

Resetting the Biological Clock in *Gonyaulax* with Ultraviolet Light^{1, 2}

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Many physiological processes show regular daily fluctuations in rate. Much information is now available to show that these daily fluctuations continue to occur in constant light and constant temperature. Thus they do not merely reflect changes in the environment but must be controlled endogenously. The diurnal rhythms, as these phenomena have been called, are so common and the processes involved, so varied, that a general mechanism for time keeping, a biological clock, has been postulated (7). The solar time at which the maximum biological activity of a process occurs may be shifted in a predictable way by exposure to light, and the biological clock, like its mechanical counterpart, can be reset. This property is of considerable practical importance since it enables biological time to be adjusted to external solar time.

In spite of extensive studies, the mechanism of the postulated biological clock is unknown. The ability of light to reset the diurnal rhythms offers a possible point of attack for the examination of the mechanism of time keeping, since light must presumably alter some clock component to reset or shift the phase. Studies of the effects of visible light on phase shifting have not as yet revealed the nature of this change. It is possible to shift the phase of at least one of the biological clocks, that controlling mating behavior in *Paramecium bursaria* (2, 3), with ultraviolet irradiation. Therefore, the possibility that the biological clock in *Gonyaulax* can also be reset by ultraviolet light has been investigated, with the hope that such a study might reveal additional clues to the mechanism by which phase is changed, and hence to the operation of the biological clock.

Materials and Methods

The photosynthetic dinoflagellate *Gonyaulax polyedra* was cultured in sea water enriched with nitrate, phosphate, iron, EDTA, and soil extract as described previously (10). During growth, the cultures received 12 hours light at about 500 ft-c alternating with 12 hours darkness, and the temperature

was $20 \pm 2^\circ$. When the population reached 5000 to 7000 cells per ml, the cultures were removed from the light-dark schedule at the beginning of a light period, and were placed in constant light of 50 ft-c intensity. This was the lowest light intensity which allowed the cells to survive by photosynthesis. In one experiment, (fig 1 and 4), 90 ft-c constant light was used. Cool white fluorescent lamps served as light sources and the intensity was measured with a Weston Illumination meter. In *Gonyaulax*, rhythmicity is not inhibited by constant light of these intensities, (4).

Irradiation with ultraviolet light was carried out at different times during the first 24 hours after the cultures were placed in constant light. Cells were irradiated as follows: a 60 ml aliquot of the cell suspension was placed in a shallow glass container where the depth of the suspension was 2 mm. In this position, cells were irradiated with the light from a 15 w Sylvania Germicidal A lamp in a parabolic reflector at a distance of 50 cm. Nearly all the light emitted by this lamp is of wave lengths shorter than 270 m μ , and, according to the specifications of the manufacturer, 98 % lies at 254 m μ . The intensity at the level of the cell suspension measured with a calibrated ultraviolet photometer (AV971, Hanovia Chemical and Manufacturing Company), was 150 ergs per cm² second. Ten experiments in which the effect of ultraviolet light on the phase of the rhythm in luminescence was tested were performed.

In one experiment (fig 1 and 4), an equivalent Westinghouse lamp (Q157P) in a parabolic reflector was used, also at 50 cm from the surface of the cell suspension.

Following irradiation, which was of 2 or 4 minutes' duration in most experiments, the cell suspension was returned to a beaker and then dispensed in 2 ml aliquots into test tubes. Unless otherwise indicated, these tubes were returned to constant light (50 ft-c intensity) at 20° until used for the measurement of luminescence, usually several days following irradiation to allow for recovery of luminescence. Recordings of the luminescence in response to stimulation were made as previously described (10). Cell division was detected by examining the contents of similar tubes for the presence of cells in pairs (11).

