CHAPTER #

The Mechanism of the *Gonyaulax* (*Lingulodinium*) Circadian Clock: Interpreting the Inputs

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Abstract

The several bioluminescence rhythms have been widely used to characterize the response to various stimuli of the *Gonyaulax* circadian clock. In particular, *Gonyaulax* responds to changes in light intensity with a phase response curve (PRC). The magnitude and sign of the phase shifts depend on the light intensity and the spectral quality of the light in addition to the time at which the cells are exposed. Different drugs can affect the timing of the cells by themselves, and can act to modulate the response of the clock to light. One important class of drugs, the protein synthesis inhibitors, produces PRCs superficially similar to those induced by light, with the difficulty in removing the drug after the pulses an important difference between the two treatments. The *Gonyaulax* clock is temperature compensated with a Q_{10} of 0.85.

Introduction

Unicellular organisms and microalgae, such as the marine dinoflagellate *Gonyaulax polyedra* (now *Lingulodinium polyedrum*) are unfettered by the complications of multicellularity and provide excellent model systems for mechanistic studies of biological rhythms, notably circadian.[1-3] Rhythms with other periodicities can also be studied in such systems; a related dinoflagellate, *G. tamarensis*, (now *Alexandrium* spp.) exhibits also an endogenous circannual rhythm.[4,5] This is a remarkable feature for a single cell and could provide a model system for studies of the cellular basis for annual rhythms.

As in many organisms, many aspects of the physiology and biochemistry of *Gonyaulax* exhibit circadian rhythmicity, with several different acrophases.[6] Some of these, such as the glow and flashing rhythms[7] or the flashing and aggregation rhythms[8] may desynchronize and exhibit different periods under constant conditions. This implicates two or more quasi-independent interconnected oscillators,[9] which may have different phase response curves (PRCs) under certain conditions.[10] Spontaneous desynchronization of different rhythms also occurs in higher organisms, including humans,[11] where, as for *Gonyaulax*, the factors responsible are not well established. Thus, it appears more suitable to model circadian systems as a network of normally coupled oscillatory elements rather than to invoke a single "master" circadian oscillator. Knowledge of the mechanism of desynchronization of the putative elements in a simple system should lead to a better understanding of the clock mechanism.

An important feature of the *Gonyaulax* system, which has enhanced its value as a model organism, is that it possesses a built-in reporter system in the form of bioluminescence exhib-

The Circadian Clock in Eukaryotic Microbes, edited by Fred Kippert.

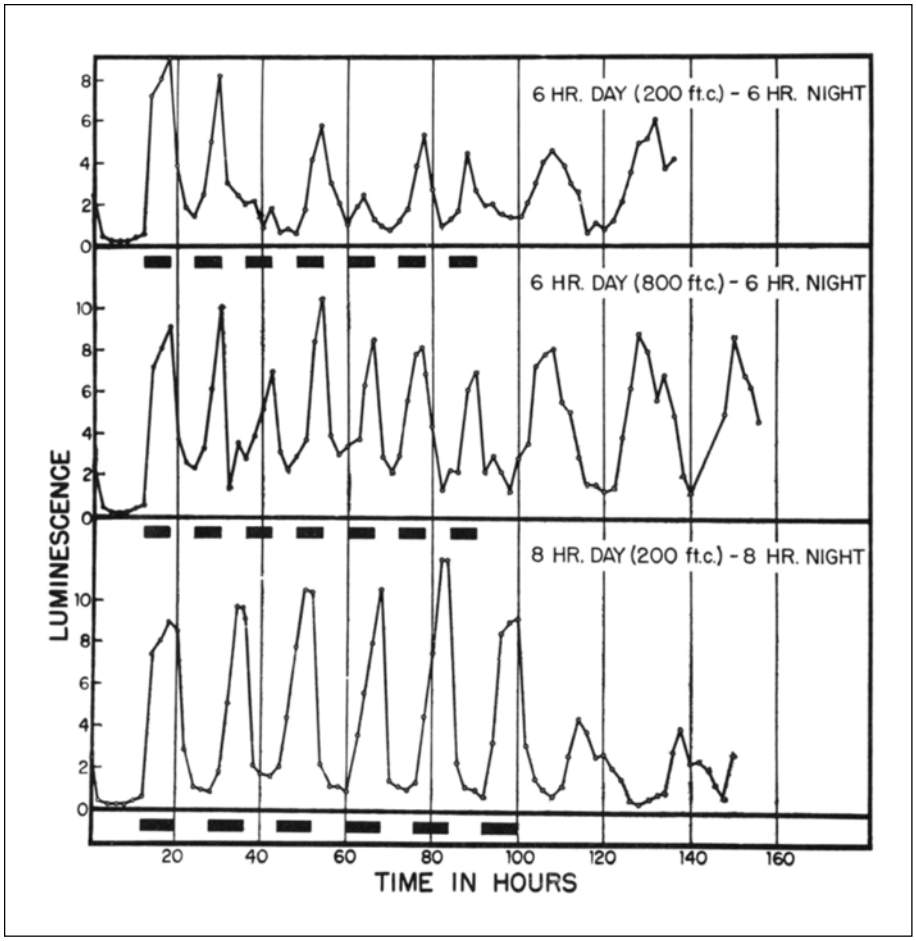

Figure 1. Entrainment by light-dark cycles.
Entrainment of the luminescence rhythm to 12-hour cycles of light-dark (L:D 6:6), shows that entrainment occurs with bright but not dim light. In contrast, dim light is sufficient to entrain to a 16 hour cycle (L:D 8:8). Reprinted from Hastings JW, Sweeney BM. The *Gonyaulax* clock. 1959 In: Withrow RB, editor. Photoperiodism in Plants and Animals. Washington, D.C.: Withrow.

iting rhythmicity controlled by the circadian clock. The automation of the measurement of luminescence,[12] which was refined repeatedly and computerized in the late 1970s,[13-15] facilitated studies of the circadian system, its key features and the effects of light, temperature and inhibitors. This chapter will focus on the insight concerning the clock mechanism that studies of the bioluminescence rhythm in this system have provided.

Bioluminescence itself is a marker for more than one rhythm. Light emission typically occurs in *Gonyaulax* as a brief (~100 msec) bright (10^9 photons) flash upon mechanical stimulation, but spontaneous flashing also occurs, as well as a very dim spontaneous glow (max ~10^4 photons sec^{-1} $cell^{-1}$), which is emitted for several hours each cycle. Recordings of spontaneous emissions from a culture in constant darkness show that both the frequency of flashes and total light are greatest in the middle of the night phase and that the glow acrophase occurs a few hours later, at the end of the night phase. A computerized system records all three measures of

