were previously reported to result in both advances and delays (without altering the character or the rhythm), and with a breakpoint at about h 15 (based on time of beginning of pulse; Dunlap *et al.* 1980). Since we have used the procedure of beginning drug pulses at any point from the time of transfer of cells to constant conditions and 36 h later, it was of interest to continue determinations of a PRC for a given phase-shifting agent under the same conditions over the first two cycles in constant conditions (Fig. 7). The response to cycloheximide (and thus the progress of the circadian oscillator) is the same in the second cycle of constant conditions as in the first, except for a transient during the initial hours of the first cycle. Thus, for the

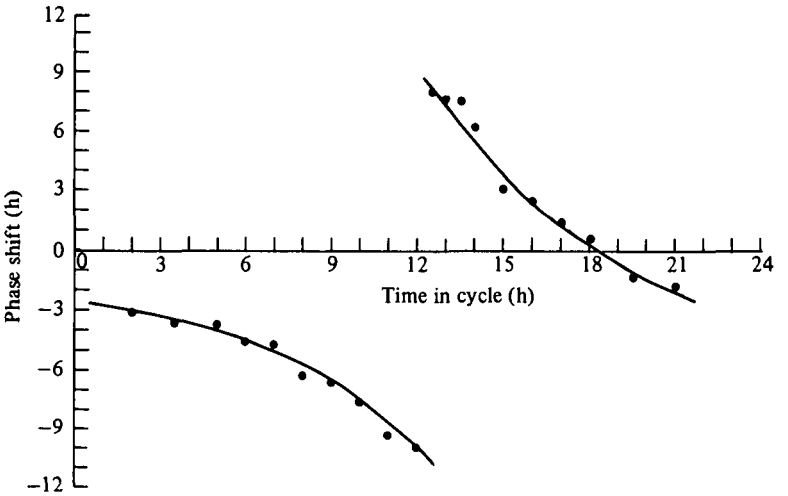

Fig. 6. A phase-response curve for 1 h pulses of 30 μM streptimidone. Abscissa, beginning of time of pulses in hours since cells were transferred to constant conditions. Ordinate; phase shift in hours. Temperature and light intensity as in Fig. 1. (Experiment 68.)

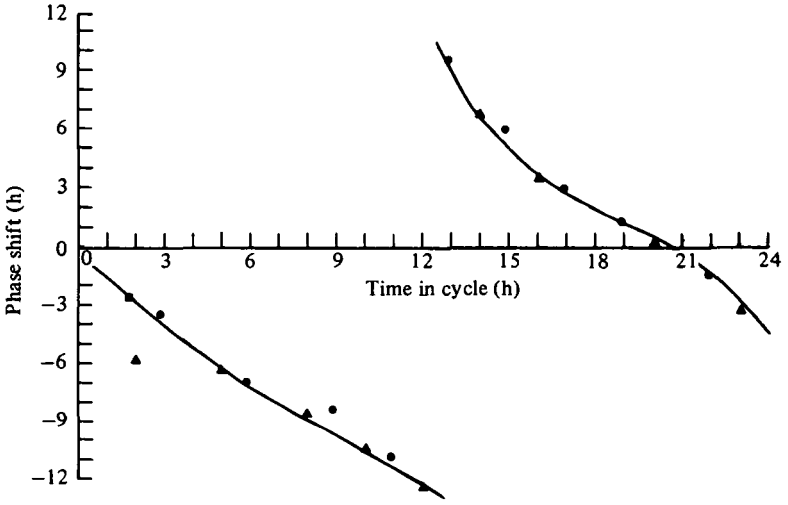

Fig. 7. A phase response curve over more than two cycles for 2 h pulses of 5 μM cycloheximide. Ordinate, phase shift in hours; abscissa, time in hours, modulo tau (23·2 h). ▲, first cycle; ●, second cycle; ■, third cycle. Temperature and light intensity as in Fig. 1. (Experiment 44.)

purpose of determining the PRC for different drugs, pulses given at any time in the first two cycles should give comparable results if calculated and plotted in circadian time. With regard to the transient, Krasnow *et al.* (1980) showed that the release from light–dark cycles to constant conditions elicited transients in both the glow and spontaneous flashing rhythm, that these differed quantitatively for the two rhythms, and that their magnitude depended on the light intensity of the constant conditions used. Since the glow and spontaneous flashing transients were different, and of opposite sign, a transient in the underlying pacemaker must be smaller, as the present two-cycle PRC demonstrates.