Results

In *Gonyaulax*, the immediate effect of ultraviolet irradiation was a reduction in the amount of lumi-

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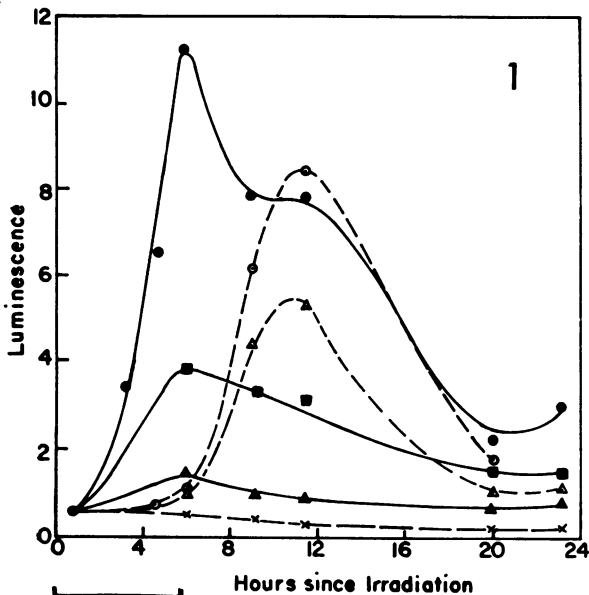


FIG. 1. Inhibition of luminescence in *Gonyaulax polyedra* immediately following irradiation with ultraviolet light for zero (●, ○); one (■); 2 (▲, △); and 4 minutes (×). Cells were grown with alternating light and darkness, 12 hours each, and were irradiated in the middle of a light period. For 6 hours immediately following irradiation with ultraviolet light, represented by a bar under the abscissa, one set of aliquots (●, ■, ▲, ×) was placed in the dark, and one (○, △) in the light from cool white fluorescent lamps 500 ft-c (12,500 ergs per cm² sec) in intensity. After this time, all samples were illuminated continuously with fluorescent light at 90 ft-c.

nescence. The effect was roughly proportional to dosage in the range of 1 to 4 minute exposures (fig 1). Cell division also showed a transitory inhibition immediately following irradiation. The reduction in luminescence following ultraviolet light was partially reversed by subsequent exposure to visible light. Experiments to test for photoreversibility were carried out as follows: immediately after ultraviolet irradiation, a portion of irradiated and unirradiated aliquots were placed in darkness for 6 hours, while corresponding aliquots of unirradiated cells and of cells irradiated 2 minutes with ultraviolet light were placed in bright visible light (500 ft-c) for 6 hours. Subsequently all cells were kept in continuous light (90 ft-c). At intervals, aliquots were removed for the measurement of luminescence (fig 1). When the maximum luminescence attained during the first 24 hours in unirradiated cells is compared with that of cells irradiated for 2 minutes with ultraviolet light, it is apparent that the effect of ultraviolet light is larger when darkness follows irradiation than when cells are brightly lighted immediately after irradiation (90% as compared with 40% inhibition). This difference was still apparent in the second 24 hours following irradiation and thus cannot

be attributed to the later time at which cells in light reached maximum luminescence. This delay is the result of a shift of phase brought about by the bright illumination with visible light (4). Since light inhibits luminescence (12), the control curve for cells darkened for 6 hours then transferred to continuous light show a dip after this transfer. This dip obscures the position of the maximum in luminescence in these cells.

Whether or not a change in the time of maximum luminescence (a phase shift) had resulted from ultraviolet irradiation was uncertain from the data for the luminescence during the first 24 hours of the experiment because irradiated cells showed an indistinct maximum in luminescence. However, the ability to

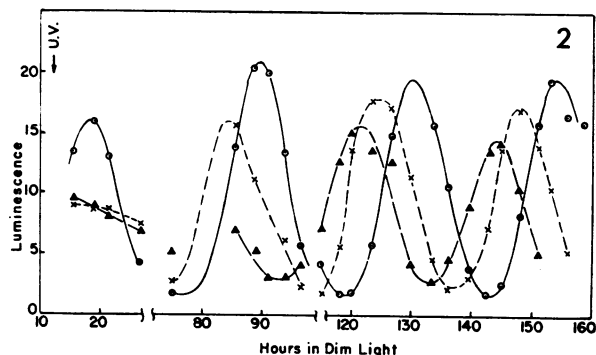


FIG. 2. Shifting the phase of the rhythm in luminescence in *Gonyaulax polyedra* with ultraviolet light. Cells were grown in alternating light and darkness, and were transferred to continuous light at the beginning of a light period. Twelve hours later, at the arrow, cells were irradiated with ultraviolet light for zero (○), 2 (×), or 4 minutes (△), and were subsequently replaced in continuous light of 50 ft-c intensity.

