# Photoactivation of Urocanase in *Pseudomonas putida*. Temperature-Compensated in Vitro Model of an Hourglass Timer†

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ABSTRACT: Purified urocanase (urocanate hydratase, EC 4.2.1.49) from *Pseudomonas putida* was inactivated in the dark in the presence of sodium bisulfite. Near-ultraviolet irradiation (6.6 J $m^{-2}$ $s^{-1}$) restored full activity in 15 min in the presence of low concentrations (5 $\mu$M) of bisulfite. If the system was kept under $N_2$ and the bisulfite concentration and temperature were properly adjusted, it was possible to demonstrate repeated cycles of enzyme activity and furthermore, to "measure" a short night and a long night. The model responded predictably to a skeleton photoperiod and to an "interrupted night". With these properties, an enzyme in a metabolic pathway could remove or produce a critical metabolite (effector or hormone) in phase with the photoperiod. This system was assembled from biological substances (urocanase, phosphate, bisulfite, urocanate, and reduced glutathione) and responded to daylight. Photoperiodic timers are characterized by temperature compensation. Previous studies demonstrated that this photoactivation was temperature independent and that the enzyme activity itself was moderately temperature dependent. In this study we showed that the enzyme inactivation was moderately dependent on temperature and that the two temperature dependent reactions tended to compensate each other. We conclude that urocanase can serve as the basis for an in vitro temperature-compensated biochemical model of an hourglass timer derived from physiological components and conditions. This model timer has a minimal light requirement and it measures the dark period; it thus resembles the photoperiodic timer in *Megoura*.

Photoperiodism is a biological response to the duration of the light (or dark) period. The relative length of day and night is the environmental factor which provides the most reliable information on the passing of the seasons of the year. The ability to discern and predict the seasons confers an advantage on the organism. It can respond appropriately to present or anticipated conditions (e.g., temperature, light, food supply, water supply, etc.) that are favorable or adverse. In photoperiodism, named by Garner and Allard (1920), the length of day or night is measured accurately over a wide range of environmental temperatures. This lack of temperature dependence is remarkable if the time measurement is assumed to be based primarily on some chemical process. Chemical reaction rates normally double or triple for a temperature rise of 10 °C.

The molecular mechanism of photoperiodism remains unknown, although a protein in plants, phytochrome, is known to detect the changes in light for photoperiodism (Vince-Prue, 1975). Two hypotheses have been proposed to explain the phenomenon. One of these is based on the endogenous circadian rhythm observed in most organisms. The other is a nonoscillating interval timer which has been designated the "hourglass" in the literature. An hourglass is a timer that measures one time interval; measurement of another interval is only initiated by resetting the timer (e.g., by turning over the sand hourglass or winding the spring). Lees (1965, 1968, 1971) and Hillman (1973b) have reported evidence that an hourglass timer is involved in aphids. Photoperiodism in a number of insects appears to involve an hourglass component (Lees, 1972).

Bünning (1936) proposed that an endogenous circadian rhythm (biological clock) was the timekeeper. There is convincing evidence that photoperiodism in unrelated organisms does involve such an oscillator (Bünning, 1969; Pittendrigh, 1966). However, Lees (1972) has contended that "the role of circadian rhythms in animal photoperiodism has been exaggerated". In this paper, we do not offer evidence for or against these two hypotheses; however, we do report a simple biochemical model that has the characteristics of the hourglass.

Urocanase (urocanate hydratase, EC 4.2.1.49) has properties which make it suitable for a model of an hourglass timer. Purified inactive urocanase, the second enzyme in histidine catabolism, from *Pseudomonas putida* (Hug and Roth, 1971) can be photoactivated by near UV[1] (Roth and Hug, 1972) and, therefore, by sunlight. This photoactivation is independent of temperature (Hug et al., 1971). The dark reversion to the inactive enzyme form is stimulated by sodium bisulfite (Hug et al., 1977). The *P. putida* urocanase reaction exhibits only a moderate temperature dependence (Hug and Hunter, 1974; Cohn et al., 1975). The purpose of this study was to assemble a biochemical in vitro model of an hourglass timer from urocanase under physiological conditions and to examine experimentally the responses of this model to photoperiods and temperature. There are three reactions involved in this model:

