Aspects of Clock Resetting in Flowering of Xanthium

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Summary. Flowering is induced in Xanthium strumarium by a single dark period exceeding about 8.3 hours in length (the critical night). To study the mechanism which measures this dark period, plants were placed in growth chambers for about 2 days under constant light and temperature, given a phasing dark period terminated by an intervening light period (1 min to several hrs in duration), and finally a test dark period long enough normally to induce flowering. In some experiments, light interruptions during the test dark period were given to establish the time of maximum sensitivity.

If the phasing dark period was less than 5 hours long, its termination by a light flash only broadened the subsequent time of maximum sensitivity to a light flash, but the critical night was delayed. In causing the delay, the end of the intervening light period was acting like the dusk signal which initiated time measurement at the beginning of the phasing dark period.

If the phasing dark period was 6 hours or longer, time of maximum sensitivity during the subsequent test dark period was shifted by as much as 10 to 14 hours. In this case the light terminating the phasing dark period acted as a rephaser or a dawn signal.

Following a 7.5-hour phasing dark period, intervening light periods of 1 minute to 5 hours did not shift the subsequent time of maximum sensitivity, but with intervening light periods longer than 5 hours, termination of the light acts clearly like a dusk signal. The clock appears to be suspended during intervening light periods longer than 5 to 15 hours. It is restarted by a dusk signal. There is an anomaly with intervening light periods of 10 to 13 hours, following which time of maximum sensitivity is actually less than the usual 8 hours after dusk.

Ability of the clock in Xanthium to be rephased, suspended, restarted, or delayed, depending always upon conditions of the experiment, is characteristic of an oscillating timer and may confer upon this plant its ability to respond to a single inductive cycle. It is suggested that phytochrome may influence only the phase of the clock and not other aspects of flowering such as synthesis of flowering hormone.

Certain short-day plants such as Xanthium strumarium, Pharbitis nil, and Lepin perpusilla exhibit a photoperiodic response to a single cycle; that is, they will flower after exposure to a single dark period longer than the critical night. It has, therefore, seemed reasonable to assume that the clock which measures this dark period had some similarities to an hourglass mechanism. For example, a substrate might decay over a period of time, and when a certain level had been reached, other processes might be initiated which eventually led to flowering. This idea would apply equally well to plants requiring more than 1 cycle. The daily light period might reinitiate the process (invert the hourglass). Indeed, such a suggestion has been formulated by Borthwick and Hendricks (1, 6). They proposed that the governing reaction was the dark conversion of phytochrome from P_h to P_r.

In the endogenous control of leaf movements and other circadian rhythms, several workers including Bunning (3) and Pittendrigh and Bruce (9), have suggested an oscillating timer. Bunning (2) has favored the idea that this same clock may also control timing in photoperiodism, and Hamner (4) has championed this idea. Thus a plant that exhibits both leaf movements and photoperiodism might use the same clock for both functions. Such a mechanism might seem plausible in plants that need a cycling series of proper photoperiods before induction occurs, but in plants that are induced after only 1 short day, the evidence for an oscillator has not been apparent. In fact Moore, Reid, and Hamner (8) found no evidence for a rhythm in flowering of Xanthium, using the experiments which have indicated such a
rhythm in flowering of other plants, such as 24-hour cycles in flowering with longer and longer dark periods. They suggest that the rhythm damps out in 1 cycle.

If an oscillator which damps out in 1 cycle were a part of the timing processes of *Xanthium*, an investigator might expect to observe certain characteristics besides the rhythmic flowering with increasing night length sought by Moore, Reid, and Hammer (8). Surely a variability in sensitivity to light interruptions during the daily cycle should be apparent (13,15). Much is also known about the characteristics of clock resetting in the circadian rhythms (2), and some of these characteristics might also appear.

The experiments reported in this and an earlier paper (13) show that *Xanthium strumarium* does exhibit an apparent cycle of sensitivity to light. Furthermore, clock resetting may be induced by a light interruption given at the proper times during an inductive dark period. At one time, such a light interruption tends to replace the clock. In other circumstances, it may delay the clock. Following prolonged light and probably dark periods, the clock may become suspended (another way of saying the rhythm damps out after 1 cycle), and it is restarted again by a dark period.

The mechanism of action of a light interruption of an otherwise inductive dark period has long puzzled workers in this field. Experiments discussed in this paper suggest that only the clock is affected.