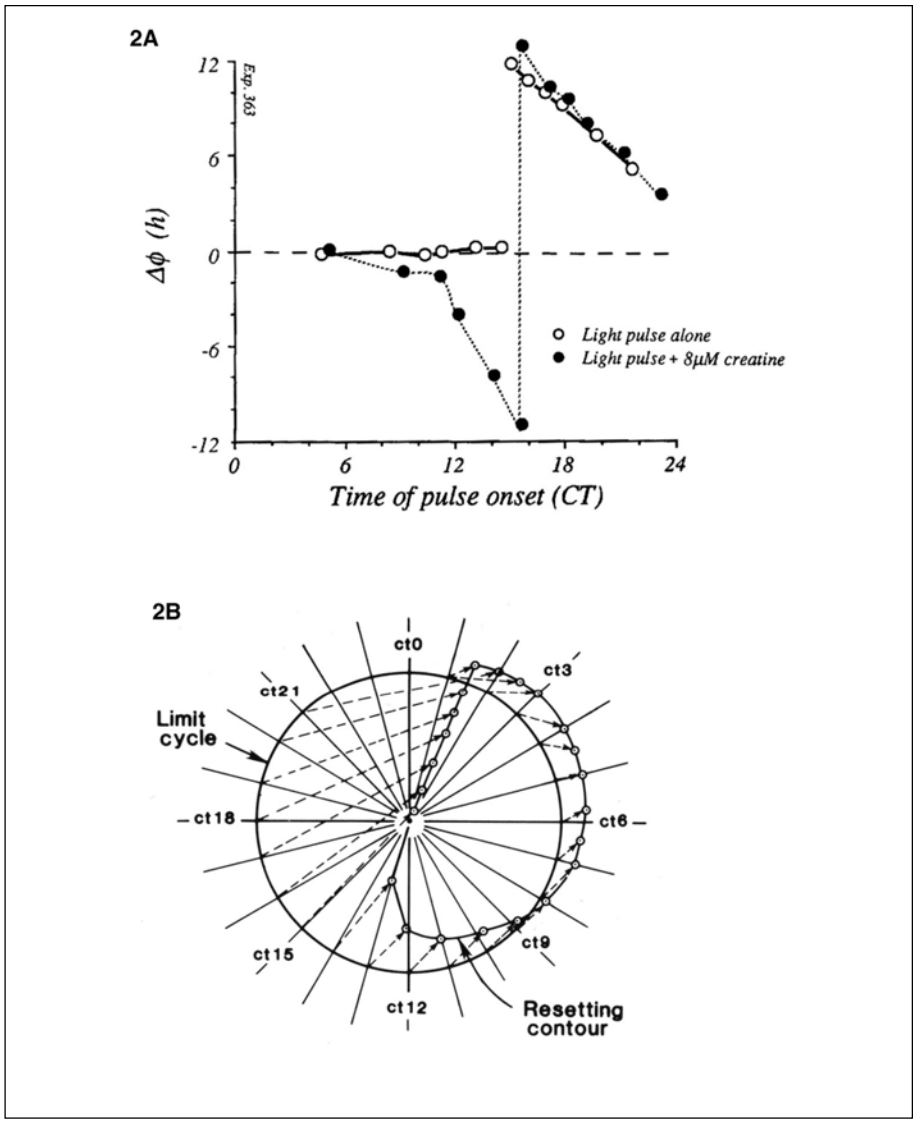

Figure 2. Phase response curves and their interpretation by a limit cycle
(A) Phase response curves for strong (150 μE) 4 hour pulses of blue light alone (open circles) and the same with 8 μM creatine added 2 days prior to pulses. Cultures were kept in constant red light (RR) before and after the light pulses. Reprinted from Roenneberg T, Taylor WR. Light-induced phase responses in *Gonyaulax* are drastically altered by creatine. J. Biol. Rhythm 1994; 9:1-12 (B) Limit cycle description for phase shifting by pulses of blue light of *Gonyaulax* cells in RR background, as in the experiment in (Fig. 2)A with light alone. Circle is the limit cycle, with the spokes (isochrons) radiating from the singular point in the center. Light pulses at each of the phases of the limit cycle shift the phase (dashed lines) to points (open circles) on the resetting contour. The numbers around the limit cycle are the phase of the oscillator at that position in the circadian time; the oscillator is precessing in a clockwise direction. Reprinted from Johnson CH, Hastings JW. Circadian phototransduction: Phase resetting and frequency of the circadian clock of *Gonyaulax* cells in red light. J. Biol. Rhythm 1989;4:417-437

the rhythms from as many as 60 cultures for days or weeks. The acrophase of the glow peak is particularly well defined and accurate to within a few minutes a day,[16] so this was used in most of the studies to be cited. It is important to note the rhythm measured is an average resulting from the behavior of the population of cells. While single cells are rhythmic, the bandwidth of the glow peak is wider in a population, suggesting that different cells in a population have slightly different phases.[17,18] However, this effect may be mitigated by cell-to-cell communication of phase information, as mixing two out of phase cultures results in the adoption of a compromise phase.[19]

Light: Phase-shifts, Entrainment and Effects on Period

The Phase Response Curve (PRC)

The origin and evolution of the circadian timing mechanism can be attributed to the action of environmental light/dark cycles[20] which forces an organism to adapt to periodic changes in its environment. The endogenous free-running period of the rhythm (i.e., under constant conditions) is slightly different from 24 hours (typically 23 or 25 hours, depending on light, temperature, etc). To adjust to an imposed light-dark cycles of 24 hours, the endogenous clock must either advance or delay its phase once every cycle. In fact, light dark cycles of different lengths can entrain the rhythm to adopt the period of the entraining cycle, up to a limit defined by the ability of light to shift (reset or alter the phase of) the rhythm. One of the first studies on entrainment in *Gonyaulax* demonstrated that the magnitude of such phase shifts is dependent upon the intensity and duration of the light exposure, a matter that was ignored for a long time in the field. This can be clearly observed from the effect of light intensity on the limits of entrainment. For example, *Gonyaulax* can entrain to a 12 hour cycle (LD 6:6) in bright but not in dim light (Fig. 1),[21] a fact also rather poorly appreciated in the literature. Although entrainment by a 12 hour cycle has been referred to in a recent text[22] as "frequency multiplication",[23] it does fall within the theoretical limits of entrainment for *Gonyaulax*, and possibly for other cases as well. Frequency demultiplication has not been reported in *Gonyaulax.*

An explanation of phase shifts needed for entrainment was first provided by the demonstration in *Gonyaulax* that single light pulses given to cells, maintained otherwise in constant conditions, can result in phase shifts in the rhythm and the underlying oscillator, and that such shifts are different in both sign and magnitude when applied at different times in the circadian cycle.[6] This light phase response curve (PRC) indicated that a single light pulse could reset the rhythms to any of all possible new phases. It also showed the apparent discontinuity between advances and delays, now termed the breakpoint (dotted lines, (Fig. 2A). Interestingly, studies on *Gonyaulax* also first demonstrated that strong (type 0) and weak (type 1) PRCs relate to the intensity of the stimulus.[24]

In the limit cycle model for circadian oscillations[24] resetting by light perturbations alone, such as those in (Fig. 2A), can be depicted as shown in (Fig. 2)B. In theory, a light pulse at a certain intensity and duration, given at a critical time, should be able to drive the state variables to a center point in the diagram. This is where the amplitude of the oscillation is nil, thus without a defined phase, as when standing on the North Pole, where one's longitude is not defined. Evidence that this is so for *Gonyaulax* was obtained by varying the time in the cycle when a light pulse of a given intensity was applied, and by varying the intensity at a specific time in the cycle.[25] Light of a particular intensity given at a time corresponding to the breakpoint resulted in a sharp decrease in the amplitude of the glow rhythm peaks, suggestive of just such an effect on the majority of cells in the population.

Action Spectrum for Phase-Shifting

Phase shifting by light depends not only on its intensity and duration, but also on the spectrum of the incident light, as related to the absorption spectrum of the primary photore-

