The Action Spectrum for Shifting the Phase of the Rhythm of Luminescence in *Gonyaulax polyedra*

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ABSTRACT The action spectrum for changing the phase of the rhythm of luminescence in the marine dinoflagellate *Gonyaulax polyedra* has been determined. Maxima in effectiveness were found at 475 and 650 mμ. The significance of these findings is discussed.

The luminescence of the photosynthetic marine dinoflagellate, *Gonyaulax polyedra*, shows a pronounced diurnal rhythm. Cultures exposed to alternating light and dark periods of 12 hours each (LD) exhibit a much greater luminescence during the dark periods (Fig. 1), and these rhythmic changes continue to occur when the cultures are transferred to conditions of either continuous dim light (100 to 200 foot-candles) or darkness. In dim light, the rhythm of luminescence has been observed to continue undamped for more than 2 weeks, with a period of approximately 24 hours. In the dark, however the amplitude diminishes (Fig. 1), due to the need for light in the nutrition of this organism (Sweeney and Hastings (1957); Hastings and Sweeney (1958)).

As in the case of many other persistent diurnal rhythms (Pittendrigh and Bruce (1957); Pittendrigh (1958)), the properties of this rhythm suggest that a clock-like mechanism is involved. The period of the rhythm, for example, is essentially temperature-independent (Hastings and Sweeney (1957, 1958)). A feature which is of specific concern in the present studies is the fact that the phase, or the solar time at which a maximum in luminescence occurs, may be shifted or "reset" by a non-repeated change in light intensity (Hastings

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and Sweeney (1958)). No exposure to a new light-dark cycle is necessary. A phase shift may be brought about either by a single change in light intensity, or by a single pulse of light, as illustrated in Fig. 1. The fact that light perturbations of this sort shift the phase indicates that the products of a photochemical reaction have a specific effect upon the mechanism responsible for the rhythm. Therefore, information concerning the identity of this phase-shifting reaction should be of considerable importance in gaining an understanding of the mechanism of diurnal rhythms. In order to investigate the nature of the photosensitizer in the phase-shifting reaction, a determination of the action spectrum was made.

Materials and Methods

Cultures of *Gonyaulax polyedra* were grown at 22°C. in a supplemented sea water medium, as described previously (Sweeney and Hastings (1957)). Light required for growth was provided by "white" fluorescent lamps which were scheduled by means of switchclocks to be on for 12 hours during each 24 hour period. Under these conditions, luminescence was greatest during the middle of the dark period (Fig. 1), and cell divisions occurred almost exclusively at the end of the dark period (Sweeney and Hastings (1958)). Cultures were inoculated at a density of about 1000 cells per ml. and used for experiments 6 to 10 days later at densities ranging from 7,000 to 12,000 cells per ml.

The measurements of bioluminescence were made, as described previously (Sweeney and Hastings (1957)), by measuring the total light emitted from an aliquot upon stimulation. Subsequent to the measurement, the aliquot was discarded.

The large biological spectrograph at the Argonne National Laboratory served as the source of isolated narrow spectral regions in most of the experiments. The design and characteristics of this grating spectrograph have been described previously (Monk and Ehret (1956)). The light source was a high intensity carbon arc lamp, and the slit was 2.54 cm. in width and 12.7 cm. in height. Nineteen stations were established at 25 mμ intervals, from 325 to 775 mμ, along the focal curve, with an additional station at 312.5 mμ. Each experimental determination required eight test tubes. Each station was designed to accommodate sixteen test tubes, so that determinations at a given wave length could be made at two different irradiances. In this design, each station occupied a width of 5.5 cm., corresponding to 5.5 mμ along the focal curve. Thus the station nominally at 500 mμ encompassed positions where peak energies ranged from 497.25 to 502.75 mμ.

At each station, the incident horizontal beam was reflected vertically downward into the open tops of the test tubes by an aluminum-surfaced mirror. When desired, the light intensity was reduced by the use of calibrated neutral density screens. The energy of the horizontal beam at each station was measured with a thermopile and the appropriate corrections for mirror reflectance and neutral density screens were made. The density of the cell suspensions was such that the transmittance was greater than 95 per cent at the wave lengths of maximum absorbance, so that no correction was made for absorption by cells or medium.

Experiments concerning sequential irradiation with red (660 mμ) and far red (730 mμ) light were carried out using 1000 watt incandescent light sources mounted in Kodak projectors, with appropriate interference filters in the light path, as described elsewhere (Sweeney, Haxo, and Hastings (1959)).