luminescence is recovered if ultraviolet irradiated cells are allowed to remain in constant light for several days. Consequently, the curves for luminescence 5 and 6 days after irradiation were measured and examined for phase differences. At this time, cells had recovered sufficiently to show considerable luminescence during the night phase. It was now possible to detect that a phase change had taken place as a result of irradiation (fig 2). Following a 4-minute exposure to ultraviolet light, a phase shift of 10 hours could be observed. This phase shift was proportional to dosage, 2-minute exposures giving about half as much phase shift as 4-minute exposures. The direction of the shift was clearly an advance, since maxima occurred sooner the longer the irradiation (fig 2). Similar phase advances were obtained in experiments where 2- and 4-minute exposures to ultraviolet light were given at the middle of the light period or the middle of the dark period. When the time in the luminescent cycle at which cells were exposed to ultraviolet light was varied, it was found that the amount of phase change de-

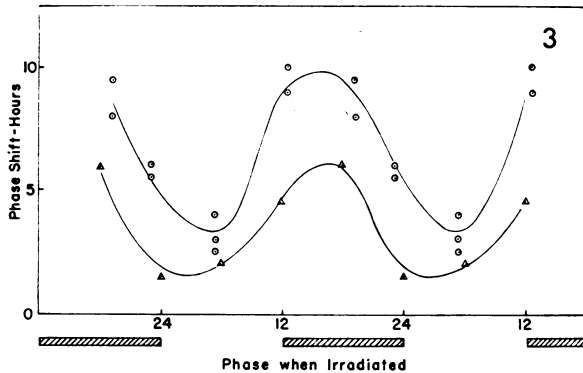


FIG. 3. Phase shift in the rhythm in luminescence in *Gonyaulax polyedra* after 4 minutes' exposure to ultraviolet light at different times in the rhythmic cycle. The data from 2 experiments are presented, (o, 7/9/59; Δ , 7/27/59). All cells were in continuous dim light (50 ft-c) for the duration of the experiment. The black bars on the abscissa represent the dark periods of the preceding light-dark schedule which determined the phase of the cultures at the time of irradiation.

pendent on when in the cycle irradiation occurred, the greatest shifts occurring in cells irradiated early in their night phase (fig 3). Such differences in sensitivity to reset are also found after illumination with visible light (4, 5). However, the maximum effectiveness appears to occur earlier in the night phase with ultraviolet than with visible light. Furthermore, while visible light may cause either an advance or a delay in phase depending on when in the cycle cells are illuminated, only phase advances are obtained with ultraviolet light.

That the phase of the rhythm in luminescence was shifted immediately by irradiation with 4-minutes' ultraviolet light was shown indirectly, since it was not possible to measure any convincing peak in luminescence during the first day following irradiation. The phase of the rhythm was determined by examining the subsequent effect of visible light on phase, utilizing the difference in sensitivity to reset by light as a measure of the phase of the rhythm. Cells which had been exposed to 2 minutes of ultraviolet light at the start of the night phase were illuminated again, this time with visible light at the middle of the night phase when the cells would have been most sensitive to reset by visible light if the cycle had not been shifted by ultraviolet exposure. In such doubly irradiated cell suspensions, the visible light no longer brought about as great a reset as in cell suspensions receiving only visible light (fig 4), but now responded to visible light with an additional phase shift equal to that of cell suspensions 5 hours more advanced in phase. The change in phase is stable, with time, being essentially the same $4\frac{1}{2}$ and $7\frac{1}{2}$ days after irradiation (fig 4).

In contrast to the clear reversal of the ultraviolet inhibition of luminescence by visible light, little if any photoreversal was observed in the phase shifting