As shown in Fig. 7, the apparent breakpoint in these experiments was at an earlier time after the beginning of constant conditions (12 h) than in the previous ones (Dunlap *et al.* 1980). This difference may be attributed to the higher concentration (5 μM *v.* 1 μM) and the longer pulse duration (2 h *v.* 1 h) used in the present study.

Puromycin (which is also an effective inhibitor of protein synthesis on 80s ribosomes) was previously reported to block cell division and to result in the abolition of the rhythmic output (Karakashian & Hastings, 1962, 1963). In those experiments, puromycin (20 μM) was given in step doses (applied and not removed); the luminescent glow was stabilized at a low level, at least for the 4 days of observation subsequent to the addition of puromycin. Pulses of puromycin (10 μM, 8 h) caused small (< 4 h) delay shifts, and were most effective at h 0–8, somewhat less at h 8–16. The shifts were judged to be 'characteristic and significant, suggesting that puromycin may act at a step closely related to the rhythmic system' (Karakashian & Hastings, 1963). In the present experiments, 4 h puromycin pulses (30 μM) were tested and confirmed to cause only small (2–3 h) shifts, with the most effective time being centred at h 10. It is difficult to know why larger phase shifts did not occur; it may be that higher concentrations are needed. Alternatively, it may be due to the fundamentally different mode of action of puromycin in inhibiting protein synthesis: it causes premature chain termination of nascent polypeptide (Vasquez, 1979). Stahr, Holzapfel & Hardeland (1980) have reported larger shifts of the *Gonyaulax* glow rhythm using puromycin pulses, but the pulses were longer. Phase shifts of 8 h required 8 h pulses.

Pulses (0·1–100 μM) of emetine, another inhibitor of 80s ribosome function (Vasquez, 1979), caused only small phase shifts (< 4 h; data not shown). The results were similar to those obtained with puromycin. Emetine (unlike cycloheximide, anisomycin, streptimidone, and puromycin) has been reported to be an irreversible inhibitor of protein synthesis in HeLa cells (Grollman, 1968). If such irreversibility occurs in *Gonyaulax*, then emetine pulses should give results similar to those obtained with continuous exposure to cycloheximide and anisomycin, a result we indeed obtained.

If the suppression of the *expression* of the rhythm by constant exposure to puromycin (Karakashian & Hastings, 1962, 1963) is due or related to its inhibition of protein synthesis on 80s ribosomes, then other such inhibitors might be expected to act similarly, and they did (Fig. 8). Continuous exposure starting at h 4 to both anisomycin and cycloheximide at concentrations ranging from 10 to 300 nM resulted in phase shifts (delays), which were more pronounced at higher concentrations, and, at the highest concentrations of anisomycin, a suppression of rhythmicity similar to that found with puromycin. Although cycloheximide was not tested at higher concentrations in this experiment the rhythmic feature of the glow is already markedly suppressed at 100 nM.

A feature of the experiments of Fig. 8 that differs from earlier results with other organisms is that the period of the *Gonyaulax* rhythm during constant exposure to the drugs was not greatly altered (Table 1). The first reported effect of cycloheximide on a circadian rhythm was a lengthening of the period of the phototactic period in *Euglena* (Feldman, 1967). The drug may be less stable in our medium, but the effect in *Euglena* was substantial and readily detectable. We did not see it in *Gonyaulax*. Similar treatments with anisomycin have also been shown to have a large effect on the period of the rhythm in *Aplysia* (Jacklet, 1980*a*), and in that case the artificial