EXPERIMENTAL PROCEDURE AND RESULTS

The experimental procedure may be best described by referring to Fig. 1. Approximately 2 days prior to the time of monochromatic irradiation, a 2 ml. aliquot of cell suspension from a culture grown in LD conditions was pipetted into each of about 800 test tubes. These were then replaced in LD conditions at constant temperature. On the day when the irradiations were to be carried out, all tubes were transferred to darkness and divided into three major groups.

The control group remained in the dark with no additional exposure to light, and was used to determine the phase of the luminescence rhythm of cells under these conditions (solid line in Fig. 1).

A second group received irradiation (from 66 to 69 hours) with white light of several intensities (fluorescent lamps; color "white"). Subsequently, they were kept in the dark at constant temperature and their luminescence measured. The phase shifts were determined from curves similar to that illustrated in Fig. 1 with a dashed line. Using these values a curve was constructed showing the relationship between the white light intensity during irradiation and the number of hours by which the phase was shifted (Fig. 2).

The third group was irradiated with monochromatic light of different wave lengths and intensities for the same 3 hour period; *i. e.*, from 66 to 69 hours. These were subsequently placed in darkness and the amount of phase shift was determined, obtained in each case from measurements of luminescence. Since the number of test tubes which could be used was limited, it was not possible to obtain sufficient data to draw curves as complete as those shown in Fig. 1. The eight tubes were used to determine the time at which a single maximum in luminescence occurred, as illustrated in Fig. 3.

A three hour light exposure at the time when luminescence was maximum was selected since in previous experiments it had been found that the maximal phase shift was thus obtained (Hastings and Sweeney (1958)).

Two experiments of this type were carried out. In the first, the first maximum in luminescence subsequent to the irradiation was measured in order to determine phase shift. In the second experiment, the cells were kept in the dark for an additional day and the second maximum in luminescence was measured. The agreement obtained between these experiments indicates that the two maxima are equally reliable as indicators of phase shift. This conclusion has also been reached from other experiments (Hastings and Sweeney (1958)).

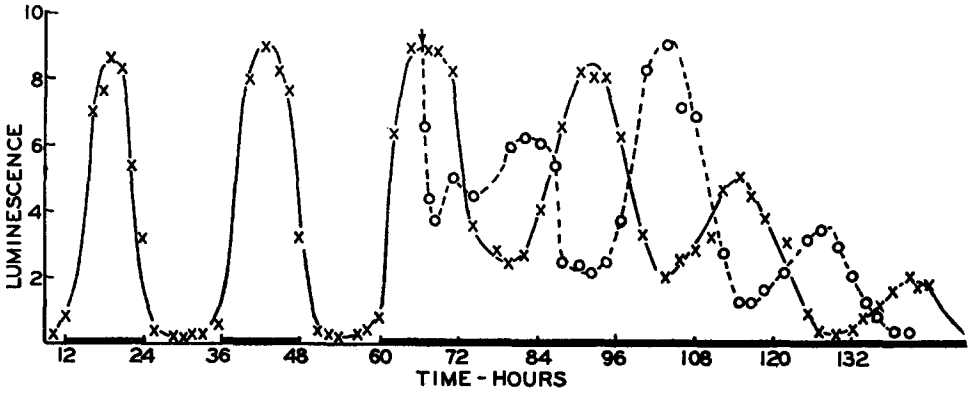

FIGURE 1. This figure illustrates shifting of the phase of the rhythm of luminescence by a single exposure of the cells to light. Phase shifting in all the experiments described was carried out in this way. The cells were grown with alternating light and dark periods of 12 hours each (LD), and were pipetted into test tubes about 2 days before irradiation, at some time between 0 and 12 hours on the graph. They were then replaced in LD conditions, and the typical rhythm of luminescence is shown. Cells remaining in the dark after 60 hours continue to show the rhythm with a period of approximately 24 hours, although the amplitude decreases (solid line). Cells treated similarly, except for a 3 hour exposure to light, between 66 and 69 hours, also show the rhythm of luminescence, (broken line), but with a shift in the phase, or time at which the maxima occur. The number of hours by which the phase is shifted is dependent upon the intensity and color of the light used for irradiation, as illustrated in the subsequent figures.