**Materials and Methods**

The methods used in these experiments have been described in some detail by Salisbury (11,12). Briefly, cocklebur plants of the Chicago strain of *Xanthium strumarium* L.4 were germinated in flats, transplanted to plastic pots, and maintained vegetative by exposure to long days (daylight extended to about 20 hrs by incandescent light). They were fertilized with ammonium sulfate every 2 to 3 weeks after transplanting. When at least 60 days old, the plants generally responded well to photoperiodic induction. For the sake of uniformity, plants were used on which the third leaf from the top (12) was from 6.9 to 8.5 cm long. Leaves below this one were removed.

In all of the experiments, the plants were placed in growth chambers (10), and temperatures were maintained between 21 ± 1°C (for the dark period) and 25 ± 1°C (for the light period). The experiment was begun by placing the plants in continuous light (fluorescent and incandescent at about 20,000 lux) for 2 days and then subjecting them to various light and dark periods depending upon the type of experiment. The last period was always a dark period which was long enough normally to induce flowering. The plants were dissected 9 days later and classified according to the arbitrary floral stages of Salisbury (12).

Certain terms need to be defined: **Phasing Dark Period.** This is the first dark period following the 2-day continuous light period. A light period, at least long enough to saturate the phytochrome system (several seconds in our system), terminates this phasing dark period. In many of our experiments the phasing dark period was 7.5 hours long, but it can be any desired length.

**Intervening Light Period.** This is the light which terminates the phasing dark period, and it may be any length from very brief in duration (in our experiments, usually 1 min) to quite long (sometimes 16 hrs or longer).

**Test Dark Period.** This dark period, following the intervening light period, may vary in length but is usually long enough normally to induce flowering (longer than 8.5 hrs).

**Time of Maximum Sensitivity to Light.** In some experiments, light interruptions are given (usually at 1- or 2-hr intervals) during the test dark period. The approximate hour at which light is most inhibitory, or stated conversely, the hour at which the flowering process is most sensitive to a light interruption, is considered to be the time of maximum sensitivity to light. This is often shortened to the time of maximum sensitivity.

**Real Time.** This is the amount of elapsed time counting from the beginning of the phasing dark period. It continues through the phasing dark period, intervening light period and the test dark period. Time after beginning of the intervening light period or time after beginning of the test dark period may also be convenient.

**Results and Discussion**

**The Basic Experiment.** In this experiment, the plants were given various phasing dark periods ranging in length from 1 to 8 hours. Each phasing dark period was followed by a 12-hour test dark period. The curve shown in figure 1 indicates that inhibition due to termination of the phasing dark period was rather suddenly more pronounced for phasing dark periods longer than 5 hours. Figure 2 shows phasing dark periods ranging from 1 to 16 hours long. The sharp drop between 0 and 1 hour was not evident in the first experiment or in any others (not shown), and the drop following the 5-hour phasing dark period is somewhat less evident in the figure. Inhibition was clearly more pronounced for phasing dark periods between 6 and 9 hours in length than for other phasing dark periods. Phasing dark periods longer than 9 hours undoubtedly allow synthesis of flowering hormone before the intervening light period, so that any effect of light on time measurement is masked.

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4 This is the strain of cocklebur long referred to as *Xanthium pennsylvanicum* Wallr. In view of the careful work of Löve and Dansereau (7), we have decided to adopt the more general term, (*X. strumarium* includes all previous "species" except *X. spinosum.* )
Fig. 1. Effect of 1-minute light interruptions terminating phasing dark periods of various lengths when followed by 16-hour test dark periods. Temperature 23°C. Bars above the figure represent the plan of the experiment: a 16-hour dark period as control; a 2-hour phasing dark period followed by a 1-minute intervening light period and 16 hours of dark; and so on for other treatments. Bars are drawn to scale but not the scale of the abscissa.

Time of Maximum Sensitivity Following 2-, 4-, and 6-Hour Phasing Dark Periods. Figure 3 shows the results of light interruptions given during the test dark periods which followed 2-, 4-, and 6-hour phasing dark periods. The curves show that the time of maximum sensitivity remained essentially the same following 2- and 4-hour phasing dark periods, namely, at the 8-hour time expected for the usual light interruption experiment, as indicated by the control line (essentially a “zero-hour” phasing dark period). There is a broadening of time of maximum sensitivity following the 2- and 4-hour phasing dark periods, especially the 4-hour phasing dark period. The 6-hour phasing dark period differs from these and the control because it shows a point of maximum sensitivity which is delayed 10 hours compared to the other times of maximum sensitivity. This, along with previously reported experiments (13), demonstrates that brief light periods which terminate phasing dark periods of 6 to 8 hours duration have a marked effect on time measurement.