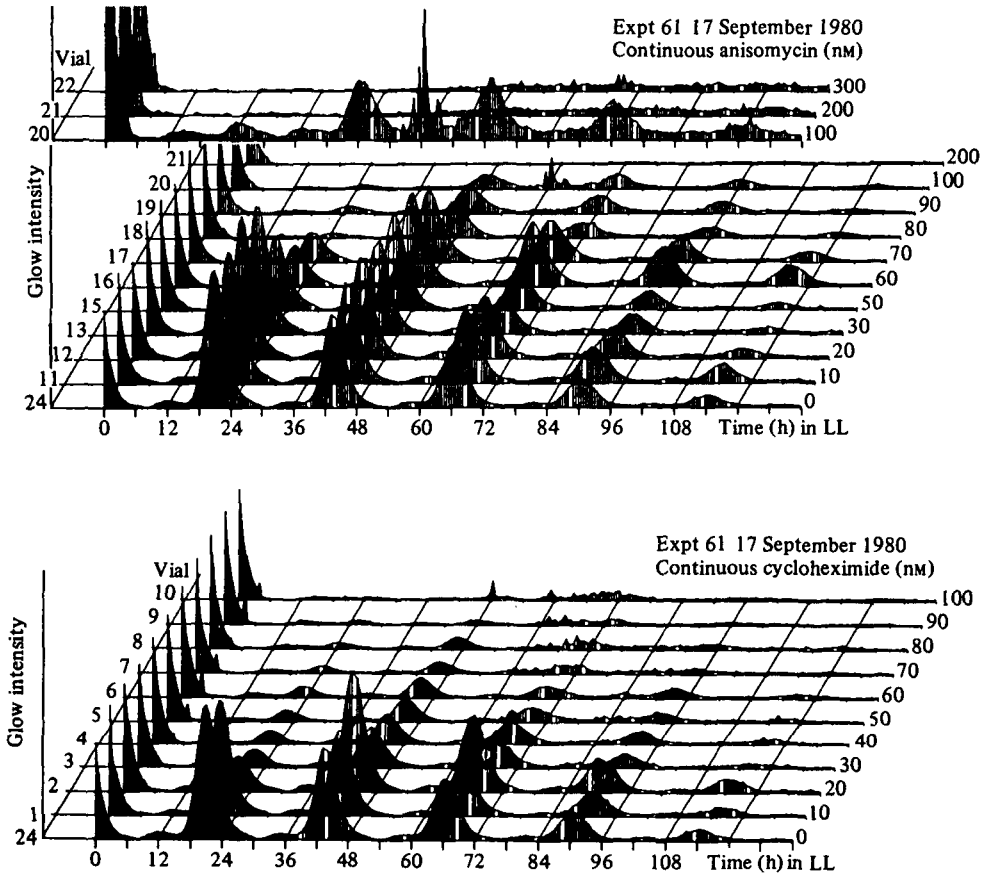

Fig. 8. The effect of continuous exposure (starting at h 4) to various concentrations (as noted on plots) of anisomycin (A) and cycloheximide (B) on the phase and amplitude of the glow rhythm. Abscissa, hours after transfer to conditions of constant dim light (LL; 35 μE m^{-2} s^{-1}) and temperature (19 °C).

sea-water medium was not so different from ours, so it is not so easy to attribute the different result to instability of the drug.

If inhibition of protein synthesis causes the phase shifting, then analogues of the inhibitors that are inactive for protein synthesis inhibition should be inactive in phase shifting. Jacklet (1980*b*) found that four such analogues of anisomycin had no effect on phase in the *Aplysia* system. We have tested these same analogues using 4 h pulses covering the entire circadian cycle (at concentrations of 0·1, 0·3, 1·0 and 3 μM) and also found them to be inactive in causing phase shifts of the *Gonyaulax* glow rhythm. Pulses at even the highest concentrations gave glow rhythms indistinguishable from controls, in clear contrast to anisomycin itself. Experiments with continuous exposure were not carried out. These experiments therefore provide additional evidence for the involvement of protein synthesis in the circadian mechanism.

