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Rhythms as Photoperiodic Timers in the Control of Flowering in *Chenopodium rubrum* L.

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Summary. In *C. rubrum*, the amount of flowering that is induced by a single dark period interrupting continuous light depends upon the duration of darkness. A rhythmic oscillation in sensitivity to the time that light terminates darkness regulates the level of flowering. The period length of this oscillation is close to 30 hours, peaks of the rhythm occurring at about 13, 43 and 73 h of darkness.

Phasing of the rhythm by 6-, 12- and 18-h photoperiods was studied by exposing plants to a given photoperiod at different phases of the free-running oscillation in darkness. The shift in phase of the rhythm was then determined by varying the length of the dark period following the photoperiod; this dark period was terminated by continuous light.

With a 6-h photoperiod the timing of both the light-on and light-off signals is shown to control rhythm phasing. However, when the photoperiod is increased to 12 or 18 h, only the light-off signal determines phasing of the rhythm. In prolonged periods of irradiation—12 to 62 h light—a "durational" response to light overrides any interaction between the timing of the light period and the position of the oscillation at which light is administered. Such prolonged periods of irradiation apparently suspend or otherwise interact with the rhythm so that, in a following dark period, it is reinitiated at a fixed phase relative to the time of the light-off signal to give a peak of the rhythm 13 h after the dusk signal.

In daily photoperiodic cycles rhythm phasing by a 6-h photocycle was also estimated by progressively increasing the number of cycles given prior to a single dark period of varied duration.

In confirmation of Bünning's (1936) hypothesis, calculated and observed phasing of the rhythm controlling flowering in *C. rubrum* accounts for the photoperiodic response of this species. Evidence is also discussed which indicates that the timing of disappearance of phytochrome P_{fr} may limit flowering over the early hours of darkness.

Introduction

Bünning, in his hypothesis of the physiological clock (Bünning, 1936, 1960), suggested that endogenous rhythms were causally involved as timers controlling photoperiodic induction of responses such as flowering. Recent reports of the existence of rhythms that control flowering (see Hammer, 1960; Cumming and Wagner, 1968) provide substantial support for the involvement of such rhythms as photoperiodic timekeeping devices. However, as stressed by Pittendrigh and Minis (1964),

final proof of Bünning's hypothesis requires an understanding of how photoperiodic cycles influence the timing (*i.e.* phasing) of a particular rhythm. The phasing of the rhythm determines whether or not light extends into the scotophil phase of the oscillation and, hence, determines whether or not photoperiodic induction results.

Previously, to ascertain how photoperiod controlled the phasing of a rhythm that might influence flowering, some workers have utilized "overt" or "indicator" rhythms such as leaf and petal movements (Bünsow, 1960; Ioffe, 1968; Halaban, 1968b; Bünning, 1969; Denney and Salisbury, 1970; Brest *et al.*, 1971) or carbon dioxide output in darkness (Hillman, 1970). However, it is evident in the experiments of Wagner and Cumming (1970) with *Chenopodium rubrum* that the phasing of at least one "indicator" rhythm—betacyanin accumulation—does not always reflect the phasing of an actual rhythm that can be observed in the capacity of this species to flower. Also, Denney and Salisbury (1970) reported that, for *Xanthium strumarium*, the response of flowering to light interruptions of darkness does not correlate with the effect of the same light treatments on rephasing of a rhythm of leaf movement. Thus, it must be questioned whether "indicator" rhythms can be used as a basis for inferring how photoperiod controls the induction of flowering.

To avoid the ambiguities and assumptions that appear to be associated with measurements of "indicator" rhythms, we have investigated photoperiodic phasing of a rhythm that directly determines the capacity of *Chenopodium rubrum* to flower. Phasing has been assessed for both single and repeated photoperiodic cycles. The results support the concept of an oscillatory timekeeping mechanism. The phasing of the timer determines the photoperiodic induction of flowering.

Materials and Methods

Seedlings of *Chenopodium rubrum* L. selection 374 (origin 60°47′N 137°32′W) were used in all experiments. Detailed descriptions of growing techniques and of the origin and characteristics of this selection have been given by Cumming (1969a).