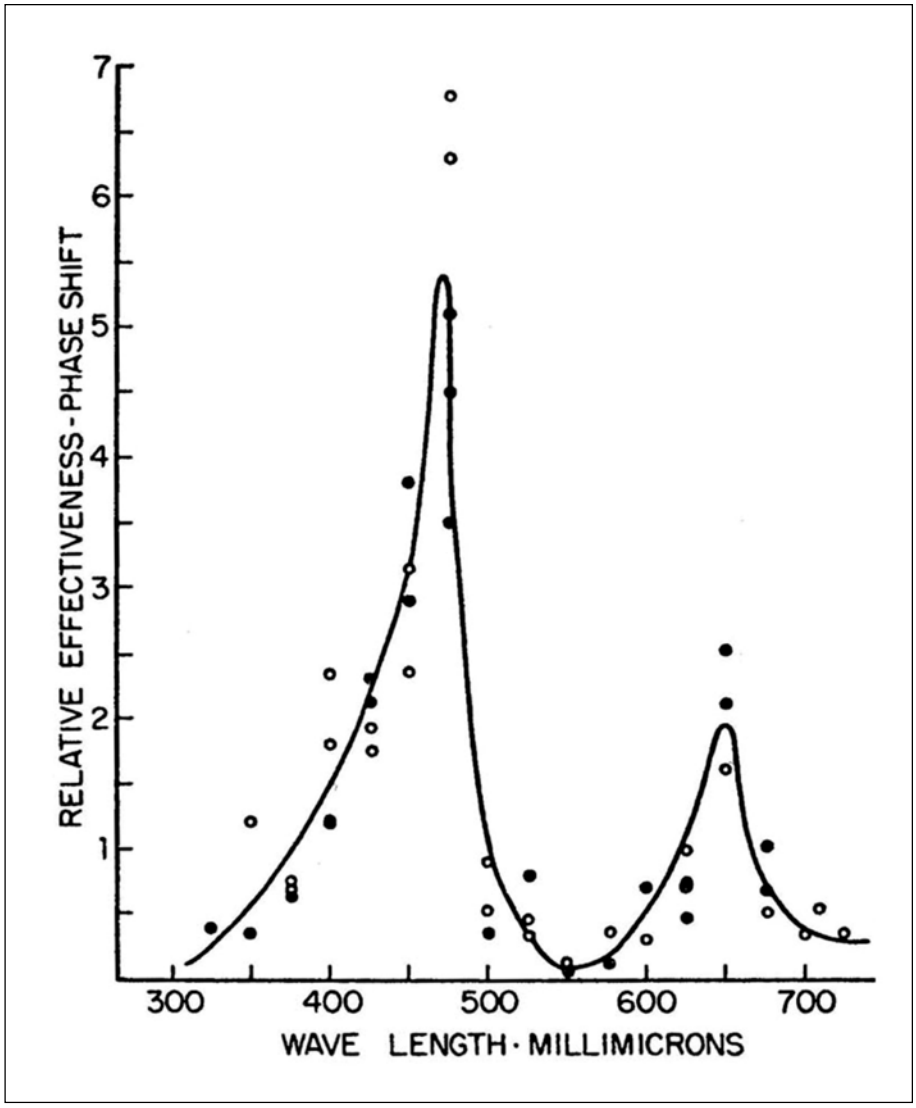

Figure 3. Sensitivity of phase shifting to light of different colors
Action spectrum for shifting the phase of the luminescence rhythm in *Gonyaulax* by single 3-hour exposures to light of equal quanta but different wavelengths. Cells were kept in DD and exposures were given at about CT 17 in the region of the PRC giving advance phase shifts. Reprinted from Hastings JW, Sweeney BM. The action spectrum for shifting the phase of the rhythm of luminescence in *Gonyaulax polyedra*. J. Gen. Physiol. 1960;43:697-706

ceptor pigment. In *Gonyaulax* the action spectrum was determined for advance phase shifts caused by 3-hour light exposures;[26] it has two peaks, at 475 and 650 nm, with blue light being about 3 times more effective than red (Fig. 3).

Although this could be due to absorption by a single pigment, two different ones could be involved, and later studies indicated that this is so. Although the protocols were somewhat

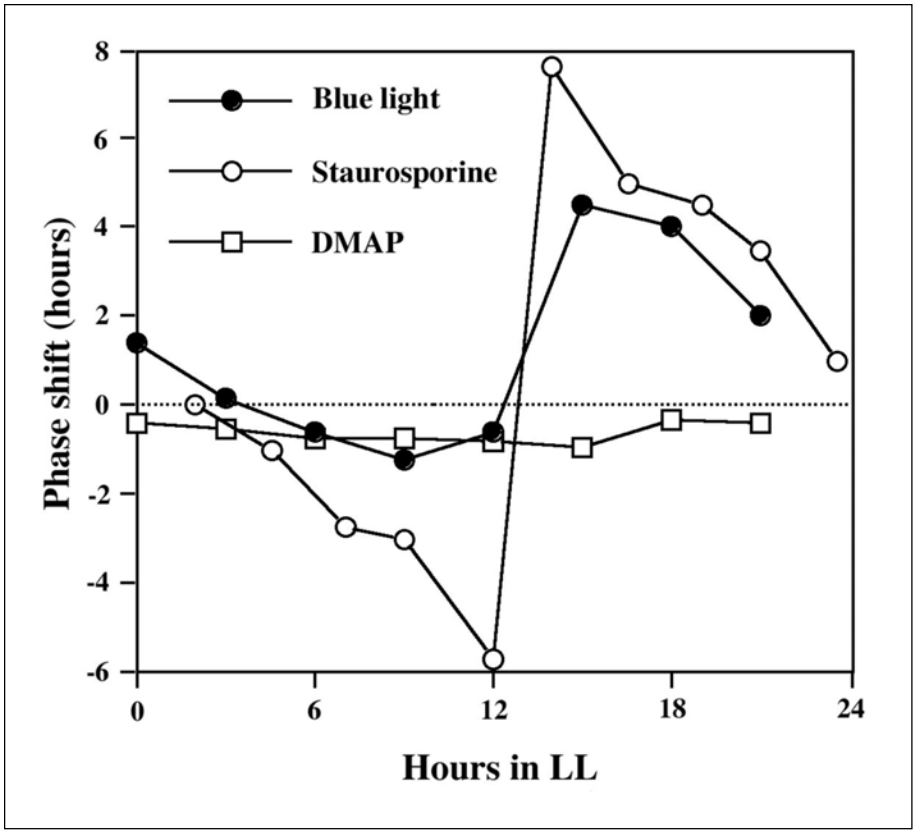

Figure 4. Effects of drugs on phase shifting by light
PRCs for 3-hour exposures to bright blue light (150 μE) of *Gonyaulax* cells kept otherwise in dim white light at 19°C (solid circles). The kinase inhibitor 6DMAP (5 mM) blocked phase shifting (open squares) while a different kinase inhibitor (staurosporine; 20nM) enhanced phase shifting by light. Data redrawn.[34,35]

different, PRCs determined separately for broad spectra blue and red light differ quantitatively and qualitatively, indicating that different pigments and pathways are involved.[27] Also, several drugs have been found to alter the PRC, and some have an effect in only blue light (see below), indicative of separate receptors and pathways for the two colors of light.

Effects of Background Light on the Light PRC

The PRC is a measure of the relative effectiveness of light for phase shifting at different specified times in the circadian cycle. Various factors, both physical and chemical, can affect such phase shifting. In cultures kept otherwise in darkness (DD), bright light pulses induce substantial advances (8 hours) and delays (5 hours), and this results in a prototypical type 0 PRC.[6] However, when cells are kept in a background of either constant dim white (WW) or red (RR) light, the PRCs are very different.[25,28] Unlike cells in DD, phase delays are not observed or are very small, while phase advances can be up to 12 hours (light pulse alone, (Fig. 2A)