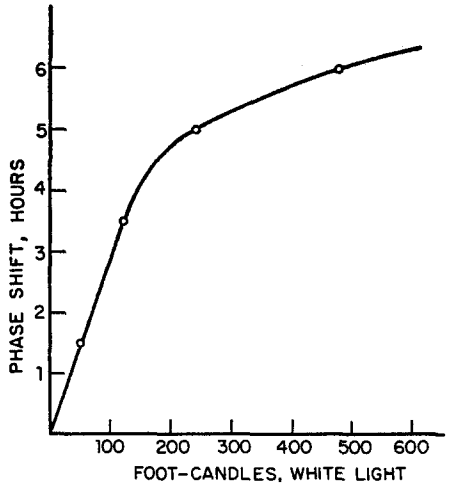

FIGURE 2. The relationship between the intensity of white light and the number of hours by which the phase was shifted in the first of the two experiments. A 3 hour light exposure between 66 and 69 hours (Fig. 1) was used. The first maximum in luminescence subsequent to irradiation was measured to determine phase shifts. The exact shape of this curve has been found to vary from experiment to experiment.

In making calculations for the action spectrum the curve for the effect of white light obtained in the same experiment was used to correct values for monochromatic light.

The time at which the maximum in luminescence occurred following each different irradiation is illustrated in Fig. 3. Details concerning the curves are explained in the legend. Curves for the effect of the second intensity are omitted for some wave lengths, as are those for irradiations at 700 mμ and at longer wave lengths, which caused no measurable phase shift in this

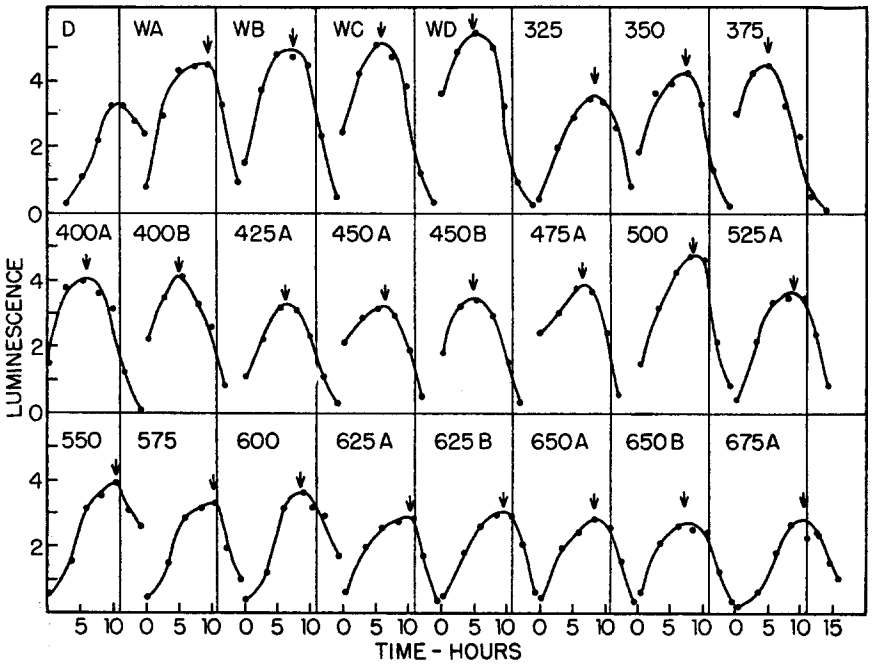

FIGURE 3. This figure shows curves for luminescence as a function of time in the first of the experiments. The curves were used to estimate the phase of the luminescence rhythm, and the arrow above each curve indicates the time of maximum luminescence. To facilitate comparison, the time at which the maximum in luminescence occurred in the dark control (upper left, labeled D) is indicated on all curves by vertical lines. Zero time on the graph was 18 hours after the cells were first placed in the dark, or 12 hours after the beginning of the 3 hour light exposure. Thus the maximum in luminescence of the dark control occurred 29 hours subsequent to the time the cells were placed in the dark (see Fig. 1). The curves labeled WA, WB, WC, and WD were from cells which were exposed to white light at four different intensities, and these values are plotted in Fig. 2. All other curves were from experiments in which monochromatic light was used, the wave length being indicated above each curve. When there is a letter suffix, two intensities at that wave length were used, B having the greater energy.

experiment. The values obtained may be noted from Table I. In the second experiment, higher energies were used at 700 mμ and at longer wave lengths so that some phase shift was obtained.

