fraction analysis. X-ray diffraction patterns were recorded with a Debye-Scherrer type powder camera (114.59 mm diameter) and V-filtered Cr radiation. Exposure times from 24 to 36 hours were used at 30 kv and 20 ma. The diffraction lines obtained on the photogram were measured and the *d*-spacings and line intensities compared to the available data from the ASTM (American Society for Testing and Materials) card index of standards. Since a very close match to the compound cholesteryl stearate was obtained, a standard sample of commercially available cholesteryl stearate was prepared, and the x-ray and electron diffraction data were compared to the patterns obtained from the bone marrow and conjunctiva samples.

Crystals in the conjunctival sections exhibited a distinct morphology in electron microscope images (Fig. 1a). Similar crystals were also observed in corneal and bone marrow samples. Electron diffraction ring patterns obtained by selected-area studies of crystals in the conjunctival sections are shown in Fig. 1b. Similar ring patterns were obtained for both the corneal and bone marrow samples. The *d*-spacings obtained from the diffraction patterns for the conjunctival and bone marrow samples (Table 1) matched the *d*-spacings for a standard cholesteryl stearate pattern within 5 percent. This was within the allowable limits of accuracy of the electron diffraction method and suggests that the crystals observed in the electron micrographs were cholesteryl stearate.

Missing reflections or incomplete rings in the electron diffraction patterns are quite common, especially when the crystalline material develops a layered or textured structure (Fig. 1a). Very little information is available on the structure of cholesteryl stearate, so that indexing of its lines and electron diffraction intensities was not possible at this stage.

X-ray diffraction Debye-Scherrer data for the bone marrow and conjunctiva have also been recorded in Table 1. The *d*-spacings and intensities were found to match very closely the values of the compound cholesteryl stearate tabulated in the ASTM standards. The *d*-spacings for cystine were also checked, because we first thought that the crystals might be cystine. These values did not compare well with the values obtained for tissue or bone marrow samples. The cause of the crystalline deposition of cholesteryl stearate in a patient with a paraproteinemia is unknown, but the nature of the crystals should provide another clue toward the identification of this disease process.

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5 November 1968

## Circadian Clock Action Spectrum in a Photoperiodic Moth

Abstract. *A circadian rhythm of egg hatching in the moth* Pectinophora gossypiella *can be initiated with a brief light pulse. The action spectrum for this effect has a peak in the blue and near-ultraviolet region of the spectrum with a sharp cutoff above 500 nanometers and a more gradual cutoff below 390 nanometers.*

The moth *Pectinophora gossypiella* exhibits a circadian rhythm of egg hatching that can be induced by a single short light signal given after the midpoint of embryogenesis (*1*). This moth also undergoes a photoperiodically inducible diapause, and it is one of the few insects in which photoperiodic time measurement and the biological clock have been studied (*2*). We now report an action spectrum for the light which is effective in initiating the hatching rhythm. Ultimately, it should be of interest to compare this with the action spectrum for photoperiodic induction.

The hatching rhythm persists in continuous red light, but continuous white light damps the rhythm unless the intensity is very low. No rhythm is observed if the developing eggs are maintained at 20°C in darkness after the 5th day of development, regardless of light conditions up to that day, but a rhythm can be initiated with a relatively weak light signal, provided that the signal is given at least 132 hours after oviposition. An action spectrum for the clock controlling this rhythm can be obtained by finding the minimum energy required at different wavelengths to initiate a rhythm. In practice this amounts to finding a threshold value which separates light signals that are too weak from those of saturating effectiveness. The hatching rhythm is restricted to two, or at most three, cycles; and the possible hatching times are spread out over a considerable fraction of each cycle, resulting in a rhythm which is not very sharply defined. However, the light signal can be given at a time when the insects are relatively transparent, still immobile, photoperiodically inducible, and easily handled in large numbers in a small area which can be illuminated with the monochromatic light.

Techniques of culturing the moth and of monitoring the clock-controlled hatching rhythm have been described (*1*). Eggs from adult moths maintained on a light-dark cycle are collected from one night's oviposition. They are then kept at 20°C in the dark; hatching begins in about 10 days and continues for about 70 hours. The number of eggs hatching per hour is monitored by a special fraction collector (*1*). On the 8th or 9th day after oviposition, a rhythm is initiated by a brief light pulse (Fig. 1) always given at the same clock hour.

Light intensity was measured by means of a Yellow Springs Instrument radiometer and a Photovolt photometer model 514. Energies above 1000 erg $cm^{-2}$ $sec^{-1}$ were measured directly with the radiometer. To measure lower intensities the photometer was used in combination with neutral density filters, and the energies were calculated by extrapolation from the radiometer readings. A Carey recording spectrophotometer was used to measure the actual transmission of the neutral density filters.

