No Desynchronization among Four Circadian Rhythms in the Unicellular Alga, Gonyaulax polyedra

Abstract. *The phase relationships among several circadian rhythms in the unicellular alga* Gonyaulax polyedra *remained unchanged during several weeks under constant conditions. A 6-hour exposure to darkness shifted the phase of all rhythms similarly. The* Q_{10} *for the frequency of three of these rhythms had been shown previously to be approximately the same and furthermore less than 1. These facts may mean that all these rhythms are the outputs of a single "master oscillator."*

Circadian rhythms have been reported in a wide variety of organisms from unicells to man (*1*). A circadian rhythm is a rhythm of behavioral or physiological activity having a period of exactly 24 hours when the organism experiences the day-night, light-dark, and temperature cycles of the earth; furthermore, the rhythm persists in constant laboratory conditions, where it assumes a period characteristic of the particular organism and the ambient light intensity and temperature. This "innate" period is approximately 24 hours but rarely exactly that.

In some organisms, more than one circadian rhythm has been studied. In this case, the question arises whether all the rhythms are outputs of the same mechanism, a "master oscillator" which controls the timing of several functions, or whether each observed rhythm is the output of a different oscillator having some degree of autonomy from the other oscillators. (The word "oscillator" is used here to refer to the unknown biological mechanism which gives rise to and controls circadian rhythms; its specific nature is as yet unknown.)