In calculating the action spectrum, we have used the curve shown in Fig. 2, rather than a straight line, since it represents the relationship between

TABLE I

Wave length	Irradiance	Observed phase shift	Calculated energy required for a 2 hr. phase shift	Calculated relative effectiveness on the basis of equal No. of quanta
	ergs/mm.²/sec.	*hrs.*	*ergs/mm.²/sec.*	
325-1	10.7	2.5	8.8	0.37
325-2	10.8	2.5	8.0	0.33
350-1	18.7	3.5	10.9	0.32
350-2	18.7	7	2.3	0.12
375-1	41.0	6	6.0	0.63
375-2A	16.0	5	4.2	0.72
375-2B	32.6	7	4.0	0.76
400-1A	11.3	5	3.3	1.2
400-1B	23.0	6	3.3	1.2
400-2A	10.5	6	1.4	2.4
400-2B	4.5	4	1.8	1.8
425-1A	4.5	4.5	1.7	2.1
425-1B	9.2	5.5	1.8	2.3
425-2A	4.5	4	1.8	1.9
425-2B	9.2	5.5	2.0	1.7
450-1A	4.0	4.5	1.5	2.9
450-1B	8.1	6	1.2	3.8
450-2A	2.5	3	1.5	2.4
450-2B	3.7	4.5	1.2	3.2
475-1A	3.6	4.5	1.4	3.5
475-1B	7.3	6.5	0.9	5.1
475-2A	2.3	5	0.60	6.3
475-2B	3.3	6	0.56	6.8
500-1	19.5	2.5	15.6	0.33
500-2A	7.7	3	4.7	0.87
500-2B	15.6	3.5	7.6	0.53
525-1A	6.6	2	6.6	0.80
525-1B	13.4	4.0	6.5	0.81
525-2A	6.6	1.5	9.4	0.45
525-2B	13.5	2.0	13.5	0.31
550-1	15.5	0.5	62.0	0.089
550-2	15.6	1.0	33.0	0.133
575-1	12.4	0.5	49.5	0.12
575-2	12.5	2.0	12.5	0.37
600-1	10.8	2.5	8.6	0.70
600-2	10.9	1.5	15.5	0.31
625-1A	3.5	0.5	14.0	0.45
625-1B	7.2	1.5	9.6	0.66
625-2A	3.6	1.5	5.1	0.98
625-2B	7.2	2.0	7.2	0.70
650-1A	3.2	2.5	2.6	2.5
650-1B	6.5	4.0	3.1	2.1
650-2A	3.2	2.0	3.2	1.6
650-2B	6.5	3.5	3.2	1.6
675-1A	2.5	0.5	10.6	0.68
675-1B	5.1	1.5	6.8	1.0
675-2A	2.5	0	—	—
675-2B	5.1	1.0	10.8	0.50
700-1A	2.1	0	—	—
700-1B	4.2	0	—	—
700-2	5.3	0.75	15.0	0.37
712.5-2	11.4	2.0	11.4	0.57
725-1A	1.5	0	—	—
725-1B	3.2	0	—	—
725-2	4.0	0.5	17.0	0.34
750-1	3.5	0	—	—
750-2	3.5	0.5	14.9	0.40
775-1	3.3	0	—	—

the energy of white light and amount of phase shift in hours. The magnitude of the phase shift was corrected to equal incident quanta. The data for all determinations are shown in Table I and are plotted in Fig. 4. It is evident that there are two maxima in effectiveness, at 475 mμ and at 650 mμ. Light at the red maximum, however, is considerably less effective than light at the blue maximum. The ineffectiveness of light at wave lengths longer than 700 mμ, shorter than 350 mμ, and in the vicinity of 550 mμ, is quite apparent.

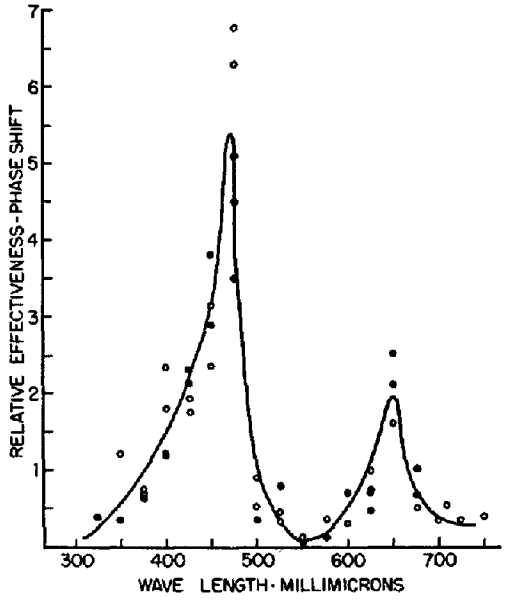

FIGURE 4. The action spectrum for phase shifting in *Gonyaulax*, calculated as the relative effectiveness of equal incident quanta at each wave length. First experiment, ●; second experiment, ○.