Before sowing, the seed was cleaned, washed for 30 min in 5% "Aerosol" (American Cyanamid Co.), and treated for 30 min with 10% Javex. About 100 seeds were sown on seven layers of 4.25 cm diameter filter paper (Whatman No. 2) in a 6 cm Petri dish. The filter paper had been moistened previously with an excess of distilled water and the dishes, water and filter paper autoclaved.

Germination required a daily temperature cycle of 32.5°C for 12 h and 10°C for 12 h. Continuous cool-white fluorescent light (Westinghouse F20 T12 CW) was imposed during the germination period and was of about 700 ft-c at 32.5°C and 500 ft-c at 10°C. Almost complete germination resulted after 4.5 days; the temperature was then changed to 20°C at a light intensity of 600 ft-c. Luminous energies were measured with a selenium photovoltaic cell (Weston Illumination Meter Model 756). A luminous energy of 600 ft-c was equivalent to a radiant energy between 400 and 900 nm of 1037 $\mu w/cm^2$: measured with an ISCO SR spectroradiometer (Instrument Specialities Co., Lincoln, Nebraska).

The first application of the Hoagland's nutrient solution was made 4.5 days after sowing. "Sequestrene" (Geigy) was added as chelatingagent (6.0 mg/ml). In one series of experiments the Hoagland's solution was supplemented with 0.25 M glucose. In all experiments Hoagland's solution was also applied at the end of darkness and after a further 2 to 3 days.

The first dark period (of a single or daily series) was begun 5.5 days after sowing, *i.e.*, after 1 day at 20° C and 600 ft-c. The temperature during darkness was 20° C. The plants were then returned to 600 ft-c fluorescent light at 20° C. Interruptions of the dark period with single or multiple exposures to 115 μw/cm^2 red light was from red fluorescent tubes (GE F30 T12 R.RS) filtered through a layer of No. 14 ruby cinemoid. Cool-white fluorescent light (600 ft-c) was sometimes used since responses were comparable for red or fluorescent light (King, 1971). The temperature under the fluorescent lamps could be maintained at 20° C but when prolonged irradiations were given under the red lamps, the temperature was 26° C. These differences in temperature were of no importance to the responses reported (King, 1971).

One week after the end of the dark period the percentage of flowering plants was determined using a dissecting microscope at 25X magnification. Fifteen plants were sampled from each of two dishes and the average value of the per cent flowering is reported. For any specific level of flowering in a particular treatment, a constant value resulted for measurements of per cent flowering made 6 or more days after the end of the dark period (King, 1971). Plants kept in continuous light, or given a dark period shorter than the critical length of about 7.5 to 8 h, showed no sign of floral initiation after 3 weeks. All curves have been fitted by eye to the data.

Results

The Photoperiodic Response of C. rubrum

The per cent flowering of *C. rubrum* was assayed following one to eight daily photocycles of various durations (Fig. 1). A 12-h photoperiod was maximally inductive and there was little or no flowering in photoperiods <6 h or >18 h. In another experiment, six but not three cycles of an 18-h photoperiod were slightly inductive when given after a single 12-h photocycle. Flowering was $62\pm3\%$ after one 12-h photocycle and $84\pm3\%$ following six additional 18-h photocycles. Six or more photocycles of <6 h light per day caused severe etiolation and sometimes death of the seedlings. Photosynthesis may often be limiting to growth and flowering of *C. rubrum* (Cumming, 1967) and this may account for the evidence in Fig. 1 and in later figures of a damping out of the flowering response with increasing durations of darkness.