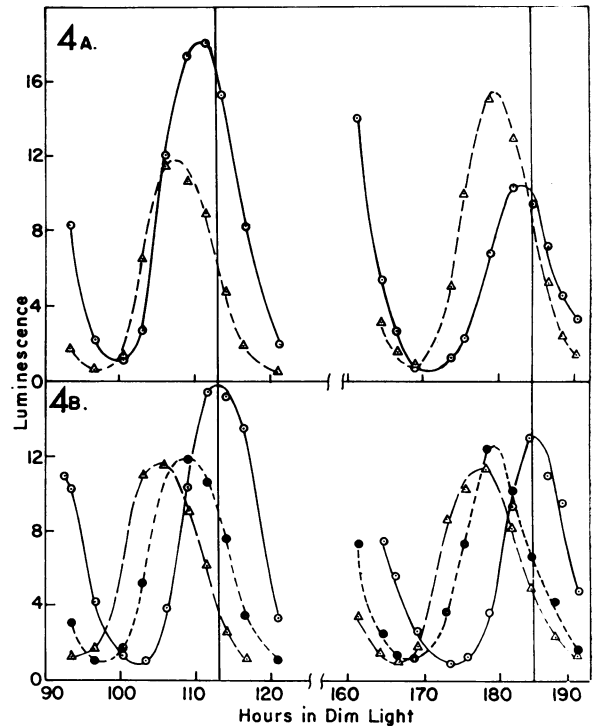


FIG. 4. Shifting the phase of the rhythm in luminescence in *Gonyaulax polyedra* by consecutive exposures to ultraviolet and visible light. The curves show the luminescence $4\frac{1}{2}$ and $7\frac{1}{2}$ days after irradiation. The luminescence of the unshifted controls is represented in curve B, o, and the positions of the maxima in this control curve are marked by vertical lines to facilitate comparison. Cells of the top curves (A) were illuminated for 3 hours with visible light (800 ft-c) at the middle of the night phase (curve A, Δ) and 5 hours later (curve A, o). The bottom curves (B) represent the luminescence of cells with no irradiation (curve B, o); with 2 minutes' ultraviolet light at the beginning of the night phase (curve B, \bullet); with 2 minutes' ultraviolet light at the beginning of the night phase followed by 3 hours' bright visible light (curve B, Δ) at the time corresponding to the middle of the night phase of unirradiated cells. The phase shifts are as follows: visible light at midnight, 6 hours' advance; visible light 5 hours later, 3 hours advance; 2 minutes' ultraviolet light, 5 hours' advance; followed later by 3 hours visible light, 8 hours advance.

effect of ultraviolet irradiation (fig 5). When irradiation was immediately followed by darkness, 2-minute exposures to ultraviolet light shifted the phase by about $5\frac{1}{2}$ hours, while the phase shift in cells exposed to bright white light immediately after irradiation was $4\frac{1}{2}$ to 5 hours, not a significant difference.

In *Gonyaulax*, luminescence is not the only process which shows a diurnal rhythm and is therefore presumably under the control of the biological clock. Both cell division (11) and photosynthesis (6), are strongly rhythmic. Does ultraviolet light effect these rhythms or does it specifically effect lumi-

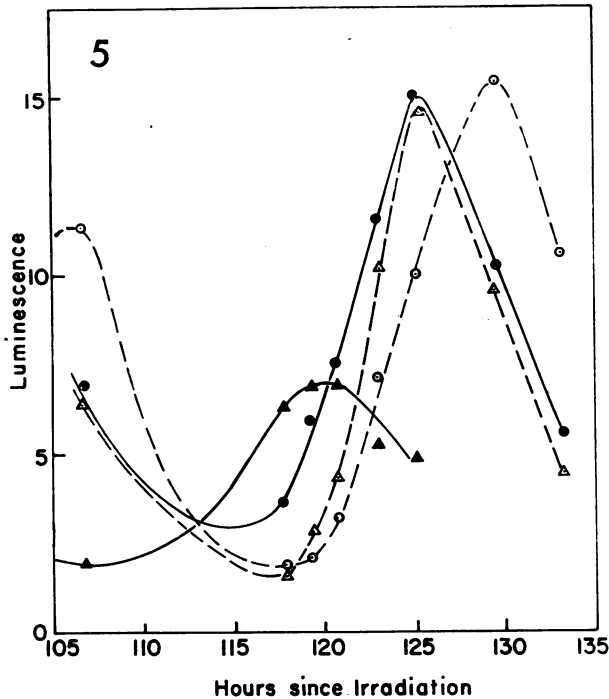


FIG. 5 (left). Lack of photoreversibility in the phase shifting effect of ultraviolet light on the rhythm in luminescence in *Gonyaulax polyedra*. These curves represent data from the same experiment as those of fig 1, but show the rhythm in luminescence 5 days after irradiation. Cells in darkness (●), and in bright light of 500 ft-c. (○) for 6 hours at the start of the experiment; cells irradiated for 2 minutes with ultraviolet light then placed in darkness (▲) or in bright light (△) for 6 hours. When darkness follows irradiation, the phase shift due to ultraviolet light is $5\frac{1}{2}$ hours while the phase shift is $4\frac{1}{2}$ to 5 hours in cells brightly illuminated following irradiation with ultraviolet light. This difference is not significant.