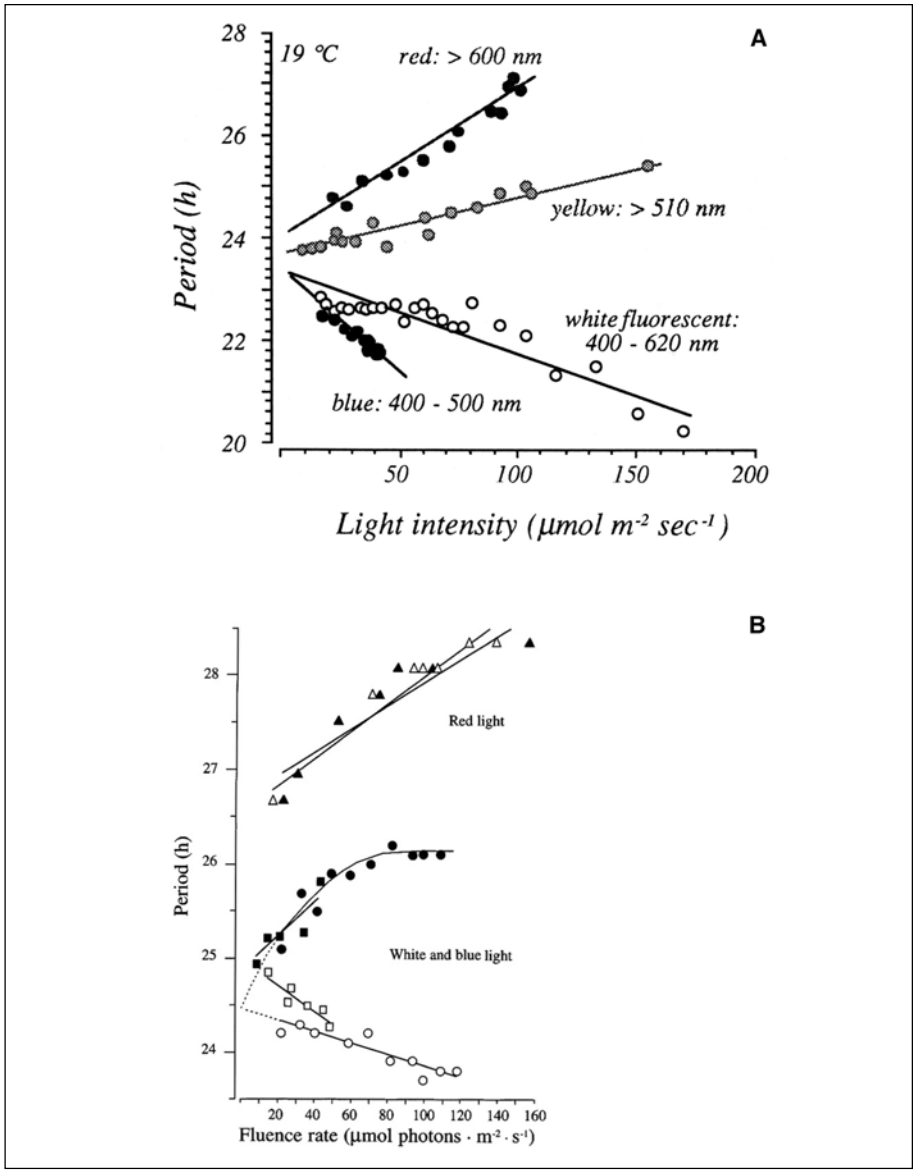

Figure 5. Effect on period of different colored light. (A) Effects of intensity (abscissa) of the continuous light to which *Gonyaulax* cells are exposed on the period of the rhythm of luminescence. The period is lengthened with increasing intensities of red light but shortened with increases in blue light intensity. Reprinted from Roenneberg T, Hastings JW. Two photoreceptors control the circadian clock of a unicellular alga. Naturwissenschaften 1988;75:206-207 (B) The action of allopurinol (1 mM; solid symbols) on the period-intensity relationships of the glow rhythm of *Gonyaulax* in red light (triangles) as compared to white (circles) and blue light (squares) on the period of the rhythm of luminescence . The action of white or blue light is blocked by allopurinol, but not that of red light. The fact that the curves do not extrapolate to the same value at zero influence was unexplained. Reprinted from Deng T-S, Roenneberg T. Photobiology of the *Gonyaulax* circadian system. II. Allopurinol inhibits blue-light effects. Planta 1997;202:502-509

Effects of Creatine and Drugs on the Light PRC

Curiously, the light-PRC may be dramatically altered by several types of drugs. One important example is creatine, initially discovered as a substance with a strong effect on the period of the rhythm.[29] Creatine allows delays to be fully expressed in an LL background, so that the light pulses result in a prototypical type zero PRC, with a dead zone in the day phase and both 12 hour delays and advances in the night phase (Fig. 2A).[30] The explanation for this remarkable effect remains unknown, but it may very well relate to an effect on protein phosphorylation, which for other systems is an important player in the progression of clock proteins involved in the feedback loop of the molecular oscillator.[31,32] In higher organisms, creatine serves as a shuttle molecule in phosphate transfer from ATP produced in mitochondria to sites of ATP utilization elsewhere in the cell.[33]

While creatine is not known to occur in *Gonyaulax*, the possibility that it has an effect on phosphorylation is supported by the finding that an inhibitor of protein kinases, 6-dimethyl amino purine (6-DMAP) at mM concentrations completely blocks both advance and delay phase shifts caused by light, in both the presence and absence of creatine (Fig. 4).[34] By itself, DMAP induces small phase delays that are not dependent on the phase of drug administration, attributable to its effect on the free running period (see below).

Staurosporine, a more specific protein kinase inhibitor, increases (at nM concentrations and in the absence of creatine) the magnitude of both delays and advances in light-induced phase shifting (Fig. 4).[35] The difference in the actions of 6-DMAP and staurosporine may be because they act on different target clock protein kinases. Many other protein kinase inhibitors are without effect on the *Gonyaulax* circadian system,[36] so the targets may be inferred to be specific.

Okadaic acid, cantharadin and calyculin, inhibitors of protein serine/threonine phosphatases, also cause pronounced increases in light-induced phase shifts, altering as well the time in the cycle when the maximum shift occurs .[37] These varied effects suggest that protein phosphorylation may be involved not only in regulating the activity of clock proteins, but also steps in the circadian light transduction pathway.

Effects of Light on Period

As for rhythms in other organisms, the free running period (FRP) of the bioluminescence rhythm changes as a function of the (constant) light intensity.[6] Such an effect may be explained on the assumption that constant light has a tonic phase-shifting effect, such that the progression of the oscillator is accelerated in the phase-advance region and decelerated during the phase-delay region of the PRC.[22] If the PRC is asymmetric, the FRP will change with intensity. However, in *Gonyaulax* this effect depends also on the spectral quality of the light.[29] With an increasing intensity of blue light the period becomes increasingly shorter, while with an increasing intensity of red light it becomes longer (Fig. 5A).

This effect is attributed to a tonic effect of light mentioned above, and to the fact that the PRC for blue light has advances more pronounced than delays, while delays are more pronounced in the PRC for red light.[27] This is further supported by the observation that the xanthine oxidase inhibitor allopurinol blocks the period-shortening effect of white (or blue) light as well as the phase advances it provokes (Fig. 5B).[38] Allopurinol does not affect period lengthening nor phase shifting by red light, which supports the idea that red and blue light act through different transduction pathways.[39] It is also interesting that xanthine oxidase activity itself is rhythmic, with a peak during the subjective day.[40] It is possible that a product of this enzyme is involved in blue light reception.

Many brief dark pulses (2-3 min each, 20- 30 per cycle), well outside the limits of entrainment, can also alter the period by an hour or more, as compared to cultures maintained in constant light.[14] This is not attributable to an intensity effect, since the differences are less than 10%, but to repetitive small phase shifts.

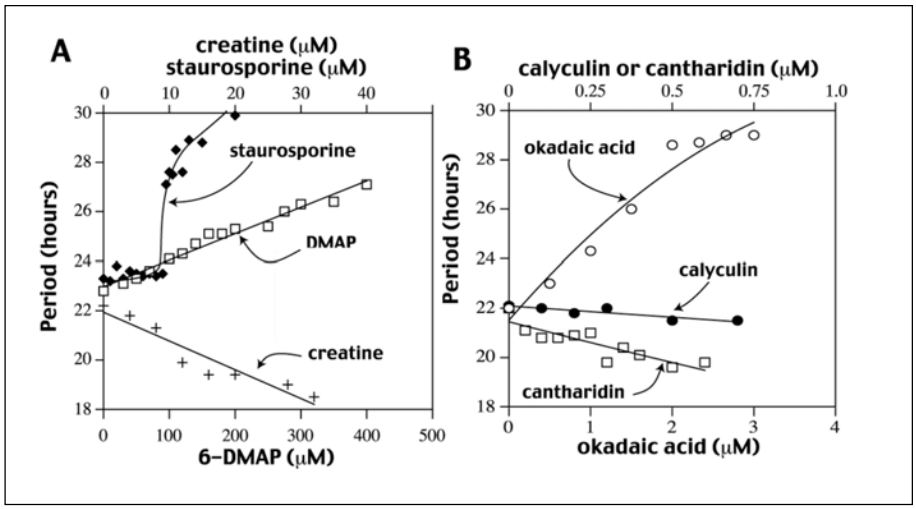

Figure 6. Effect on period of protein kinase or protein phosphatase inhibitors. (A) The effects of two protein kinase inhibitors[34,35] and creatine, as well as (B) three inhibitors of phosphoprotein phosphatases on the period of the *Gonyaulax* luminescence rhythm. [37] Data redrawn from combined references.