In figure 4, time of maximum sensitivity during a long test dark period following a 2-hour phasing dark period (as in fig 3) is compared with results when the test dark period is simply terminated by returning plants to light. That is, critical dark period, another indication of time measurement (9, 11), is determined following the 2-hour phasing dark period and compared with time of maximum sensitivity. Results of these 2 treatments are again compared with the “zero-hour” phasing dark period. The light period after 2 hours of darkness does affect the critical night, causing it to shift by about an hour (not the “expected” 2 hrs), and time of maximum sensitivity is again broadened. In several experiments not shown, the delay in the critical night was always evident and sometimes nearly equaled the length of the phasing dark period (up to 4 hrs).

Fig. 2. Effect of a 1-minute light interruption terminating phasing dark periods of various lengths when followed by a 16-hour dark period. Temperature 23°C.
Effects of Intervening Light Period Following a 7.5-Hour Phasing Dark Period. Having ascertained the effect of phasing dark periods of various lengths, we returned to a study of the 14-hour time of maximum sensitivity following a 7.5-hour phasing dark period. Our reason for doing this was that 7.5 hours is the approximate time when inhibition by a light interruption is most pronounced, providing test dark periods are fairly long (13). Thus the results are often more clear-cut.

Intervening light periods of varying lengths were given following the 7.5-hour phasing dark period. These light periods were then followed by test dark periods which were interrupted at 2-hour intervals. This indicated the time of maximum sensitivity during the test dark period for each intervening light period. Critical night had already been studied in such an experiment (13). The results were then plotted in terms of real time, as in figure 5 which shows only a few representative results. Times of maximum sensitivity for all experiments are indicated in figure 6. It was evident that the time of maximum sensitivity occurred at the same time for intervening light periods that do not exceed 5 hours. Light duration appears to have no effect up to 5 hours.

It was thought that if the first 5 hours of the 14 hours required before maximum sensitivity is reached were a phase in which light is at least innocuous, then a skeleton photoperiod consisting of 2 brief intervals of light corresponding to the beginning and the end of the real photoperiod (intervening light period) should have the same effect upon time of maximum sensitivity as the continuous-light real photoperiod.
For this experiment we used a 3-hour photoperiod and a 3-hour skeleton photoperiod. The results (fig 7) indicate that the time of maximum sensitivity is the same in each case. Light is innocuous during a 3-hour intervening light period.

The effect of a 12-hour skeleton photoperiod was also investigated, as shown in figure 8. The results for the skeleton and the real photoperiods were not the same. Probably the second light interruption acts as a replacer (dawn) rather than a simulated end of a 12-hour intervening light period (dusk).

Further Implications of the Results. From the experiments reported here (figs 1-4) it is evident that the timing mechanism or clock which controls flowering in *X. strumarium* is only delayed by a light interruption during the first 4 hours of phasing darkness, and typically by an amount less than or at most equal to the phasing dark period. It is very strongly affected soon after the fifth hour of phasing darkness, however. In this respect, the clock has the aspects of a relaxation or oscillator mechanism (3).

By a delay following phasing dark periods of 4 or 5 hours or less, we mean that the cycle is not changed to a new phase, but that the current phase is only shifted. There is still a requirement for 7.5 to 8.5 hours of darkness following the light flash before induction occurs if critical night is studied. Even this delay is seen to be partial, however.

We found that the time of maximum sensitivity was shifted from the usual 8 hours (approx.) to about 18 to 22 hours (real time) when a brief light period was given after about the fifth hour of phasing darkness. In terms of time after such a light interruption, time of maximum sensitivity comes 10 to 14 hours later instead of an expected 8. At first it was thought that time measurement was being slowed down (13), but the present experiments make it clear that the change in time of maximum sensitivity is a real phase shift and not just a delay. The brief light period given after 5 or 6 hours of phasing darkness apparently replaces the clock from a phase during which light is most inhibitory, to one during which light is innocuous [promotive in some experiments (13)]. These would be the photophil and skotophil phases of Bunning (3), but we found little or no real promotion during the so-called photophil phase. In this rephasing, light acted as a *dawn signal*, advancing the cycle, so that the dawn state comes several hours earlier.

Thus the clock must go through about a 5-hour light-innocuous state (minimum), then a light inhibitory state of about 8.5 hours, before induction can occur. If this is an integral part of the timing mechanism in *Nasturtium*, then the possibility of an oscillating mechanism is likely. Such an oscillation would normally be obscured because cocklebur requires only 1 such oscillation to induce flowering. Therefore, the importance of the dark period emphasized by Hammer and Bonner (5) and most subsequent authors is diminished somewhat by the likelihood that both light and darkness appear to be equally necessary in flowering of cocklebur as well as in plants that require a number of cycles to induce flowering.