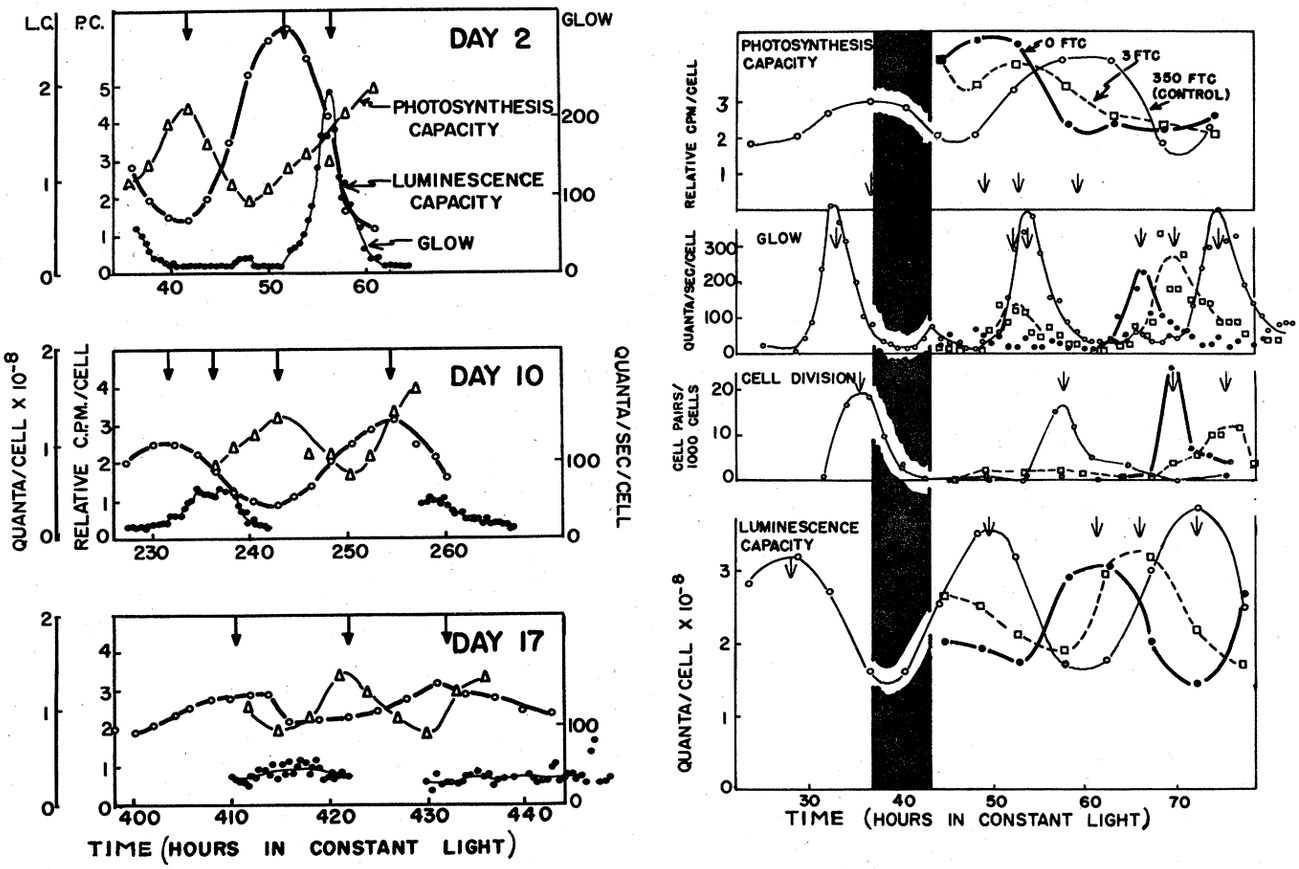

Fig. 1 (left). Luminescence capacity (*L.C.*), glow, and photosynthesis capacity (*P.C.*) rhythms after 2, 10, and 17 days in constant light (200 footcandles cool white fluorescent). Arrows locate rhythm maxima, estimated visually. A flask containing 1200 ml of culture was transferred from a light-dark, 12 hour–12 hour, cycle at the end of a light period (time 0) into constant light. At the times shown, samples for assay of all three rhythms were withdrawn. Glow samples were placed upon the scintillation counter turntable 32 minutes apart (if three replicates) or 24 minutes apart (if four replicates) and the glow intensity was thus measured every 32 or 24 minutes in between times of sampling the flask (every 2 to 3 hours). Three replicates for each rhythm were assayed for the day 2 and day 17 measurements; four were done for the day 10 measurements. Some glow points are missing because of turntable malfunction. The culture increased linearly in cell number from 4,500 cells per milliliter on day 2 to 16,000 on day 17. The cell division rhythm was not measured but in a similar experiment was not detectable 17 days after time 0. Fig. 2 (right). Phase shift of four rhythms caused by a 6-hour dark interruption. The dark interruption is represented by a vertical black swath. Ten-milliliter samples from a light-dark, 12 hour–12 hour, culture were pipetted into vials during the light-on phase. At the end of the next light-on phase the vials were placed on the turntable in constant light of 350 footcandles (*FTC*) and the turntable was started. Sampling began as shown after 23 hours. The exposure to a lower light intensity was given to appropriate vials by putting them into transparent containers wrapped with the appropriate number of white towels (3 footcandles) or by putting them in a light-tight box (0 footcandle). In assaying for the rhythms, samples were removed from the vials in a rotating schedule; only those vials used for measurement of glow intensity were undisturbed. All determinations except those of the number of cell pairs were made in triplicate from vials spaced around the turntable and, except for the glow intensity values, were averaged.

Comparison of several rhythms in *Drosophila* has shown no indication of the presence of more than one controlling oscillator (*2*). In man, however, there is evidence for the existence of more than one oscillator. The rhythm of body temperature was immediately entrained by (forced to assume the same period as) a newly begun 21- or 27-hour activity routine, while the rhythm of potassium excretion maintained the 24-hour period of the original routine (*3*). The body temperature and activity rhythms in some individuals had different periods under constant conditions (*4*). The activity rhythm was entrainable by a wider range of light-dark cycle frequencies than the temperature rhythm (*5*). These are three examples of the desynchronization of rhythms—that is, of instances where the frequency of one rhythm differs from that of another.

Circadian rhythms also occur in isolated tissues in vitro (*6*). Along with the desynchronization between rhythms seen in man, this fact indicates that in a multicellular organism there can be a number of semiautonomous oscillators. Could such a situation also occur in an organism consisting of but a single cell? This report describes attempts to desynchronize several rhythms manifested by a unicellular organism. All such attempts have been unsuccessful. Therefore there is no evidence that these rhythms are controlled by more than one oscillator.