A slide containing eggs was transferred from the dark rearing box to the exposure box and exposed to light of a particular wavelength and intensity for intervals of 7 seconds to several minutes. It was then transferred in the

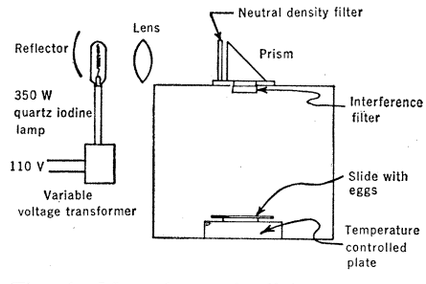


Fig. 1. Monochromatic light source. A partially collimated light beam is projected through a 1-inch (2.54-cm) diameter interference filter onto the specimen. Oriel Optic filters with total bandwidth of 100 Å at one-half peak transmission were used.

dark to a dark box with a fraction-collector. Generally, six slides were used in each experiment, one serving as a dark (DD) control handled in the same way as the other slides but without exposure to light.

In Fig. 2 one can compare the results of experiments in which we used a relatively high-energy monochromatic light pulse, no light pulse (DD control), a light signal just above threshold, or a signal below threshold. One can visually judge whether a signal was above or below threshold. We have also analyzed the results by computer, with a rather unsophisticated statistical approach since the nature of the data does not justify a more rigorous analysis.

The results from ten DD controls were pooled and smoothed to obtain an "average DD control." The time scales in different experiments were normalized by making the median times coincide, so that the zero reference time point is the median of the hatching distribution. Individual experiments can be compared with the master control in the following way. (i) The median of any experiment is made to coincide with the median of the master control. (ii) Each ordinate of the master control is multiplied by the ratio of the total in the individual experiment to the total in the master control; that is, the ordinates are normalized so that the total number in the experimental and the control are the same. (iii) The deviation of each ordinate from the corresponding ordinate of the master curve is computed. (iv) The sequence of deviations in each experiment represents a time series, and an auto-correlation analysis can be done by computing the covariance of the sequence lagged by 0, 1, 2 hours, . . . up to 15 hours. If there is a periodic component with a circadian period, then the typical correlation diagram would show positive correlation with zero lag, zero correlation with a quarter cycle lag, and negative correlation with a half cycle lag. If there is no periodic component, only the zero lag correlation should be significantly different from zero (Fig. 3).

No rhythmic component is indicated for either the DD control or the subthreshold signal, whereas a periodic component with an indicated 20-hour period is found if the signal is above threshold (Fig. 3). The indicated period length is typically short relative to lengths of most circadian periods, primarily because the interval between peaks is biased by the distribution of developmental times.

Following the approach just described, we determined the action spectrum (Fig. 4). The curve in Fig. 4 is drawn so that signals judged to be