In figure 6, the time of maximum sensitivity is plotted as a function of the length of the intervening

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3 The term “phase” in Bunning’s terminology refers to a portion of a cycle. In modern circadian rhythm usage, it refers to a point on a cycle. We will use the word “state” to imply a portion of a cycle.
light period. When plotted in this way, the first 5 hours of intervening light show a straight line of slope zero (real time), after which the line has a slope of 1 except for an interesting dip from 10 to 13 hours. The zero slope during the first 5 hours indicates, as mentioned above, that increasing the intervening light period to 5 hours has no effect on timing. The slope of 1 indicates that the time of maximum sensitivity remains constant at about 8 hours after the beginning of the dark period. This is also evident when data are plotted in terms of time after beginning of the test dark period, in which case a slope of zero indicates no effect of the previous light period on time of maximum sensitivity during the test dark period.

The dip between 10 and 13 hours indicates that these intervening light periods result in times to maximum sensitivity less than the usual 8 hours after beginning of the test dark period. This agrees with Salisbury’s (13) previous observation that critical night is less than 8 hours following a 12-hour intervening light period. The explanation for this phenomenon remains to be verified, but it is as though the tendency for the clock to oscillate were so strong that it begins to go into the dark status after about 10 hours of light. By the end of 13 hours of light, the clock is re-entrained to the light state. Formation of an inhibitor after 12 hours was suggested in the last paper (13), but this does not agree with the present data, namely the 8-hour time of maximum sensitivity following intervening light periods of 6 to 9 hours.

The broad implication of figure 6 is that the beginning of the intervening light period following phasing dark periods of 6 hours or longer acts as dawn, rephasing the clock so that a period of maximum sensitivity will occur about 14 hours later. This effect remains unchanged as the intervening light period is extended to about 5 hours. After this time, a return to darkness acts like a true dusk signal. Starting and phasing the clock so that a time of maximum sensitivity will occur about 8 hours later. We might imagine that the clock has been “suspended” after 5 hours until it is “started” by a dusk signal. [It is possible that the clock can also be suspended during long dark periods. See Moore, Reid, and Hammer (8).]

In this species, we could say that the operation of the clock can be delayed during the first few hours of phasing darkness (a dusk effect); it can be rephased after 5 hours of phasing darkness (a dawn effect); it can be suspended (as with intervening light periods exceeding 5 hrs); and it can be restarted following suspension (also a dusk effect). These interesting properties of cocklebur (especially suspension and restarting) may confer upon this plant its nearly unique ability to flower in response to a single dark period following continuous light. These properties are illustrated in figure 9.

Workers in this field have long wondered about the mechanism of a light interruption during an other-

wise inductive dark period. Did it reset the clock or did it inhibit flowering only to an extent determined by the phase of the clock? Salisbury (12) decided against resetting because he assumed that resetting could only mean a delay as used in the sense of this paper, and if that is all that there were to it, why should an interruption after 8 hours of a 20-hour dark period be inhibitory? The remaining 12 hours should provide plenty of time for a critical night and for synthesis of flowering hormone.

The concept of rephasing introduced in this paper, however, makes it possible to explain most light interruption experiments on the basis of effects on the clock alone. Assume that flowering hormone synthesis depends only upon phase of the clock, and that this phase is only achieved 14 hours after a dawn signal or 8.5 hours after a dusk signal. The interruption after say 4 hours inhibits with fairly short dark periods (e.g., 16 hrs) because time for flowering hormone synthesis is shortened by up to 4 hours. The interruption at 8 hours requires at least 14 more hours of darkness before the clock will be in the phase allowing flowering hormone synthesis, or a total of at least 22 hours. A light interruption after 8 hours in a 24-hour dark period does allow flowering (13). If the interruption comes after flowering hormone synthesis has been initiated (after 8.5 hrs), it will always act as a dawn signal, rephasing the clock and thus changing its phase so that flowering hormone synthesis is no longer allowed.

*Fig. 9. A schematic representation of the features of time measurement in flowering of Xanthium as presented in this paper.*
Two kinds of experiments do not readily fit this approach as yet. It is not clear why threshold light should inhibit flowering but apparently not influence time measurement (11), nor why far-red light should be inhibitory during the first part of long but not short dark periods (9a). We hope to investigate these problems by the approaches described here and based upon the hypothesis that the effects of a light interruption of a dark period may be interpreted only as effects upon time measurement. That is, far-red-receptive phytochrome (Pfr) may influence only the clock and not synthesis of flowering hormone.

**Literature Cited**