$$E_{inactive} \xrightarrow{h\nu} E_{active} \quad \text{(photoactivation)} \qquad (1)$$

$$E_{active} \xrightarrow{HSO_3^-} E_{inactive} \quad \text{(dark reversion)} \qquad (2)$$

$$\text{urocanate} \xrightarrow{E_{active}} \text{imidazolonepropionate}$$

$$\text{(enyme reaction)} \qquad (3)$$

Reaction 1 "turns over" the hourglass by restoring the timer to the initial condition (photoactivation of the enzyme, E). Reaction 2 is the timing mechanism and, in the analogue, corresponds to sand running through the hourglass (dark in-

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[1] Abbreviation used: UV, ultraviolet.

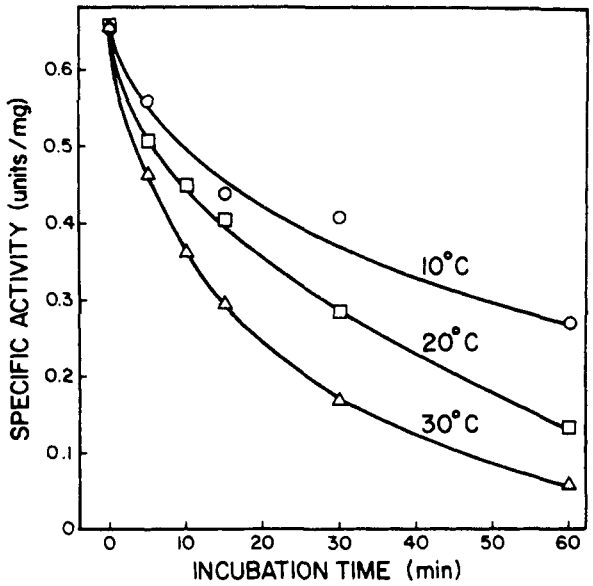


FIGURE 1: Effect of temperature on the dark inactivation of urocanase (reaction 2) in the presence of bisulfite. Purified urocanase (7.5 $\mu$g/mL) in 33 mM potassium phosphate buffer (pH 7.4) was treated (under $N_2$) with 25 $\mu$M sodium bisulfite for the time and temperature indicated. Samples (1.0 mL) were withdrawn and assayed at once as described in Materials and Methods.

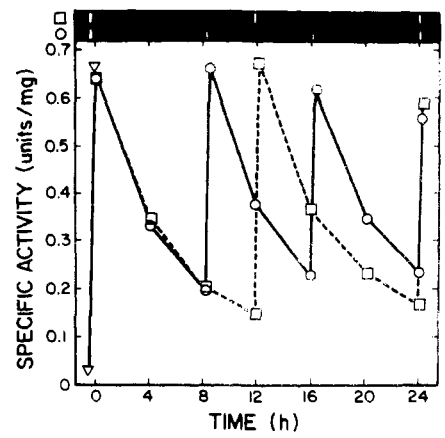


FIGURE 2: Urocanase as a model of an hourglass timer. Two 50-mL Erlenmeyer flasks each contained in a total volume of 45 mL: 7.5 $\mu$g/mL of purified urocanase; 33 mM phosphate buffer (pH 7.4); 5 $\mu$M sodium sulfite; 3.3 mM reduced glutathione; the solution was saturated with $N_2$ and kept in darkness at 20 °C. Near-UV irradiation, described in Materials and Methods, was given for 15 min at 0, 8, 16, and 24 h for the short night cycle (○) and 0, 12, and 24 h for the long night cycle (□). At the indicated times, samples were withdrawn and assayed as described in Materials and Methods. Each point is the mean of duplicates.

activation). Reaction 3 is the "display" of the timer (reaction catalyzed by enzyme E). The lack of temperature dependence results from the interrelationship of reactions 2 and 3 (compensation, discussed later) and the temperature independence of reaction 1.

## Materials and Methods

*General. P. putida* A.3.12 was grown aerobically in a medium which contained 0.2% L-histidine. Growth conditions, cell harvesting, and storage have been described (Hug and Roth, 1971). Protein was measured by the method of Warburg and Christian (1941). Urocanate was from Calbiochem and reagent grade chemicals were used.