As might perhaps be expected, single hour-long dark pulses that interrupt the day phase also provoke phase shifts, principally phase advances, with a maximum response at roughly CT 8.[14,41] However, the absence of light does not produce a PRC that is simply an inverted image of the light-PRCs. The mechanism underlying these phase shifts is thus unclear. As mentioned above, phase shifting by light may require signaling mechanisms mediated by protein phosphorylation, as some protein kinase inhibitors block phase shifts and others as well as phosphatase inhibitors increase the amplitude of the shifts.[34,35,37]

Effects of Drugs and Other Substances on the Circadian System and the FRP

Creatine and Nitrate

An important discovery was that creatine shortens the period of the circadian glow rhythm; it can be shortened by as much as 5 hours in a concentration-dependent manner (Fig. 6A).[42] Pulses of creatine do not result in phase shifts, so this effect cannot be attributed to differences in phase shifting, as with light. For drugs, tonic and phasic effects can be clearly distinguished.

An endogenous substance, named gonyauline,[43] has been shown to act as creatine does. However, when identified, gonyauline was found to be cyclopropanecarboxylic acid, structurally related to methionine. Gonyauline may mediate between amino acid metabolism and the circadian system, and the similarity of its effect to that of creatine may constitute molecular mimicry.

Inorganic nitrate has effects on both the phase and period of the *Gonyaulax* rhythms. When the algae are grown in seawater without added nitrate, the FRP is an hour shorter than that of controls containing the normal nitrate supplements.[44] This shorter period may cause the algae to advance the time of their descent to deeper water, where nitrate is more abundant.[45] Furthermore, under these conditions nitrate can also provoke phase delays at the end of the night,[46] a delay that might allow the alga to remain longer in a nitrate-rich area before rising to the

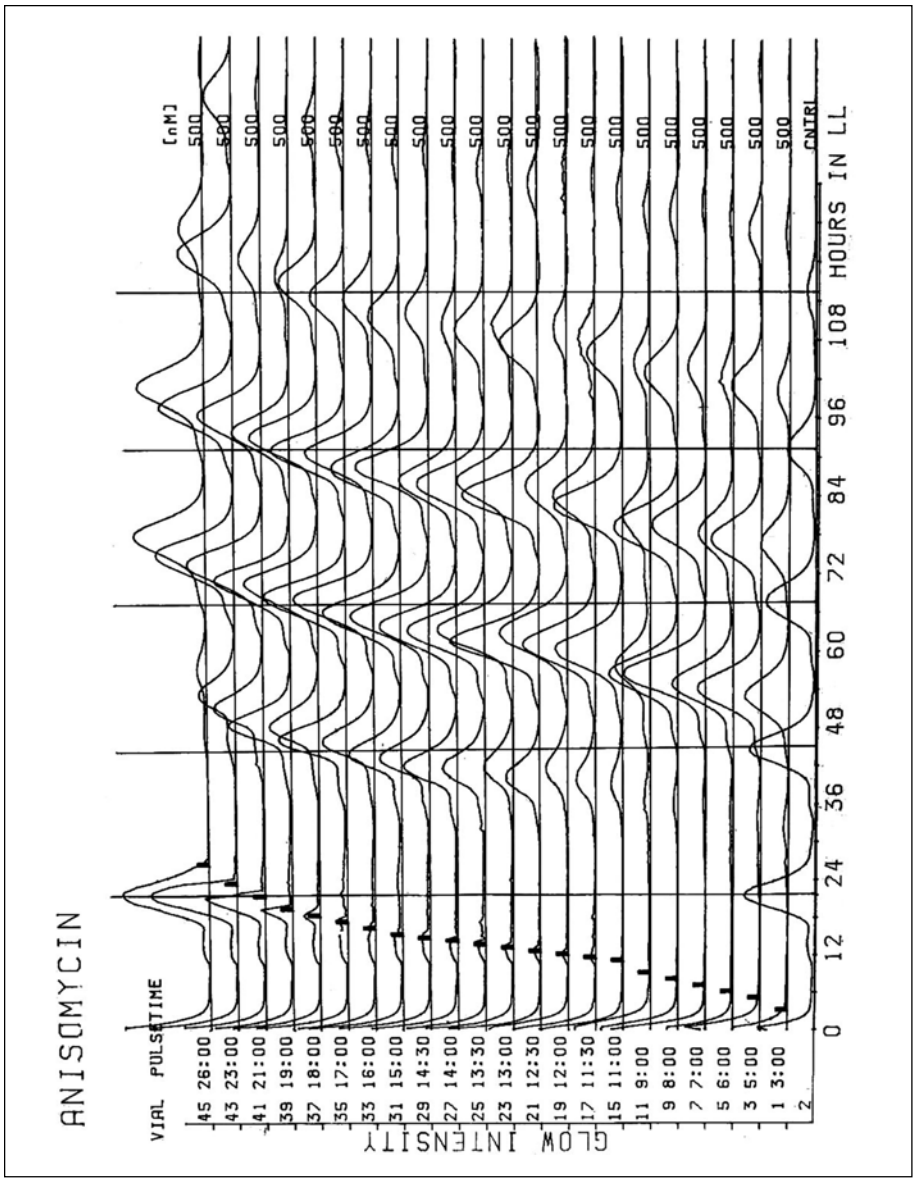

Figure 7. Effect of anisomycin on the bioluminescence glow phase
Phase shifts in the rhythm of luminescence in *Gonyaulax* following 2 hour pulses of 0.5 μM anisomycin given at different times in the cycle, with cells maintained in constant dim light (LL). Reprinted from Taylor WR, Krasnow R, Dunlap JC, et al. Critical pulses of anisomycin drive the circadian oscillator in *Gonyaulax* towards its singularity. J. Comp. Physiol. 1982;148:11-25

surface. Nitrate does not provoke phase shifts when sufficient nitrate is present in the medium. Nitrate reductase exhibits a circadian rhythm in its activity.[47]

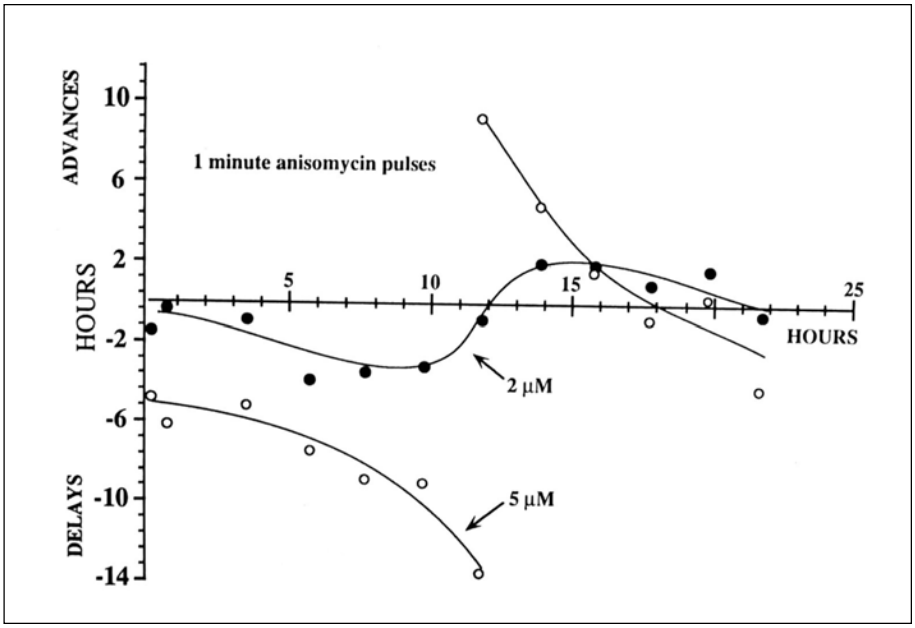

Figure 8. Strong and weak phase resetting by anisomycin. (A) Phase response curves for one minute pulses of 2 µM and 5 µM anisomycin, showing weak (type 1) and strong (type 0) PRCs. Ordinate, phase shift in hours; abscissa, time of pulses in hours since cells were transferred to constant dim light. Reprinted from Taylor WR, Hastings JW. Minute-long pulses of anisomycin phase-shift the biological clock in *Gonyaulax* by hours. Naturwissenschaften 1982;69:94-96