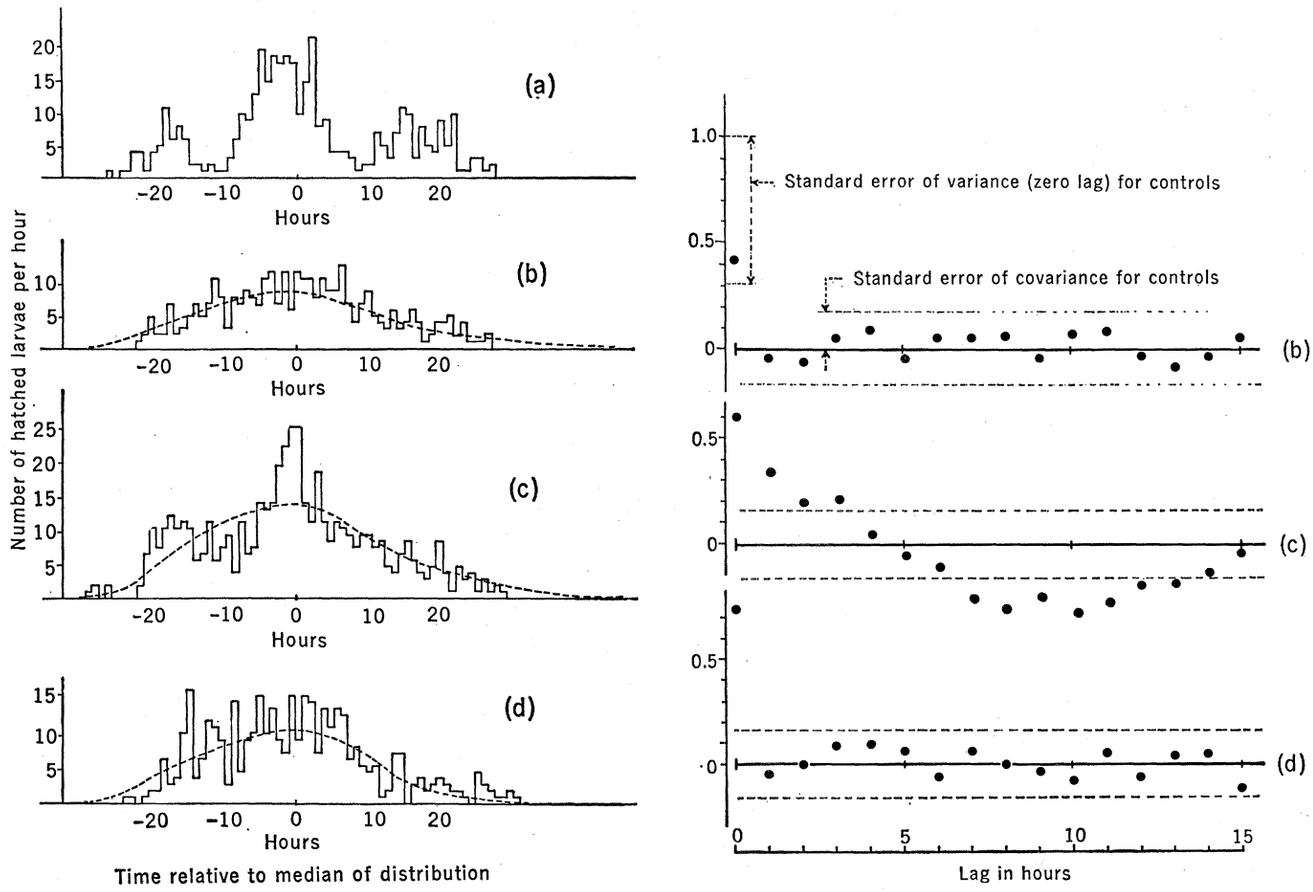


Fig. 2 (left). Hourly hatching of larvae of *Pectinophora* at 20°C. The time scale is plotted relative to the median of the distribution, which generally comes about 265 hours after oviposition. Except as noted the cultures were in complete darkness. (a) A light signal (480 nm, $2.7 \times 10^6$ erg cm$^{-2}$) was given to the developing eggs 89 hours before the median of the distribution; total hatch, 393; median at hour 267; (b) a DD control culture which received no light signal; total hatch, 329; median at hour 273; (c) 89 hours before the median, a light signal (480 nm, 33.5 erg cm$^{-2}$) was given; total hatch, 536; median at hour 267; (d) 61 hours before the median, a light signal (480 nm, 8.6 erg cm$^{-2}$) was given; total hatch, 417; median at hour 263. Fig. 3 (right). Correlations for the results shown in Fig. 2, b–d. The abscissa is the lag in hours, and the ordinate is expressed relative to the mean value for ten DD controls. The mean variance (zero lag) for ten controls is taken to have a value of 1 on the scale shown, and the standard error of this variance is 0.69. The mean covariance (lags 1 through 15) for all ten controls is almost 0 ($-9 \times 10^{-4}$), and the standard error of the covariance is 0.18. These limits are shown.