*Enzyme and Assay.* Enzyme purification and the assay of urocanase at 30 °C by measurement of the absorbance of urocanate (277 nm) have been described (Hug and Roth, 1971). The total volume for assay was either 1.0 mL in a water-jacketed microcuvette or 3.0 mL in a standard cuvette. However, the concentrations in the assay mixture were the same. A unit of enzyme activity is defined as the amount of enzyme which converts 1 $\mu$mol of substrate per min at 30 °C.

*Temperature.* A constant-temperature bath (±0.01 °C control) and circulator were used to hold the water-jacketed cuvettes and sample at the indicated temperatures. The temperature was monitored with thermistor probes.

*Hourglass Experiments.* Urocanase was placed in a 25- or 50-mL Erlenmeyer flask (closed by a rubber septum stopper) at assay conditions in phosphate buffer. Samples were withdrawn and $N_2$ gas was transferred by needle through the rubber stopper. The flask was saturated with $N_2$ and incubated in a water bath. Sodium bisulfite was added to stimulate the inactivation of urocanase. Since bisulfite is oxidized by air, its concentration is somewhat uncertain. Precautions were taken to use freshly prepared bisulfite stored under $N_2$. Oxygen was purged from flasks in the hourglass experiments by addition of reduced glutathione and $N_2$. Reduced glutathione did not inhibit enzyme activity or restore activity to the inactive form of the enzyme. Irradiation of the flask contents was carried out by two 15-W GE black-light lamps with near-UV filter at 10 cm, 6.6 J $m^{-2}$ $s^{-1}$ (Hug et al., 1971). In one group of experiments, the daylight through a north window was the light source (Figure 4). Samples (1.0 mL) removed for assay were placed in a 30 °C bath briefly (~1 min) to bring to assay temperature (30 °C) and the assay was initiated at once with 100 $\mu$M urocanate. In one series of experiments (Figure 5) on temperature compensation, the sample was assayed at the incubation temperature of the dark inactivation reaction (reaction 2). Sulfite can replace bisulfite.

## Results

*Effect of Temperature on Dark Inactivation.* The rate of conversion of active enzyme to the inactive enzyme (reaction 2) in the presence of sodium bisulfite was dependent on temperature as would be anticipated (Figure 1).

*Hourglass Model.* When dilute purified urocanase is placed in buffer under anaerobic conditions in the presence of sodium bisulfite, the inactivation proceeds in the dark (Figure 2). The concentrations of bisulfite and temperature were adjusted with two goals: (1) conditions that would enable the reaction to measure the length of "typical" nights of 8-16 h; (2) a reaction rate that could be readily overcome by the photoactivation (reaction 1) with daylight, or black light lamps. The system at 20 °C with 5 $\mu$M bisulfite met these goals. We demonstrated that the timer could be cycled (Figure 2) and that the model differentiated a long night from a short night. In this experiment, day lengths were only 15 min in length. The timer starts at the light-off signal (dusk) and is reset by the light-on signal (dawn). During a 12-h night, the urocanase activity fell below 0.2 unit $mg^{-1}$ for 4 h, whereas during 8-h nights the activity did not fall below this arbitrary threshold. In a 16-h night (not shown), the activity remained below the assumed threshold (0.2 unit $mg^{-1}$) for an 8-h duration. Thus, the length of night was translated into a change in the rate of a reaction which could affect the concentration of a hormone or effector in an actual timer.

*Effect of Daylight and Night Break.* In the previous experiment (Figure 2), we employed relatively intense near-UV light and "day lengths" of 15 min. In order to test the system in a more natural time and light environment, the model was exposed to daylight in a north window at 20 °C for 8 h and kept