Gonyaulax polyedra is a bioluminescent marine dinoflagellate about 40 μm in diameter. Four circadian rhythms have been studied: (i) luminescence capacity, the amount of light released in a burst when 1 ml of 0.06*N* acetic acid is injected into a 1.5-ml culture sample; most of this light can also be elicited by passing a stream of air through a sample until the bright cell flashes so stimulated finally cease (*7*); (ii) luminescence intensity or "glow," a low-intensity, continuous emission, monitored on vials of cells by a modified automatic scintillation counter (*8*); (iii) time of cell division, measured by counting the number of cell pairs in 0.1-ml samples of the culture; each pair represents a cell fission completed less than half an hour earlier (*9*); (iv) photosynthesis capacity, measured as the amount of ^{14}C from carbonate fixed by cells in light of saturating intensity for photosynthesis (*10*). To measure photosynthesis capacity, a 5-ml (Fig. 1) or a 2-ml (Fig. 2) culture sample in a scintillation vial was injected with 0.062 or 0.037 μc of $Na_2{}^{14}CO_3$ (New England Nuclear Corp). After 10 or 15 minutes at 1100 footcandles (11,800 lu/m^2) of cool white fluorescent light, the uptake of carbonate was stopped by filtering the cells (Fig. 1) or by adding 0.4 ml of 1*N* HCl and filtering (Fig. 2). The cells were washed once with 5 ml of medium (Fig. 1) or once with 2 ml of 0.1*N* HCl and twice with 2 ml of water (Fig. 2). The 2.4-cm diameter Whatman filter papers on which the cells had been collected were air-dried and counted in a Nuclear-Chicago gas flow counter. The method used to gather the data shown in Fig. 1 caused less leaching of photosynthetic counts during the washing.

Cultures were grown on light-dark, 12 hour–12 hour, cycles routinely as previously described (*11*). The experiments described here were done at 20.5°C in a temperature-controlled, electrically shielded room. Intensity of fluorescent lights was measured with a Weston illumination meter, model 756.

If the different rhythms in *Gonyaulax* were each caused by a different underlying oscillator and if the periods of these oscillators were slightly different, the phases of the rhythms would drift apart in constant environmental conditions. We looked to see if this would happen.

Figure 1 shows measurements made on three rhythms 2, 10, and 17 days after the start of constant conditions. There was no significant difference in the phase relationships among the three rhythms after 10 days, nor was there any among the remaining two (the glow rhythm has dampened) after 17 days. Another experiment in which the glow and luminescence capacity rhythms were measured after 2 and 15 days in constant conditions also showed no change in the phase relationship between those two rhythms.

The phases of the rhythms, then, did not drift apart noticeably in constant conditions. The implication is that the periods of the three rhythms did not differ noticeably and that therefore there is no evidence that more than one underlying oscillator is involved.

A second kind of experiment involved shifting the phase of all the rhythms by dark treatment. In a circadian rhythm which is "free-running" under constant conditions, a phase shift can typically be induced by interrupting the constant conditions with an increase or decrease in light intensity for a few hours (or even minutes). Whether the phase is advanced or delayed, and by how much, depends upon the point during the cycle at which the interruption comes. We measured the effect of a 6-hour exposure to darkness (0 footcandle) or to very dim light (3 footcandles) upon all four rhythms simultaneously. The direction of the resulting shift, advance or delay, would be that direction in which the 0 footcandle exposure (being the greater change in light intensity) gave a greater shift than the 3-footcandle exposure gave.

The results are shown in Fig. 2. The phases of all four rhythms were advanced by the treatment. The magnitude of the advances was similar for all four rhythms for both exposures. The shift for the glow rhythm would have resembled a delay had the 3-footcandle exposure not been included.

The similarity in the response of all four rhythms following 6 hours of lower light intensity provides no indication that the four rhythms are not under the control of one oscillator.

The evidence presented here is consistent with the hypothesis that the glow, luminescence capacity, cell division, and photosynthesis capacity rhythms in *Gonyaulax* are all outputs of a single "master" oscillator. Other evidence supporting this hypothesis is that the luminescence capacity and cell division rhythms responded similarly to a phase-shifting exposure to ultraviolet light (*12*) and that the Q_{10} for the effect of temperature on frequency was approximately the same for the three rhythms tested; in fact, the frequencies were slightly *lower* the higher the temperature, rather than being higher as is more common for circadian rhythms (*13*).

A second explanation consistent with all presently available information is that there are several equivalent, tightly coupled oscillators responsible for the several *Gonyaulax* rhythms. Even if a stimulus did not affect all such oscillators identically, an immediate subsequent mutual entrainment would wipe out the initial tendency of the oscillators to dissociate.