Rhythmicity in the Flowering of C. rubrum

A rhythm in the capacity of *C. rubrum* to flower was obtained when seedlings were given a single dark period of varied duration that interrupted continuous white light (Fig. 2). The values in Fig. 2 are averages from three experiments carried out at different times during the course of this work. The shape of the curve and timing of the peaks of the

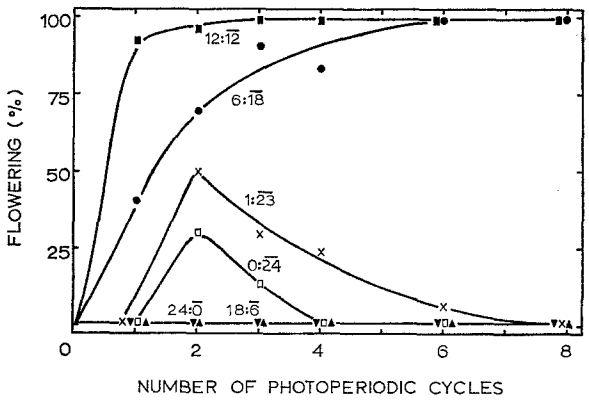

Fig. 1. Flowering response of *C. rubrum* to increasing numbers of daily photoperiodic cycles of various durations of the photoperiod. Bar *e.g.*, 12:12̄ indicates daily hours darkness.To restrict flowering in this experiment, the Hoagland's solution was applied at 20% of its normal strength

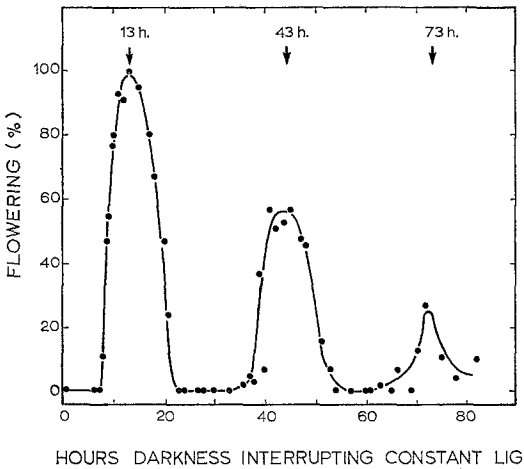

Fig. 2. Flowering response of *C. rubrum* following a single dark period of varied duration beginning 5.5 days after sowing that interrupted continuous fluorescent light (600 ft-c). One week after darkness flowering was assessed for each of six to seven dishes: 15 plants were assayed per dish. Arrows indicate peak timing of a rhythm with a period of exactly 30 hours

oscillation is identical to the many published curves for this selection of *C. rubrum* (Cumming *et al.*, 1965; Cumming, 1967, 1969b). The period of the oscillation was about 30 hours. From the three different experiments, the average times of peak capacity to flower occurred at 13.0± 0.7 h, 44.0± 1.2 h and 71.0± 0.9 h in darkness for the first, second and

third peaks of the rhythm, respectively. The position of the third peak of the rhythm is often difficult to define accurately. Subsequently, for simplicity, the period of the free-running rhythm has been taken as 30 h with peak times at 13, 43 and 73 h in darkness.

Rephasing of the Rhythm by a 6-h Light Interruption

To study how light rephased the rhythm of flowering of *C. rubrum*, a single 6-h exposure to red light was administered to different groups of plants at various times, beginning from 0 to 36 hours in darkness. Thus, according to its timing, each light interruption impinged on a different phase of the free-running control rhythm. To assess rephasing of the oscillation, the plants were then returned to darkness for various durations.

As compared with the phasing of the 30-h rhythm of flowering of the non-irradiated control plants (upper curve Fig. 3), according to its timing, a 6-h light exposure differentially shifted the position of the peaks of the rhythm. Light exposures commencing between 0 and 29.9 h in darkness acted on different phases of a complete 30-h cycle of the rhythm. Thus light exposures beginning at 30, 33 and 36 h in darkness impinged on the oscillation at the equivalent of hours 0, 3 and 6 of the next 30-h cycle. Rephasing was greatest when light impinged on the positive slope of either the first peak—hour 0 to 12—or second peak—hour 30 to 36—of the control rhythm. There was little or no rephasing of the rhythm when light coincided with the control peak and less rephasing on the negative than on the positive slope of the oscillation. There was equivalent rephasing of the next and subsequent peaks of the rhythm.