If protein *synthesis* plays a critical role in the circadian mechanism, then intracellular *degradation* of protein might also be involved. A number of different speci

Table 1. *Lack of effect of cycloheximide (vials 1–10) and anisomycin (vials 11–23) upon the period of the glow rhythm in* Gonyaulax *under constant conditions (data is taken from experiment 61, some results of which are plotted in Fig. 8)*

Vial no.	Conc. (nM)	No. of peaks	Average period length ± S.D.
1	10 CHX	5	23·65 ± 0·65
2	20 CHX	5	23·18 ± 1·02
3	30 CHX	4	23·47 ± 1·40
4	40 CHX	4	23·43 ± 1·25
5	50 CHX	4	23·67 ± 0·91
6	60 CHX	4	23·57 ± 1·11
7	70 CHX	4	23·50 ± 1·22
8	80 CHX	2	22·40 ± —
9	90 CHX	2	22·40 ± —
10	100 CHX	2	23·50 ± —
11	10 ANI	5	23·32 ± 0·85
12	20 ANI	5	23·73 ± 0·85
13	30 ANI	4	23·73 ± 0·85
14	40 ANI	4	23·53 ± 0·45
15	50 ANI	5	23·85 ± 0·97
16	60 ANI	5	23·50 ± 0·99
17	70 ANI	5	23·52 ± 1·19
18	80 ANI	5	23·90 ± 0·72
19	90 ANI	5	23·93 ± 1·04
20	100 ANI	4	23·77 ± 0·83
21	200 ANI	—	— —
22	300 ANI	—	— —
23	1000 ANI	—	— —
24	0	5	23·38 ± 0·62
25	0	4	23·83 ± 0·57
26	0	5	23·83 ± 0·78
27	0	5	23·53 ± 0·75
28	0	5	23·50 ± 0·73
29	0	5	23·50 ± 0·65
All controls with no. 25		29 (23)	23·504 ± 0·627 ± 0·131 (S.E.)

models that include synthesis and degradation as important parts of the oscillator could be proposed. A possible way to test such models would be to inhibit intracellular protein breakdown at different phases of the circadian cycle. We carried out such experiments with the protease inhibitors leupeptin (0·5–25 μM) and PMSF (3 μM to 3 mM); there were no phase changes in the glow rhythms following their pulsed (4 h) application. With leupeptin, the amplitudes of the glow peaks were unaffected, indicating that the inhibitor might not have penetrated the cells. However, glow amplitude was clearly affected by PMSF, suggesting that it did penetrate the cells.

Although the effects of cycloheximide, streptimidone and anisomycin on the phase of the glow rhythm indicated that they had penetrated cells, it was desirable to ascertain if they were capable of inhibiting growth, an expected consequence of the inhibition of protein synthesis. Side effects (e.g. inhibition of DNA synthesis, Grollman, 1967) may also be induced by protein synthesis inhibition. Growth curves for cultures exposed to varying concentrations (continuous exposure) of the three drugs are shown in Fig. 9. Streptimidone inhibited growth by more than 50% at 1 μM, and completely at 3 μM. Anisomycin was found not to inhibit gowth at 0·03 μM, but to block growth at 0·1 and 0·3 μM; it resumed after several days (a longer delay at the