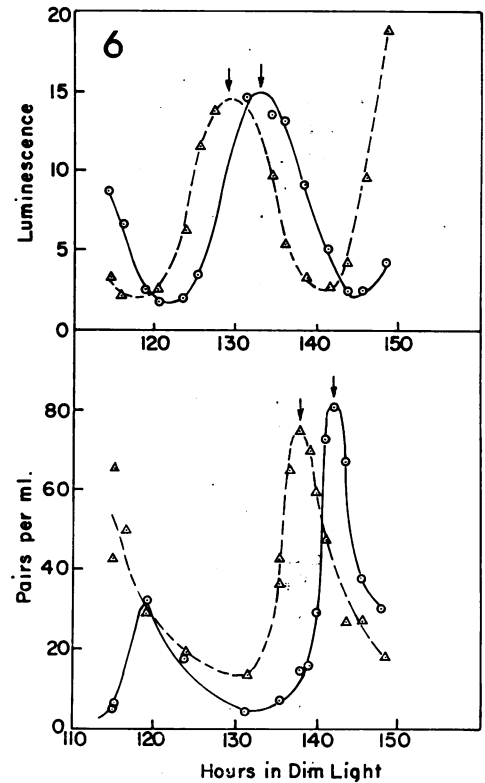


FIG. 6 (right). The change in the phase in the rhythm in luminescence in *Gonyaulax polyedra* caused by 2 minutes' exposure to ultraviolet light, compared with the effect of the same irradiation on the rhythm in cell division in the same cell suspension. Top curves, luminescence in unirradiated (○) and irradiated (△) cells; bottom curves, cell division as measured by the number of paired cells in unirradiated (○) and irradiated (△) cells. The phase of each rhythm is advanced by 4 hours. An arrow marks the maximum in each curve.

nescence? To answer this question, the rhythm in cell division and in luminescence were examined in samples of the same cell suspension after irradiation with ultraviolet light. In both, the phase of the rhythm was advanced by irradiation and by exactly the same amount (fig 6). Thus the effect of ultraviolet light is on the phase of the biological clock, rather than a specific effect on luminescence.

Discussion

Ultraviolet light at a wave length of about 254 μ quite clearly resets the biological clock in *Gonyaulax*. The reset differs from that brought about by visible light in several interesting ways. With ultraviolet light, very much shorter exposures bring about a given change in phase: at the most sensitive time in the rhythmic cycle 45 times longer exposures are

required in the visible than in the ultraviolet to produce 10 to 12 hours phase change, and the total dose relationship is about 1.3×10^8 ergs/cm² visible light compared with about 3.6×10^4 ergs/cm² in the ultraviolet. All phase changes following exposure to ultraviolet light are advances, while visible light causes both advances and delays, the type depending on the time in the cycle when exposures occur. The fact that all phase changes are clearly advances no matter when during the cycle irradiation occurs makes untenable the interpretation that exposure to ultraviolet light returns the rhythm toward a zero point. The difference in the degree of the phase shift with time in the cycle must reflect a different condition of the clock mechanism. The effectiveness of ultraviolet light suggests that this difference lies in the protein or nucleic acids which thus must form part of the clock machinery. The lack of photoreversal in the

resetting effect suggests that it is a cytoplasmic effect and does not reside in the nucleus. Absence of photoreversibility has been shown for effects of irradiation of the cytoplasm in the eggs of *Habrobracon* (14), and in *Paramecium* (1), and photoreversibility has been traced to DNA (8,9). That nuclear DNA is not involved in timekeeping was also postulated from experiments with enucleated *Acetabularia* (13).

Summary

Short exposures (2-4 minutes) to ultraviolet light bring about stable changes in the phase of the rhythms of luminescence and cell division in *Gonyaulax polyedra*, presumably resetting the biological clock. This effect is complete shortly after the end of irradiation, and does not appear to be photoreversible, unlike the inhibition of luminescence by ultraviolet light which is reversed by visible light.

The amount of change in the phase of the rhythm depends upon the length of the exposure to ultraviolet light and the time in the diurnal cycle at which irradiation occurs.

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