Rephasing apparently shifted both the peaks and troughs of the rhythm (Fig. 3). To examine this point further, the amplitude of the flowering response was enhanced by applying a solution of 0.25 M glucose to the seedlings one day before darkness. The timing of both peaks and troughs of the rhythm was identical in the presence or absence of glucose (Fig. 4a, *cf.* Fig. 2). Six hours of fluorescent light induced the same degree of rephasing as a lower intensity of red light (Fig. 4b, *cf.* Fig. 3). Compared to the non-irradiated control plants, it is clear (Fig. 4a and b) that the 6-h exposure to light rephased the continuing oscillation.

For at least 100 h in darkness 80% to 100% of the plants progressed regularly through maxima and minima in their capacity to flower. Therefore, the per cent of flowering plants in the population is representative of the capacity of any individual plant to flower and, hence, of the endogenous rhythm in each plant.

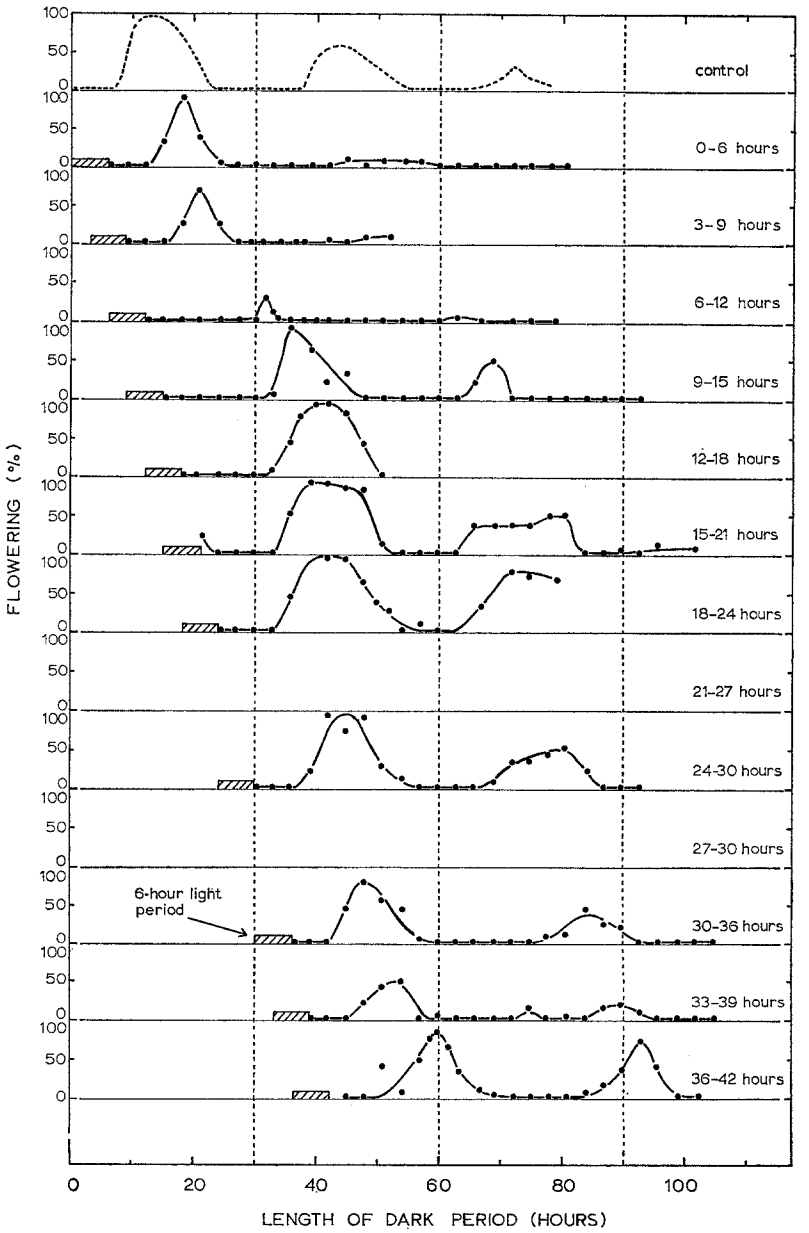

Fig. 3. Rephasing of the rhythm of flowering of *C. rubrum* by exposure to 6-h red light (115 μw/cm^2) administered at different phases of the control rhythm. Vertical lines, 30-h segments of the time scale. Upper curve: control rhythm redrawn from Fig. 2