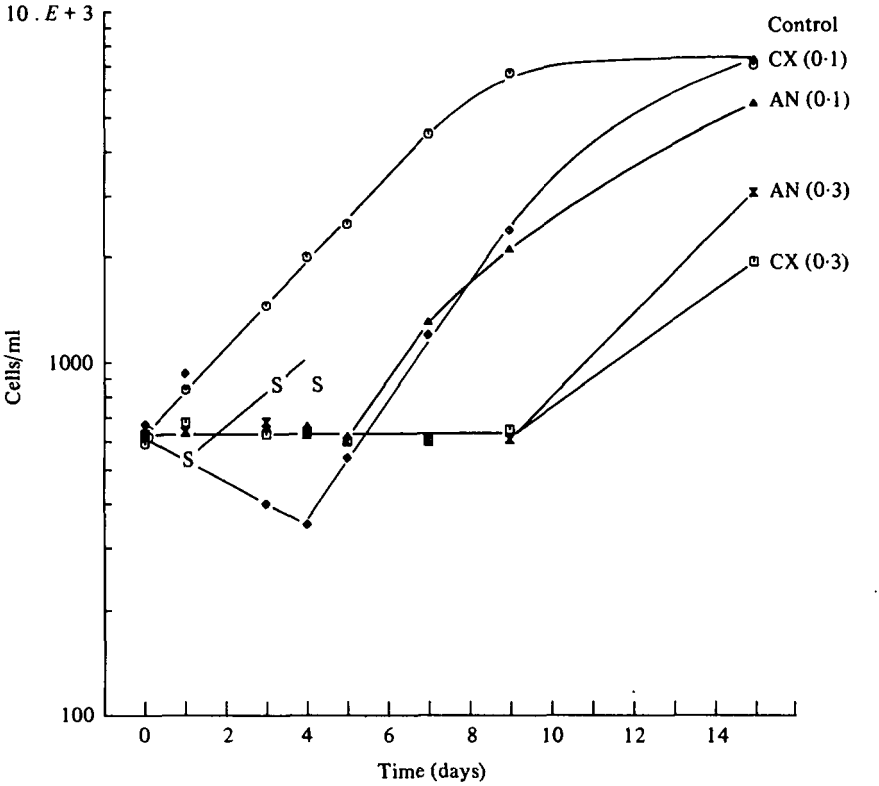

Fig. 9. The effect of 1·0 and 3·0 μM streptimidone, 0·03, 0·1, 0·3 and 1 μM anisomycin and 0·1 and 0·3 μM cycloheximide on growth of 750 ml cultures of *Gonyaulax* under LD 12:12, temperature 21 ± 2 °C. Ordinate: cell numbers per ml; abscissa, time, days (1 December 1980 and 10 February 1981).

higher concentration). The cells were killed at 1 μM anisomycin. Cycloheximide also inhibited growth at 0·1 and 0·3 μM, and in both cases growth resumed after a few days. The latter two drugs are somewhat unstable at alkaline pH (8·2 here); which could explain resumption of growth. Another possibility is that the cells developed resistance to the effects of the drug and are no longer inhibited, as has been reported for *Tetrahymena* with cycloheximide (Wang & Hooper, 1978).

DISCUSSION

The above and earlier results show that several different inhibitors of protein synthesis on 80s ribosomes (some of which have dissimilar structures and different sites of action) cause large phase shifts in the circadian glow rhythm in *Gonyaulax*. Ideally it would be valuable to have information concerning the degree of inhibition of protein synthesis by the different drugs at the various doses used, to discover whether this correlates with the effects on the circadian rhythm. Unfortunately, protein synthesis on 80s ribosomes is not easily measured in *Gonyaulax* because of the presence of bacteria in the cultures. Walz & Sweeney (1979) nevertheless suggested that the maximal effect on phase shifting occurred at cycloheximide concentrati

where they estimated the inhibition of protein synthesis to be only about 5%. Our experiments did not permit us to confirm this. In other systems there is evidence of a correlation between inhibition of protein synthesis on 80s ribosomes and phase shifting of the clock (Jacklet, 1977; Nakashima *et al.* 1981*a*).

Streptimidone (a glutarimide antibiotic similar to cycloheximide) is less effective than cycloheximide at the same concentration in inhibiting protein synthesis (Obrig *et al.* 1971); the same was true of its effectiveness in causing phase shifts of the glow rhythm in *Gonyaulax*. However, at higher concentrations it was fully as effective. Both of these antibiotics are known to block protein synthesis on 80s ribosomes by binding to the 60s subunit (Rao & Grollman, 1967).

The structure of anisomycin, as well as its mechanism of action, is different from that of the glutarimide antibiotics (Grollman, 1967; Vasquez, 1979). Anisomycin also binds to the 60s subunit, blocking the transpeptidation reaction of 80s ribosomes (Pestka, 1971). Thus, anisomycin and the glutarimide antibiotics both block elongation, but through different mechanisms. Puromycin, which is not very effective in causing phase shifts in *Gonyaulax*, has a structure very different from anisomycin, cycloheximide and streptimidone, and inhibits by substituting for the incoming amino acyl tRNA and causing premature chain termination (Vasquez, 1979). Although 80s ribosome function and/or protein synthesis is evidently of central importance in the circadian mechanism, more knowledge is required concerning the mechanisms of action of these and other (structurally different) inhibitors that affect protein synthesis, its post translational modification, and degradation.