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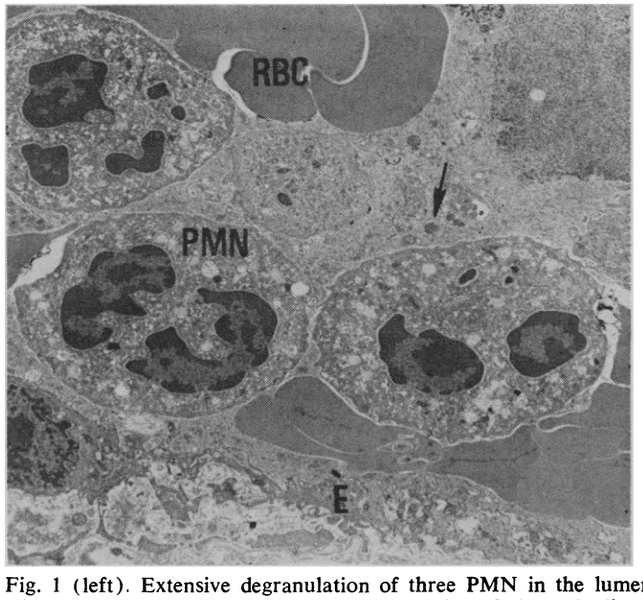
Intravascular Degranulation of Neutrophils: An Important Factor in Inflammation?

Abstract. *Polymorphonuclear leukocytes are degranulated in the lumens of vessels in synovial membrane in humans with various types of inflammatory arthritis and in dogs with synovitis induced by urate crystals. This degranulation, accompanied by the release of lysosomal enzymes and vasoactive materials, may be an important part of the mechanism resulting in vascular injury.*

In studies with electron microscopy of venules in synovial membrane in inflammatory conditions we have noted both degranulation (Fig. 1) and occasional fragmentation of polymorphonuclear leukocytes (PMN) in the lumens. Such intravascular degranulation, not previously reported in human disease, has been found in gouty arthritis, ulcerative colitis, serum sickness, rheumatoid arthritis, and undiagnosed acute arthritis. Degranulation has been seen in vessels where there is no other evidence of vascular injury but has most commonly been associated with changes in the adjacent vessel wall. Vascular alterations accompanying such degranulations include endothelial necrosis; gaps between endothelial cells; fibrin infiltration of the vessel wall and deposition of PMN, erythrocytes, and cell debris; and electron-dense deposits between endothelium and pericytes. Not all PMN were degranulated and most other cells in the specimens showed preservation of structure. Identical preparative techniques (*1*) have shown no PMN degranulation in degenerative arthritis or in normal animal joints but intraluminal PMN are less common in these conditions.

In order to study the intraluminal degranulation we produced an experimental synovitis in dogs by injection of sodium urate crystals. Sterile (pyrogen-free) crystals of monosodium urate in physiologic saline (4 ml) (*2*) were injected into one knee joint of each of ten mongrel dogs (who weighed between 18 and 25 kg) while the dogs were under sodium pentobarbital anesthesia. Tissue samples from each knee injected with urate were examined at various intervals with both light and electron microscopy. After 3 minutes and after 10 minutes, the synovia injected with urate crystals appeared normal, but



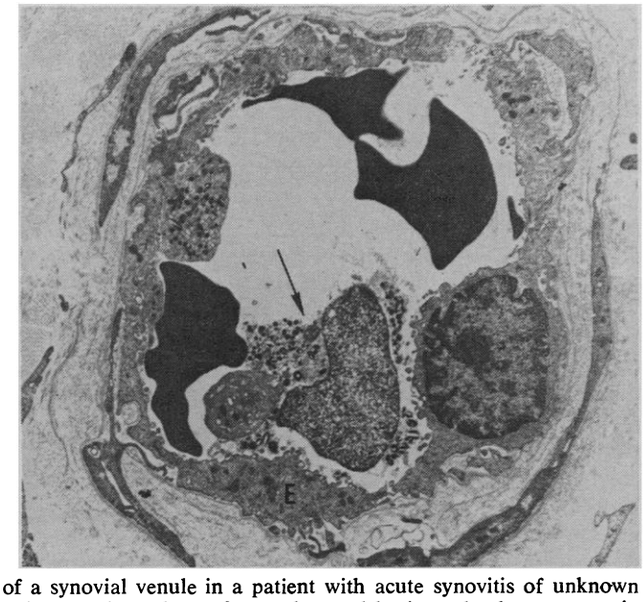


Fig. 1 (left). Extensive degranulation of three PMN in the lumen of a synovial venule in a patient with acute synovitis of unknown etiology. Degranulation is shown by the rarity of dense bodies, by increased numbers of vacuoles, and by irregular lucent areas in the cytoplasm of the PMN. Free cellular organelles present in the lumen, including mitochondria (arrow), are evidence of cell fragmentation; *RBC*, erythrocytes; *E*, venular endothelium (× 3000). Fig. 2 (right). Fragmentation of PMN (arrow) in lumen of an intact venule in dog, 30 minutes after injection of urate crystals into the knee. The PMN cell membrane has been lost and dense bodies lie free in the lumen; *E*, venular endothelium (× 6000).