Membrane Active Agents

Among the molecules inducing phase shifts, one of the most provocative is the potassium ionophore valinomycin. This drug induces both phase advances and delays of almost 4 hours magnitude each, and the PRC superficially resembles that of dark pulses.[48] The phase shifts were thought to be related to a rhythm in the potassium concentration inside the cells and gave rise to "membrane models" for the circadian clock.[49,50] These invoked ion gradients across a membrane in the generation of the circadian rhythm and predicted that agents that affected membranes would also cause phase shifts. Indeed, phase shifting by the membrane active reagents such as ethanol,[51] aldehydes[52] or vanillic acid[53] were subsequently reported. Lastly, the membrane potential was also found to exhibit a circadian variation, consistent with a role of ion gradients in the clock mechanism.[54] The explanation for the action of valinomycin has not been explained, and no further studies of this have been reported.

Protein Kinase and Protein Phosphatase Inhibitors

While the circadian system in *Gonyaulax* is unaffected by many types of drugs,[12] important effects of inhibitors of protein kinases and phospho-protein phosphatases are now known. Chronically administered, such inhibitors have large concentration-dependent effects on the FRP but do not by themselves cause phase shifts or alter phase other than by virtue of that due to the effect on period, as was found for creatine. Staurosporine, 6-DMAP and okadaic acid increase the FRP from about 23 h to more than 30 h, while cantharadin results in a decrease of about 2 h and calyculin is without effect (Fig. 6A, B). As mentioned above, this may be due to

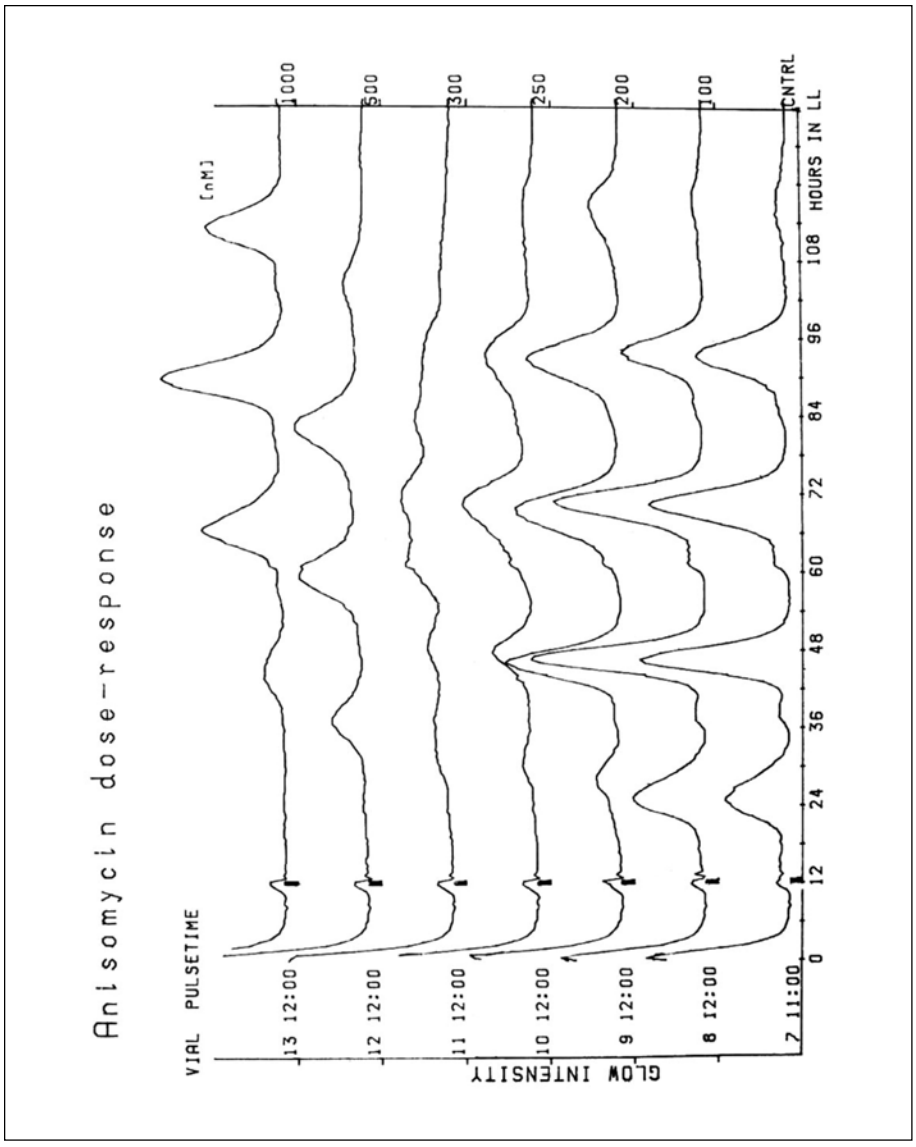

Figure 9. Critical doses of anisomycin induce arrhythmicity.
One hour pulses of different concentrations of anisomycin (from 0.1 to 1.0 μM; right ordinate) given at hour 12 cause a decrease in amplitude and a loss of rhythmicity at 0.3 μM, but not at higher or lower concentrations. Reprinted from Taylor WR, Krasnow R, Dunlap JC, et al. Critical pulses of anisomycin drive the circadian oscillator in *Gonyaulax* towards its singularity. J. Comp. Physiol. 1982;148:11-25.

effects on the phosphorylation of clock proteins involved in the feedback loop of the molecular oscillator, and may also target steps in the circadian light transduction pathway.