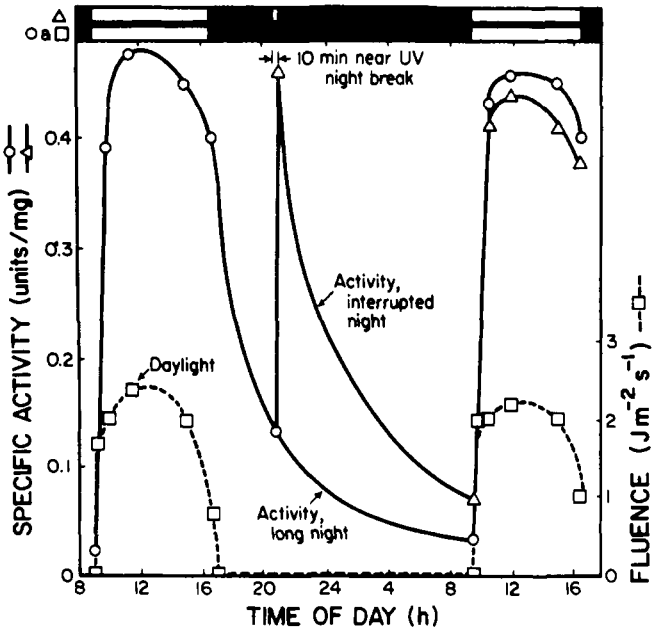


FIGURE 3: Hourglass model with natural light and "interrupted night". The experimental conditions were the same as given in Figure 2 except day long exposure to natural daylight was used for the photoperiod (see Materials and Methods). The night interruption was a 10-min irradiation with near UV as described in Materials and Methods. The experiments were carried out on February 10-11, 1977, two clear winter days. (○) Enzyme activity during natural daylight and long night. (△) Enzyme activity after night interruption. (□) Daylight fluence (long wave UV meter).

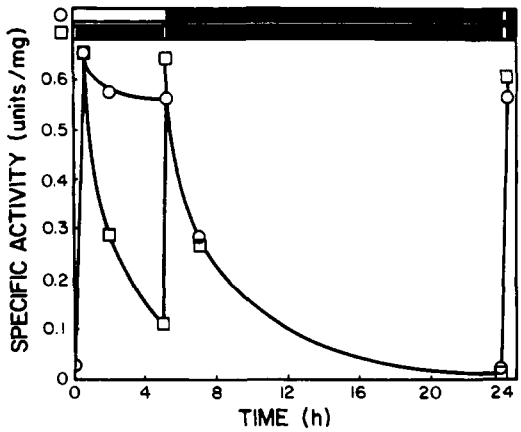


FIGURE 4: Skeleton photoperiod compared with continuous light period. The experimental conditions were the same as given in Figure 2. Irradiation by near UV, as given in Materials and Methods, are indicated: skeleton photoperiod, two 15-min irradiations (□); 5-h continuous photoperiod (○). Each symbol is the mean of duplicate assays.

in total darkness for a period of 16 h. With these conditions the enzyme activity reached a very low level after the long night (16 h, Figure 3). However, when the night was interrupted by a 10-min light break, the timer was "restarted" and the enzyme did not reach the low activity approached by the long night. Thus, the interrupted long night was measured as a short night. Note that the natural dawn to dusk light intensity changes are reflected in the changes in the enzyme activity before and after noon. Thus, in late afternoon under these conditions, the fading light fails to keep reaction 2 from overcoming reaction 1.

*Skeleton Photoperiod.* In photoperiodism studies, the main light period is sometimes replaced by a skeleton photoperiod consisting of a long dark period beginning and ending with a 15-min light break. Using the model, we compared *LD* 5:19 (light period 5 h; dark period 19 h) with a skeleton photoperiod