Other types of compounds also cause phase shifts in *Gonyaulax* and other systems. Ethanol acts in several systems (Keller, 1960; Bünning & Baltes, 1962; Enright, 1971; Brinkman, 1976; Sweeney, 1974; Taylor, Gooch & Hastings, 1979) and ionophores such as valinomycin have been reported to be effective in *Phaseolus* (Bünning & Moser, 1973), *Gonyaulax* (Sweeney, 1974) and *Aplysia* (Eskin & Corrent, 1977). Aldehydes such as acetaldehyde and also several different sulphydryl reagents cause phase shifts in *Gonyaulax* (Taylor & Hastings, 1979; Hastings, 1960; Taylor *et al.* 1979). Although these agents appear to be numerous and chemically diverse, it is possible that all act in one way or another by interfering with a single system, for example by affecting the synthesis of a protein (Cornelius & Rensing, 1981). Ethanol has been shown to block protein synthesis in HeLa cells (Koch and Koch); K^+ is known to influence the rate of protein synthesis in liver and *Aplysia* (Ram, 1974); and acetaldehyde and other aldehydes inhibit protein synthesis in liver (Lubin & Ennis, 1964) and hepatoma cells (Schauenstein, Esterbauer & Zollner, 1977). A key protein in the clock mechanism such as suggested by the experiments of LeLong & Schweiger (1979) could have a susceptible sulphydryl group. Continued studies of chemically induced phase shifts should attempt to examine this possibility but should not necessarily be confined to compounds that are known for their ability to interfere with or effect protein synthesis.

The finding that protease inhibitors did not affect the phase of the rhythm is not unreasonable; in some models it would be expected that degradation of a functionally important protein would take place only *after* it had fulfilled its function. Even if a protease is involved in the rhythm it might not be affected by the inhibitors we used. Another important test would be to *stimulate* intracellular protein degradation while

leaving protein synthesis unaffected. Although this may be difficult, more investigations concerned with protein breakdown are warranted.

Between synthesis and degradation, proteins can undergo post-translational modifications prior to intracellular delivery to sites where the functions are carried out. These include peptide chain cleavage, subunit, assembly, incorporation of prosthetic groups, phosphorylation, carboxylation, and methylation. Phosphorylation involving the cAMP pathway, has been postulated to be involved in the circadian mechanism (Cummings, 1975). Studies with *Chlamydomonas* (Goodenough & Bruce, 1980) and *Neurospora* (Perlman, 1981) provide some support for such a mechanism by showing that phase and period can be affected by classical methyl xanthine inhibitors of cyclic AMP phosphodiesterase. However, results utilizing similar inhibitors with *Gonyaulax* did not support this mechanism (Brass, 1977).

The drugs that resulted in strong advance shifts gave rise to clear transients in the glow rhythm in the course of the resets. This phenomenon was previously noted with cycloheximide (Dunlap *et al.* 1980) and can be seen with anisomycin as a late-occurring major peak (h 28, Fig. 1, vial 20) and with streptimidone (Fig. 5, vials 21 and 23) as peaks at about h 23, resulting in a particularly short period following the pulse. While previously no such transients were noted in the delay resets, they were clear in the present study, not only with anisomycin and streptimidone but also with cycloheximide. Although the transients in delay resets appeared less pronounced than those in advance resets, they also involved a shorter (20–22 h) first period after the shift (see Fig. 5, vial 15). In many cases it appeared that transients persisted for longer than one cycle.

It is not obvious how a brief (1 h) interruption of protein synthesis can cause very large phase shifts (both advances and delays) in the circadian clock. A simple explanation (not yet excluded) is that the drug binds avidly and exerts its action over a time interval longer than the pulse. However, this possibility is not supported by the experiments of Fig. 4(*a*) and (*b*). Moreover, the short pulse-large shift effect has been previously observed in circadian systems using light pulses, suggesting an explanation other than incomplete washout may pertain. Mathematical models of dynamical oscillators can easily accommodate large responses to small perturbations (Pavlidis, 1973; Winfree, 1980). Nevertheless, the mechanism by which a protein synthesizing system participates in the cellular oscillator is still to be clarified. If the 'essential' protein synthesis requires a long time (a major fraction of a period), and its interruption, even though brief, means that the process is obliged to restart (somewhat like a relaxation oscillator), then large delays (and advances) could be explained. But so far as is known, cellular biosynthesis of a polypeptide chain requires minutes, not hours, and biological clocks have properties that appear to be more like limit cycle oscillators. A temporal mapping of ribosomal functioning and the synthesis of proteins should help resolve some of the key questions.

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