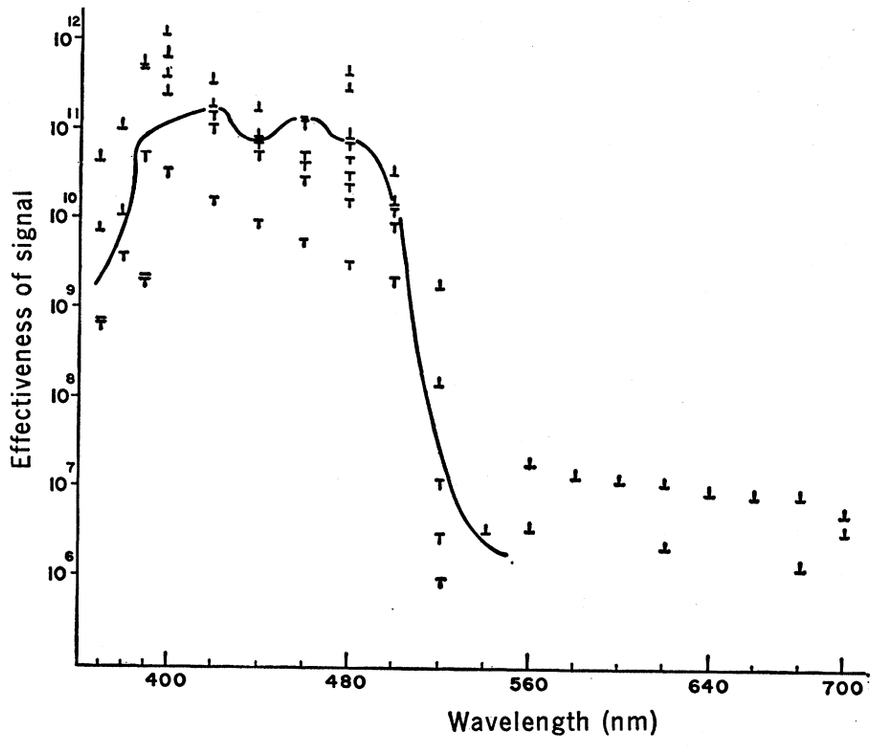


Fig. 4. Action spectrum for the initiation of a rhythm. The abscissa gives the peak transmission wavelength of the 100-Å bandwidth interference filters used. The ordinate gives the signal effectiveness, which is the reciprocal of the energy in Einstein $cm^{-2}$. Symbols used: $\perp$, signals judged to be below the threshold; $\top$, signals above threshold; $\perp\!\!\!\perp$ or $\top\!\!\!\top$, replicates giving the same results; $\frac{\perp}{\top}$, replicates giving opposite results.

subthreshold are above the curve and those which initiated a rhythm are below. Signal energies were calculated as the product of intensity and duration; for the limited range of short signal durations used in these experiments it seems likely that equal energies obtained with the use of different intensities and durations are equivalent. The computer analysis provides a quantitative measure of the proximity to the threshold, and signals close to the threshold at particular wavelengths involved intensities and durations differing by as much as 16-fold. With the exception of two experiments done at 520 nm, all effective signals were less than 2 minutes in duration. No rhythm could be initiated with wavelengths longer than 520 nm. There is a significant drop-off with wavelengths below 390 nm and above 480 nm, but the difficulty of obtaining precise thresholds precludes the detailed characterization of the curve between 390 and 480 nm. In a few instances, replicate experiments in the vicinity of the threshold gave opposite results.

The action spectrum of the *Pectinophora* clock is similar to that for the inhibition of the conidiation rhythm in *Neurospora* (*3*). The *Pectinophora* curve drops off more rapidly above 500 nm as well as below 390 nm, and between these limits the characterization of the *Pectinophora* curve is not sufficiently precise to make a close comparison between the two curves. Screening pigments could account for any observed differences. Unpublished experiments by Frank and Zimmerman (*4*) indicate that the action spectrum for phase-shifting the circadian rhythm of eclosion in *Drosophila* is also similar to that found for *Neurospora*.

Other studies of action spectra of biological clocks do not indicate any overall unity. There is a definite absence of an effectiveness of red light in *Neurospora, Pectinophora,* and *Drosophila.* By contrast, red light is effective in the photosynthetic organisms *Gonyaulax* (*5*), *Kalanchoë* (*6*), *Bryophyllum* (*7, 8*), *Oedogonium* (*9*), and *Phaseolus* (*10*), as well as the protist *Paramecium* (*11*). Whether or not the same pigment is involved in *Pectinophora, Neurospora,* and *Drosophila,* might be clarified by examining the responses in the far ultraviolet. As pointed out by Sargent and Briggs (*3*), the action spectrum for the *Neurospora* clock agrees with action spectra for other *Neurospora* photoresponses. Between 350 and 500 nm it also matches the action spectrum for the *Phycomyces* growth response (*12*). Since the *Phycomyces* curve also has a peak at 280 nm, it would be useful to know whether the *Neurospora* clock responds to the far ultraviolet. Technical limitations prevented us from extending the *Pectinophora* spectrum to shorter wavelengths, but with a modified approach this might be accomplished. Sweeney's (*13*) and Ehret's (*14*) findings that far ultraviolet light can affect the biological clocks in *Gonyaulax* and *Paramecium* should be noted in this connection.

A comparison of the action spectrum for the clock with the action spectrum for the photoperiodic response in *Pectinophora* should help to clarify the hypothetical dual role which light plays in photoperiodically responsive systems. This dual role consists of the phasing of the clock and the induction of the photoperiodic response. In addition it is necessary to determine how complex a role light plays in the clock system. The various possible light effects on the clock (initiation of a rhythm, phase-shifting a rhythm, damping a rhythm) might have fairly complicated kinetics and there may be more than one basic light effect.

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