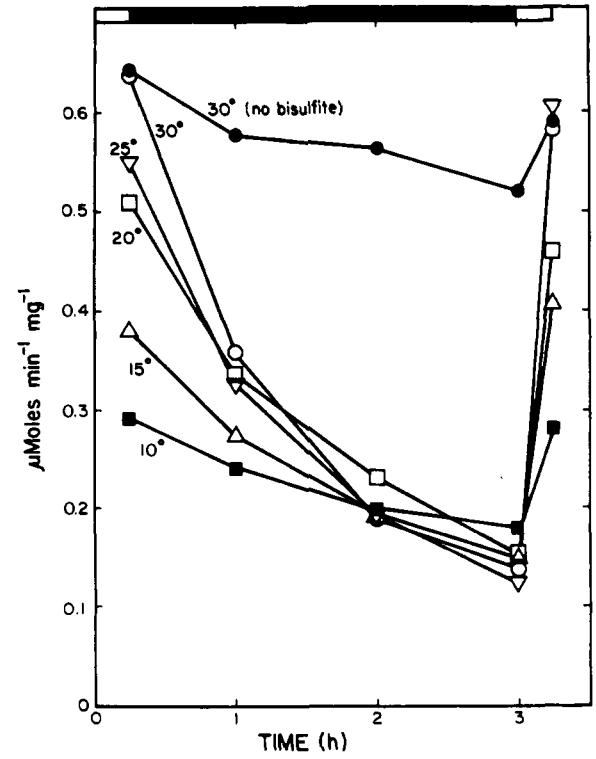


FIGURE 5: Temperature compensation between urocanase activity and dark urocanase inactivation. The enzyme with sulfite (conditions in Figure 2) was incubated at different temperatures for the indicated time and these samples were then assayed at the incubation temperature. Each symbol represents the mean of four assays from two experiments: (●) 30 °C, no sulfite; (○) 30 °C; (▽) 25 °C; (□) 20 °C; (△) 15 °C; (■) 10 °C. Flasks were exposed to irradiation by near UV (15 min) initially and at 3 h as described in Materials and Methods.

of *LDLD* $^1/_4$:4½:$^1/_4$:19. Note in both conditions the model measured the long night exactly the same even though the total light time was very different (Figure 4). Skeleton photoperiods and night light breaks are techniques used by biologists to study the nature of the photoperiodic clock.

*Temperature Compensation.* Urocanase inactivation in the dark (reaction 2) was moderately temperature dependent (Figure 1). The catalytic activity (reaction 3) was also moderately temperature dependent (Hug and Hunter, 1974; Cohn et al., 1975). The question arose whether these two reactions did not tend to compensate each other in this model. To test this concept, we compared the activity of the enzyme undergoing inactivation at several temperatures by assaying the catalytic activity at the *incubation temperature*. At zero time the activities differed, reflecting the temperature dependence of reaction 3. The enzyme was inactivated more rapidly (reaction 2) at a higher incubation temperature but the increased catalytic activity (reaction 3) at the same temperature tended to make up the loss (Figure 5). The enzyme was converted to the inactive form more slowly at a lower temperature, but the decreased activity at the same temperature tended to compensate for the higher concentration of active enzyme molecules. Obviously, in the cell, both reactions 2 and 3 are operating at the *same* temperature just as in this experiment. At five temperatures (10-30 °C), the model exhibited an activity of about 0.15 μmol $min^{-1}$ $mg^{-1}$ at 3 h (Figure 5). After inactivation for 3 h, the enzyme was irradiated and the activity was fully restored; the system was ready for another "compensated" cycle.

## Discussion

We conclude that we have assembled a biochemical model

of a temperature-compensated hourglass timer from purified urocanase, urocanate, bisulfite, light, and a suitable environment ($N_2$, phosphate buffer, pH 7.4, reduced glutathione).

Although the endogenous circadian rhythm has much support as the timer in photoperiodism (Vince-Prue, 1975), there are also reports which favor, to some degree, a role for the hourglass timer. In *Lemna,* Hillman (1971, 1976) suspects an hourglass may be involved. The aphid *Megoura* clearly does not utilize a circadian rhythm for timing photoperiodism (Lees, 1965, 1968, 1971; Hillman, 1973b). In *Pectinophora* (Pittendrigh and Minis, 1971) red light controls diapause but not other rhythms. Wagner and Cumming (1970) concluded that dark reversion of phytochrome is a timer in *Chenopodium*. Truman (1971) found an hourglass behavior of the circadian clock in the silkmoth. The tidal rhythm of *Clunio marinus* was controlled by an hourglass timer (Pflüger, 1973). Vince-Prue (1975) said, ". . . the daily alternation of light and dark periods is a universal feature of the environment to which organisms have adapted. It would not be surprising to find that different mechanisms have developed for using this environmental signal as a means for locating the time of year". Evolution may have devised more than one kind of photoperiodic timer (Pittendrigh and Minis, 1971; Hamner and Hoshizaki, 1974).