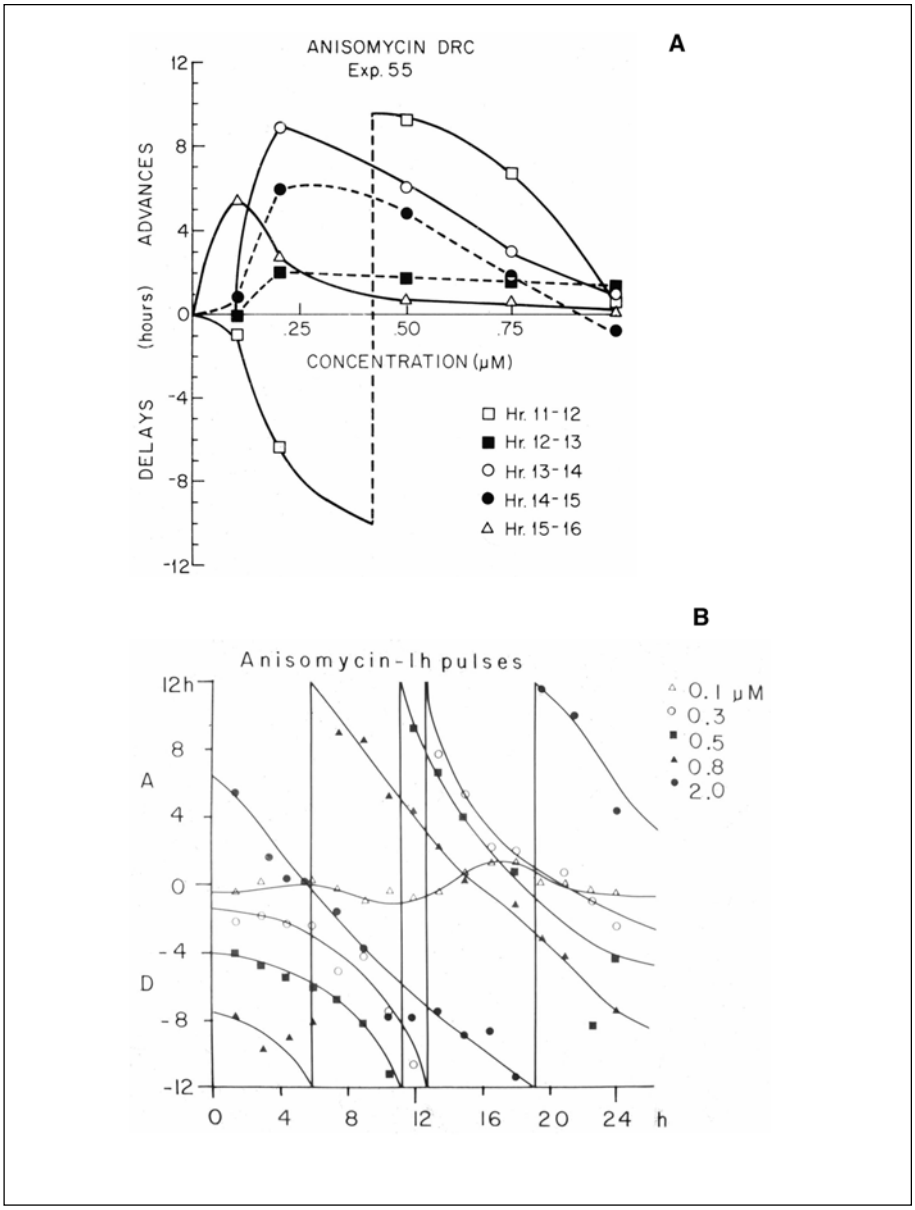

Figure 10. Dose response effects of anisomycin. (A) Dose response curves for 1 hour pulses of anisomycin, applied at different times in the circadian cycle. (B) Drug phase response curves for 1 hour anisomycin pulses at different concentrations, with a type 1 PRC at 0.1 μM and type 0 curves at all higher concentrations. The PRCs appear to move bodily to earlier phases (to the left) as the dose increases. Reprinted from Taylor WR, Krasnow R, Dunlap JC, et al. Critical pulses of anisomycin drive the circadian oscillator in *Gonyaulax* towards its singularity. J. Comp. Physiol. 1982;148:11-25.

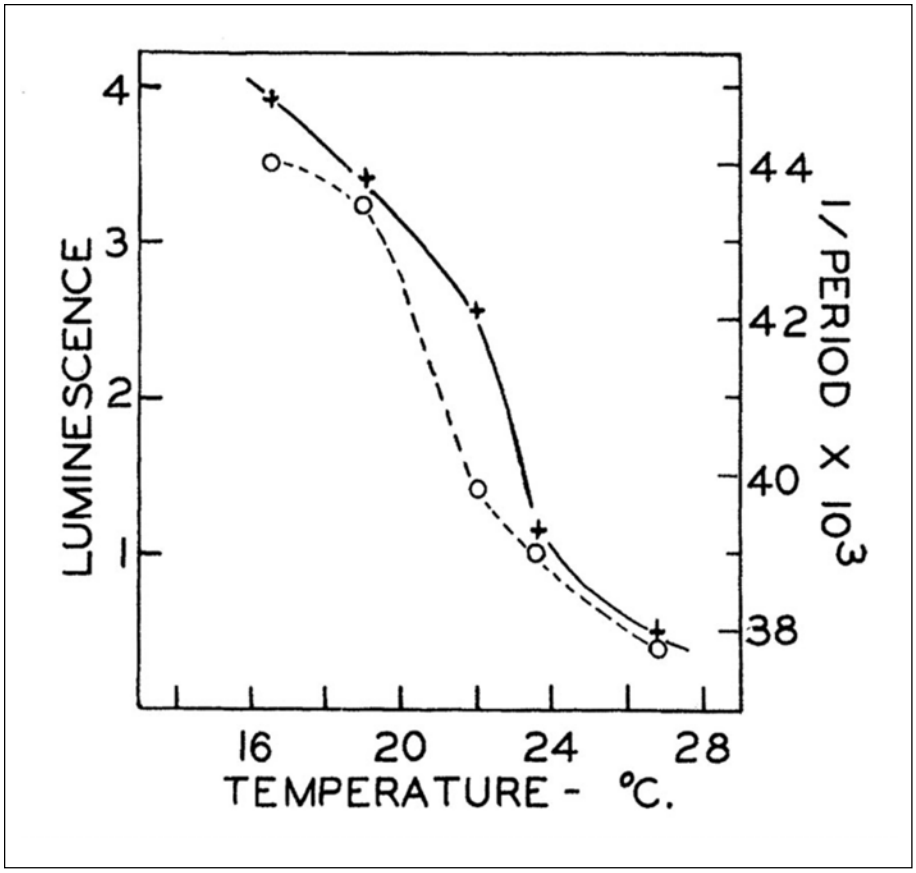

Figure 11. Temperature has similar effects on luminescence yield and period.
Plot showing the effect of temperature (abscissa) on the amount of luminescence obtained by stimulation (solid line, left ordinate) compared to its effect on period (dotted line; plotted as the inverse, right ordinate). Reprinted from Hastings JW, Sweeney BM. On the mechanism of temperature independence in a biological clock. Proc. Natl. Acad. Sci. USA 1957;43:804-811.

Inhibitors of Protein Synthesis on 80S Ribosomes

A second class of effective drugs, which have strong phase-shifting effects on the circadian clock, are those that inhibit protein synthesis on 80S ribosomes, such as puromycin, cycloheximide, streptimidone and anisomycin.[55-58] These drugs all give similar phase shifts and PRCs, so anisomycin can serve as a typical example (Fig. 7). Brief pulses of anisomycin (e.g., 1 minute, 5 μM or1 hour, 0.3 μM) can reset the rhythm to all possible new phases, and its drug-PRC can show either weak or strong phase resetting depending on the dose (Fig. 8).[59-61] Furthermore, there is a critical time and a critical dose at which the rhythm is damped or lost. The clock can be "stopped" or its amplitude diminished by such a treatment, interpreted in the limit cycle model as driving the oscillation to or near its singularity. At CT 12, corresponding to the time when delays switch to advances in the type 0 PRC, the rhythm is strongly damped with a 2-hour pulse of 0.3 μM anisomycin (Fig. 9). The concentration, duration and time of addition