The action spectrum roughly corresponds with the absorption spectrum of whole cells of *Gonyaulax* to which chlorophyll *a*, chlorophyll *c*, and peridinin contribute the gross features (Sweeney, Haxo, and Hastings, (1959)). However, the wave lengths of maximum effectiveness do not precisely correspond with the absorption maxima. In the red end of the spectrum, for example, the maximum absorption of the pigments *in vivo* falls at 680 mμ, while phase

TABLE I (*opposite*). Numerical suffix after wave length indicates first or second experiment, and letter suffix the lower (A) or higher (B) light intensity. The energy at any wave length required to shift phase by 2 hours (column 4) is calculated as follows: the intensity employed (column 1) is multiplied by the ratio of the intensity of white light causing a 2 hour phase shift in the same experiment to that causing the observed phase shift (column 3). A quantum correction (×1/wave length) is applied, and the reciprocal of the resulting value is taken as the relative effectiveness of the wave length in question (column 5), the values plotted as the action spectrum (Fig. 4).

shifting was most effectively accomplished at 650 mμ. It is possible that a discrepancy of this magnitude in the red peak could result from experimental errors, since it is apparent from Fig. 3 that the time at which a maximum in luminescence occurs could not be estimated with great accuracy. Such an explanation could scarcely account for the difference between the action and absorption spectra in the blue region, however. The pigments *in vivo* show a broad absorption with a maximum at about 440 mμ, whereas the action spectrum shows a relatively sharp peak at 475 mμ. This action spectrum might be expected if only chlorophyll *c* were involved in the reaction. From the present experiments, however, the pigment or pigments acting in photoreception for phase shifting cannot be established with certainty.

The possibility that the pigment system involved was similar to that sensitizing the large class of photoreactions induced by red light and reversed by far red light, as in the case of the germination of lettuce seeds (Borthwick *et al.* (1952, 1954)), was investigated. With *Gonyaulax* cells, the amount of phase shift resulting from exposure to red light (650 mμ) was either unaffected or slightly enhanced by subsequent exposure to far red light (730 mμ). Thus no evidence for reversal of the red effect by far red light was obtained. In agreement with the data obtained with the Argonne spectrograph, far red light alone was very ineffective in causing a phase shift.

DISCUSSION

Light has previously been shown to alter the intensity of luminescence in *Gonyaulax*. Two effects of light on luminescence, photoenhancement and photoinhibition, are described in detail elsewhere, and the action spectra for these effects suggest the participation of both the chlorophylls and peridinin (Sweeney, Haxo, and Hastings (1959)). In both cases the wave lengths of maximum effectiveness correspond more closely with the absorption spectrum than do those in the action spectrum for phase shifting described here. In addition, the region of effectiveness in the blue is broad in both cases. It is thus likely that the cellular processes and the pigments involved in these effects of light differ from those responsible for the phase-shifting phenomenon described here.

The fact that 24 hour light-dark cycles serve to establish the phase of diurnal rhythms has long been recognized. Experiments with many organisms have demonstrated that, as in *Gonyaulax*, the phase of the biological rhythm shifts in response to a new light-dark cycle which is out of phase with the preceding one (Webb (1950); Brown *et al.* (1954); Harker (1958); Pittendrigh (1958); Webb and Brown (1959)).

The fact that non-repeated light treatments are capable of changing the phase of a persistent rhythm has been pointed out as an important generaliza-

tion in recent years (Pittendrigh and Bruce (1957); Pittendrigh (1958)), and it is clear that the photosensitizing pigments involved in such phase shifting are different in different organisms. Bünning and Lörcher (1957) and Lörcher (1958) found that phase determination in *Phaseolus* is brought about by red light, with some evidence for far red reversal. In the mouse, the visual pigments of the eye act as photoreceptors for phase shifting, since the phase of the rhythm of blinded mice is not altered by light changes (Whitaker (1940)). In both these organisms, the photosensitizing pigments are evidently different from those of *Gonyaulax*.

There is thus no evidence for a photosensitizer for phase shifting common to the great variety of animals and plants which have been found to possess endogenous rhythms. It appears logical to conclude that, in different organisms, pigments which are physiologically active in various cellular processes may be utilized as photosensitizers for phase shifting.

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