The model we have presented fits Hillman's description (1973a) of the hourglass or interval timer: ". . . some process or series of processes, beginning at the start of darkness, builds up (or reduces) a crucial material to a threshold level by the time the critical nightlength (24 h minus the critical daylength) is reached. If the light goes on before that time, the whole process must start again." Our biochemical model responds to short and long nights (Figure 2), light breaks (Figure 3), and skeleton photoperiods (Figure 4). In an analysis of photoperiodism, the following points must be raised (Hillman, 1973a): (1) action of light; (2) molecular basis of the timer; (3) temperature compensation; (4) changes in metabolism; and (5) coupling of metabolic changes to flowering, gonad development, emergence, etc. This model accounts for each of these points except the last since it is an in vitro model. As for changes in metabolism, such a photoregulatory step in an amino acid pathway could, in an organism, affect many intermediates and products of the pathway including formylisoglutamine, formiminoglutamate, imidazolonepropionate, and histidine, one of which might regulate other processes in a functional timer. The concentrations of bisulfite and of urocanase have to be kept constant in this model. We accomplished this by establishing anaerobic conditions in a closed system. The cell could use well-known methods of biological regulation to accomplish this.

Photoperiodic and circadian biological timers invariably are characterized by a remarkably low temperature dependence. Hypotheses for temperature compensation have been proposed for circadian but not for hourglass timers. One suggested mechanism for the circadian clock involves a "tape reading" transcriptional model; timing is related to transcription of DNA (Ehret and Trucco, 1967). In this model, the approximate temperature independence is a result of diffusion circuits.

Hastings and Sweeney (1957) first proposed a simple temperature-compensation mechanism for circadian rhythms in terms of two chemical reactions, A $\rightarrow$ B and C $\rightarrow$ D, which are both temperature dependent. They assumed the first reaction was the timekeeper and that D was an inhibitor of reaction A $\rightarrow$ B. As the temperature rises, more inhibitor, D, is formed to oppose an increase in the rate of the A $\rightarrow$ B reaction. This suggestion was extended somewhat by Hastings (1970). The biochemical model described in this paper has essentially the same elements foreseen by Hastings and Sweeney in 1957. In each model there are two temperature-dependent chemical reactions. In response to a temperature rise, an increase in one reaction opposes the increase in the other. In their model, an increase in formation of inhibitor D opposes the increase in the rate of the A $\rightarrow$ B reaction. In our model the increase in the rate of enzyme inactivation (reaction 2) at the higher temperature opposes the expected increase in the rate of the catalytic reaction (reaction 3).

The other main hypothesis for temperature compensation has also been presented by Hastings and co-workers. In the membrane model for the circadian rhythm (Njus et al., 1974, 1976), transport of ions through the membrane is the essential element of the oscillator. Temperature compensation was envisioned as a result of changes in fluidity of lipid membranes in response to temperature changes. These changes affect the kinetics of ion transport by changing the position and orientation of membrane proteins involved in transport. The idea was based on extensive evidence that the fatty acid composition of microbial membrane lipids is adjusted in response to environmental temperature (Haest et al., 1969; Patterson, 1970; McElhaney, 1976) as a means for high (or low) temperature adaptation.

The effect of temperature on *P. putida* urocanase activity was described by Hug and Hunter (1974). The enzyme showed a remarkable ability to sustain its catalytic activity when the temperature was lowered to 0 °C. The $K_m$ increased with temperature. Results suggested the protein might resist the expected effect of temperature on the reaction by conformational change. At 17.5 °C the activation energy was 8.9 kcal $mol^{-1}$ and the $Q_{10}$ was about 1.4. We previously showed that photoactivation of urocanase from *P. putida* was not dependent on temperature (Hug et al., 1971). The dark reversion (Figure 1) has a moderate dependence on temperature. These characteristics are desirable for the temperature compensation shown in Figure 5 because the corrections needed are relatively minor. The moderate temperature dependence of the reaction catalyzed by the enzyme is compensated by the temperature dependence of the inactivation reaction of the enzyme. We suggest that the possible conformational changes which "adjust" enzyme activity as temperature is lowered (Hug and Hunter, 1974) may at the same time influence the accessibility of the active-site group in the protein which is modified by the bisulfite. It may be that these two phenomena have a common basis: temperature-dependent alteration of protein structure near the active site.