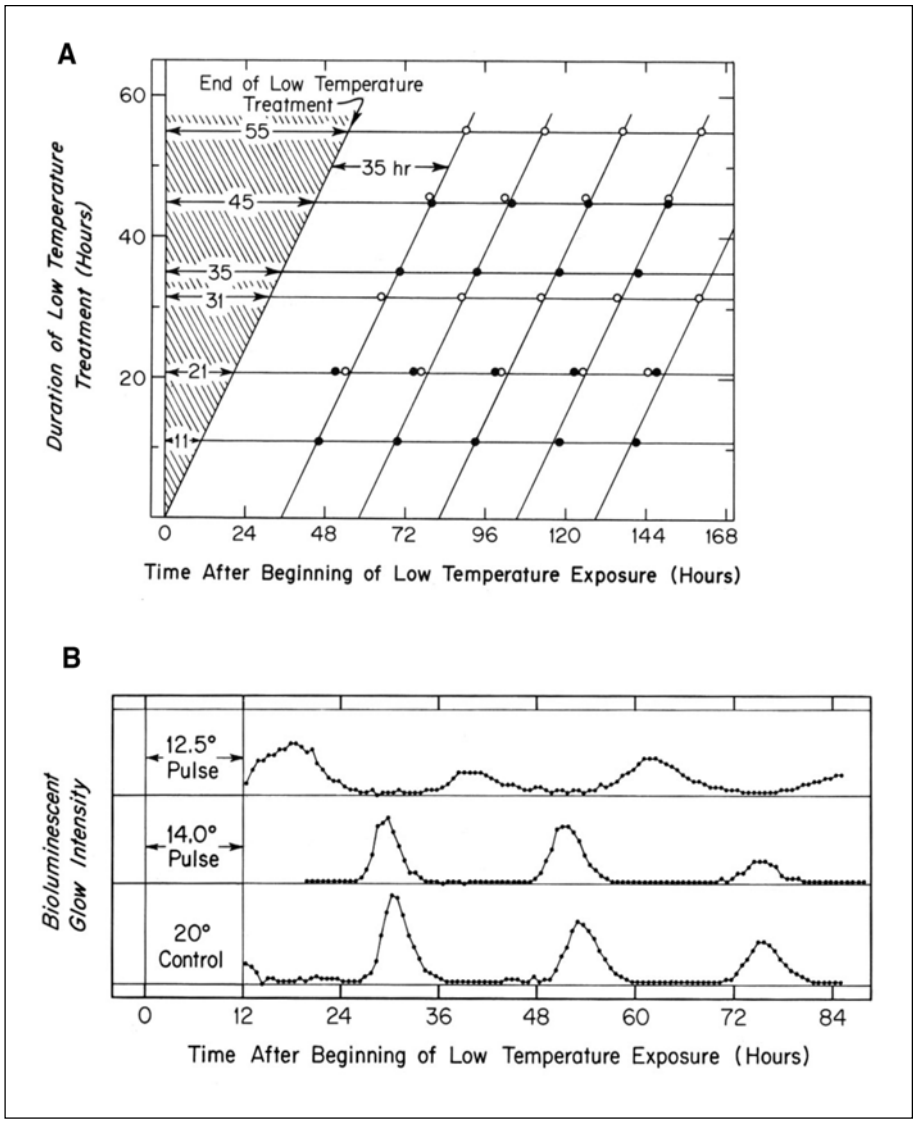

Figure 12. Effects of temperature on phase resetting. (A) The new phase of the rhythm is determined by the time at which cells are returned to 21°C after exposures to 11°C. Cultures at 21°C were transferred from LD 12:12 to LL at the end of a light period and either 8 (open circles) or 18 (solid circles) hours later moved to 11°C with no change in the illumination and kept at that temperature for the periods indicated. After returning to 21°C the glow was measured and the acrophases of the restored rhythm are shown. (B) The temperature at which the rhythm occurs is separated from the nonpermissive temperature by less than 1.5°C. Cells growing at 20°C were transferred from LD 12:12 to constant dim light at the end of a light period and immediately cooled to either 14°C or 12.5°C, maintained there for 12 hours and then returned to 20°C, still in dim light. Reprinted from Njus D, McMurry L, Hastings JW. Conditionality of circadian rhythmicity: synergistic effect of light and temperature. J. Comp. Physiol. 1977;117:335-344.

are indeed critical, as pulses of slightly lower concentrations or less duration do not produce changes in phase or amplitude while pulses of higher concentrations produce high amplitude rhythms that have experienced large phase shifts. The same protocol carried out at CT 11 or CT13 has no such effect.[59] These results provide another experimental support for a limit cycle model and the existence of a singular point in the *Gonyaulax* system.

The arhythmicity and loss of amplitude produced by the critical concentration of anisomycin does not simply reflect inhibition of the synthesis of the biochemical components to the bioluminescent system, since light emission continues at a reasonably high level. It also clearly differs from the arhythmicity and loss of amplitude caused by high doses of anisomycin in the first hours after its addition. In this latter case, it was noted that a second pulse of the drug could induce a phase shift, demonstrating that the clock is still operating and carrying a discrete phase after the initial phase shift, even though the expression of luminescence is fully suppressed.[62]

A striking feature of phase shifting by drugs is that the extent and sign of phase shifting is not a monotonic function of concentration, and that such functions differ drastically when the drug is added at different times in the circadian cycle. For example, when added at CT 11, low concentrations produce delays while higher concentrations result in advances (Fig. 10A). At CT 12 no phase shift occurs at lower concentrations, while higher ones result in small values unchanging with concentration. At CT13 advances occur, first increasing and then decreasing with concentration. Drug-PRCs can be constructed from such experiments for different concentrations, giving a somewhat remarkable family of curves (Fig. 10B).

Melatonin and Photoperiodism

Many biologists were surprised by the report that melatonin occurs in *Gonyaulax* cells. Its concentration varies ten-fold from day to night with a maximum at night, and is greater at lower temperatures.[63] The observation that a combination of low temperature and a short photoperiod provokes formation of what were referred to as asexual cysts[64] has led to the suggestion that encystment might result from elevated melatonin levels. If so, then melatonin might mediate between the circadian clock and a photoperiodic behavior that occurs in the fall each year, in which cells encyst, fall to the bottom and overwinter in the sediment. As mentioned above, an annual endogenous rhythm of cyst germination (excystment) has been observed.[4,5] Evidence has been sought without success for the possibility that melatonin or related compounds are part of the circadian clockwork in *Gonyaulax* (I. Balzer and J.W. Hastings, unpublished results). Such compounds were without effects on either period or phase, with either chronic or pulsed exposures.

Effects of Temperature

There are several aspects to the effects of temperature on the circadian bioluminescence system. First, the amplitude of the stimulated luminescence rhythm decreases as the temperature increases, whereas the amplitude of the glow rhythm is greater at higher temperatures.[65,66] Why this is so is not known, but the effect parallels the effect on the period of the rhythm, which is actually slightly longer at 26^{O} than at 16^{O} (Q_{10} = 0.85; (Fig. 11)). Thissuggested the existence of a compensatory mechanism,[65] now generally believed to be the general mechanism for circadian systems, even if still ill-defined.[22] However, this feature led to a clever test of the hypothesis that the period lengthening effect of D_2O is because it effectively lowers the temperature.[67,68] In *Gonyaulax*, D_2O has the same (concentration-dependent) period-lengthening effect as in other organisms, whereas lower temperatures shorten the period.[69]

Interestingly, the phase shifting effect of drugs is strongly dependent on temperature in *Gonyaulax*, being much more effective at lower temperatures.[58,70] This contrasts sharply with the phase shifting effects of light, which are not affected by temperature. This may suggest that light may induce changes in the phase without a requirement for new protein synthesis.

Yet another effect, reported for many different organisms, is that the expression of a circadian rhythm is conditional upon temperature, rhythmicity being absent at low temperatures.[71] In *Gonyaulax* luminescence is expressed arhythmically at temperatures at or below 12°C.[65,72] A rhythm resumes spontaneously upon return to a higher temperature, rephased to CT 12 at the time of the temperature shift-up (Fig. 12A). The transition between the two states is extremely sharp, characteristic of denaturation; at 13°C the rhythm appears normal and is not attenuated, but is completely lost at 12°C (Fig. 12B). In a limit cycle description this cannot be due to the oscillation being at the singular state, as the arhythmic state is stable at the lower temperature. It could be that the oscillation continues at a location remote from its normal limit cycle at higher temperatures, so distant that upon its return a single isochron at CT12 can essentially describe its trajectory.

Entrainment by temperature cycles, reported in several species, has not been examined in *Gonyaulax*. Low temperature pulses induce only small phase shifts (+/- 2 hours),[72] so if entrainment does occur, the limits of entrainment might be quite narrow.

Conclusion

Studies of the circadian physiology of *Gonyaulax* have provided important new discoveries along with a number of surprises. At the same time, the results obtained have been instrumental in developing our current concepts concerning how circadian clocks work and the characteristics they must possess to be useful to the organism. In many respects, the *Gonyaulax* system has proved to be a good model for the human circadian system, especially with regard to the large number of rhythmic processes and the fact that several of the rhythms can be desynchronized. One can expect that the bioluminescence system of this remarkable organism will continue to shed its light on circadian systems in the future.

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