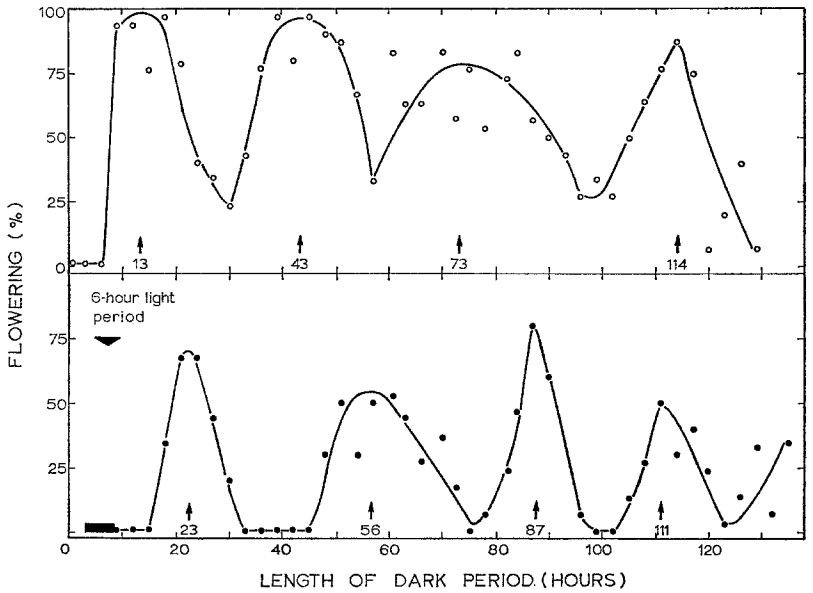

Fig. 4a and b. Flowering response of *C. rubrum* to a dark period of varied duration. Plants treated one day earlier with 0.25 M glucose in Hoagland's nutrient solution. a Free-running rhythm in darkness. b Rephasing of the free-running rhythm of flowering by a single 6-h period of fluorescent light (1037 μw/cm^2)

After 125 h in darkness the plants often died when returned to light. In long dark periods some variability in flowering and, as a result, in rhythm period was also evident in similar experiments of Cumming (1967) in which darkness was continued for as long as 10 days.

Using rhythm peak times to indicate phasing of the oscillation, Fig. 5 illustrates more directly the relationship in Fig. 3 between the time of exposure to light and the resultant rephasing of the oscillation. Data from replicate experiments have also been included and it is clear that rephasing was very consistent. For instance, in five different experiments, when a 6-h exposure to red light was given from hour 9 to 15 in darkness the average time of the first peak was 37.0 ± 0.1 h and the average flowering response was 70% (*cf.* Fig. 3).

We emphasize from Fig. 5 conclusions drawn from Figs. 3, 4 and 5:

i. The free-running, pre-existing oscillation (stippled line, Fig. 5) may be rephased by the 6-h light exposure. The amount of resetting varied with the position in the cycle at which the light period was administered.

ii. Exposure to a 6-h light interruption beginning at hour 30, 33, or 36 induced rephasing of the second peak of the control rhythm that was

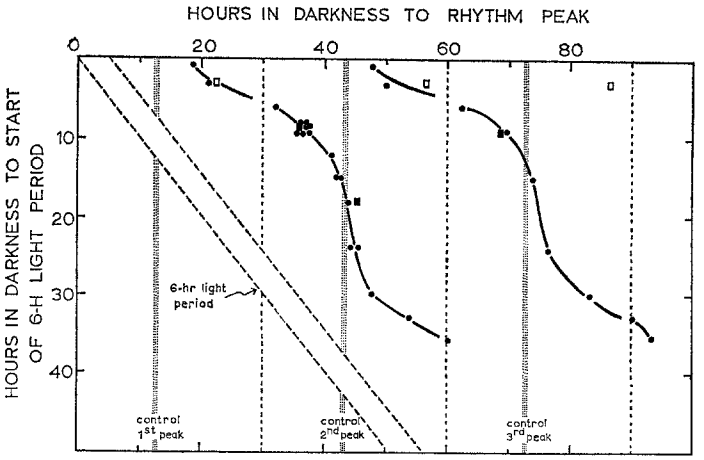

Fig. 5. Relationship between the time in darkness when a 6-h light exposure was given and the time in a subsequent dark period of maximum flowering at the peaks of the rhythm. Red light, 115 μw/cm² (●); white fluorescent light, 1037 μw/cm² (■); white fluorescent light, 1037 μw/cm², plus glucose 0.25 M (□). Representative raw data in Figs. 3, 4. Vertical dashed lines, 30-h segments of time scale. Control peak times, stippled vertical lines