No hypothesis has been proposed for the temperature compensation of the hourglass to our knowledge and, in fact, Hillman (1973a) identified the lack of temperature compensation as a "weakness" of the hourglass hypothesis. A timer as simple as our model may not account for all the responses to photoperiods such as those observed in *Megoura* (Lees, 1965; Hillman, 1973b). However, results in *Megoura* indicate an hourglass measures the dark period and that minimum light is needed to reset the timer (Lees, 1972). Although our model responds to the near-UV in daylight, the model does not function in visible light. An enzyme with a photoreceptor which absorbs visible light is needed for this feature. Yet, our model has many desirable characteristics: (1) high sensitivity to light (it responds on cloudy days); (2) temperature compensation; (3) a time scale relevant to the length of a night; (4) components of biological substances; (5) physiological conditions; and (6) the dark period is the significant one for time measurement as in insects and plants. This biochemical model (Figures 2 and 3) closely resembles a published graphic representation of an hourglass model (Vince-Prue, 1975, Figure 4.2).

Acknowledgments

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References

Bünning, E. (1936), *ber. Dtsch. Bot. Ges. 54,* 590.
Bünning, E. (1969), *Photochem. Photobiol. 9,* 219.
Cohn, M. S., Lynch, M. C., and Phillips, A. T. (1975), *Biochim. Biophys. Acta 377,* 444.
Ehret, C. F., and Trucco, E. (1967), *J. Theor. Biol. 15,* 240.
Garner, W. W., and Allard, H. A. (1920), *J. Agric. Res. 18,* 553.
Haest, C. W. M., DeGier, J., and van Deenen, L. L. M. (1969), *Chem. Phys. Lipids 3,* 413.
Hamner, K. C., and Hoshizaki, T. (1974), *BioScience 24,* 407.
Hastings, J. W. (1970), in The Biological Clock, Brown, F. A., Jr., Hastings, J. W., and Palmer, J. D., Ed., New York, N.Y., Academic Press, p 70.
Hastings, J. W., and Sweeney, B. M. (1957), *Proc. Natl. Acad. Sci. U.S.A. 43,* 804.
Hillman, W. S. (1971), *Plant Physiol. 48,* 770.
Hillman, W. S. (1973a), *BioScience 23,* 81.
Hillman, W. S. (1973b), *Nature (London) 242,* 128.
Hillman, W. S. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 501.
Hug, D. H., and Hunter, J. K. (1974), *Biochemistry 13,* 1427.
Hug, D. H., Hunter, J. K., and Roth, D. E. (1971), *Photochem. Photobiol. 13,* 171.
Hug, D. H., O'Donnell, P. S., and Hunter, J. K. (1977), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 798.
Hug, D. H., and Roth, D. (1971), *Biochemistry 10,* 1397.
Lees, A. D. (1965), in Circadian Clocks, Aschoff, J., Ed., Amsterdam, North-Holland, p 351.
Lees, A. D. (1968), *Photophysiology 4,* 47.
Lees, A. D. (1971), in Biochronometry, Menaker, M., Ed., Washington, D.C., National Academy of Sciences Press, p 372.
Lees, A. D. (1972), in Circadian Rhythmicity, Bierhuizen, J. F., Ed., Wageningen, Centre for Agricultural Publishing and Documentation, p 87.
McElhaney, R. N. (1976), in Extreme Environments, Mechanisms of Microbial Adaptation, Heinrich, M. R., Ed., New York, N.Y., Academic Press, p 255.
Njus, D., Gooch, V. D., Mergenhagen, D., Sulzman, F., and Hastings, J. W. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 2353.
Njus, D., Sulzman, F. M., and Hastings, J. W. (1974), *Nature (London) 248,* 116.
Patterson, G. W. (1970), *Lipids 5,* 597.
Pflüger, W. (1973), *Oecologia 11,* 113.
Pittendrigh, C. S. (1966), *Z. Pflanzenphysiol. 54,* 275.
Pittendrigh, C. S., and Minis, D. H. (1971), in Biochronometry, Menaker, M., Ed., Washington, D.C., National Academy of Sciences Press, p 212.
Roth, D., and Hug, D. H. (1972), *Radiat. Res. 50,* 94.
Truman, J. W. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 595.
Vince-Prue, D. (1975), Photoperiodism in Plants, New York, N.Y., McGraw-Hill, pp 104 and 194.
Wagner, E., and Cumming, B. G. (1970), *Can. J. Bot. 48,* 1.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310,* 384.