comparable to rephasing of the first peak by those interruptions beginning at hour 0, 3 and 6, respectively. This similarity of rephasing follows from the fact that the period of the pre-existing oscillation is about 30 h.

iii. When low or high intensity light terminates the normal continuous light period, rhythm phase is determined solely by the time of the light-off signal (Cumming *et al.*, 1965; King, 1971). Thus, the light-off signal controlled rephasing by a 6-h light exposure beginning at hour 0 and 30 (see *ii* above). Therefore, the changing pattern of rephasing found at other times in darkness can only be explained if the timing of both light-off and light-on cues is important.

iv. Rephasing influenced the peak immediately following the light interruption and, also, all subsequent peaks of the oscillation. The symmetry of rephasing between the peak immediately following and the subsequent peak (Fig. 5) confirms that the period of the oscillation remained close to 30 h even when the phase of the rhythm was reset.

v. The peak of the rhythm immediately following the light period was always displaced by at least 12 hours from the light period (Fig. 5). This displacement suggests that there could be obligatory preparatory steps for flowering that are only realized in darkness.

vi. There were consistent and, often, large differences in the amount and direction of rephasing of the rhythm that reflect the phase relationship between the light period and the rhythm.

In determining the direction (advance or delay) of rephasing, a phase delay of the oscillation, relative to the phase of the control, should change to an advance at a point intermediate between adjacent peaks of the control oscillation. A close approximation to these intermediate points is achieved by designating rephasing on the basis of a 30-h cycle that began at the start of darkness (dashed vertical lines in Fig. 5). In this fashion it has been possible to estimate the amount and direction of rephasing of the first—0 to 29.9 h; second—30 to 59.9 h; third—60 to 99.9 h; and fourth—>100 h—peaks of the oscillation (Fig. 5).

Aside from theoretical considerations, the designation of rephasing as a delay is unambiguous for a 6-h light period beginning at 0 and 30 hours in darkness: see part ii) and iii) above. It has also been established that a 6-h light period beginning at the ninth hour in darkness advanced the phase of the rhythm. For instance, when a continuous 6-h irradiation was replaced with a skeleton light period of 6-h, then the less frequent the light interruptions, the more the rephased-peak shifted from hour 37 back to the control peak time at hour 43 in darkness (King, 1971).

In Fig. 6 all of the data from Fig. 5 are summarized on the basis of the amount (hours) and direction of rephasing in each 30-h period relative to the time of the 6-h light exposure. It is interesting to note that greatest sensitivity to light was over the 6 to 12 hours immediately prior to the time when a peak of the rhythm would have occurred. Furthermore, there was no rephasing when plants were irradiated at the time equivalent to the peak of the control rhythm—hour 13 in darkness.

As illustrated by Pittendrigh and Minis (1964), a phase response curve, such as that in Fig. 6, is useful for predicting how photoperiodic cycles induce successive phase advances and delays of the rhythm. Moreover, they showed that when the amount of each daily advance of phase balances the amount of each daily delay then an equilibrium or steady-state will have been reached, *i.e.*, no net rephasing. This equilibrium point in Fig. 6 occurs for a light period beginning at the time of the rhythm peak. However, a further factor—entrainment of the period—must be incorporated before phase response curves can be used to assess how photoperiodic cycles control rhythm phasing. In daily photoperiodic cycles the period of the rhythm will be entrained from its free-running value to a 24-h period length. Considering the rhythm of flowering in *C. rubrum*, entrainment can be simulated by adjusting the abscissa time scale (Fig. 6) so that the 30-hour period becomes one

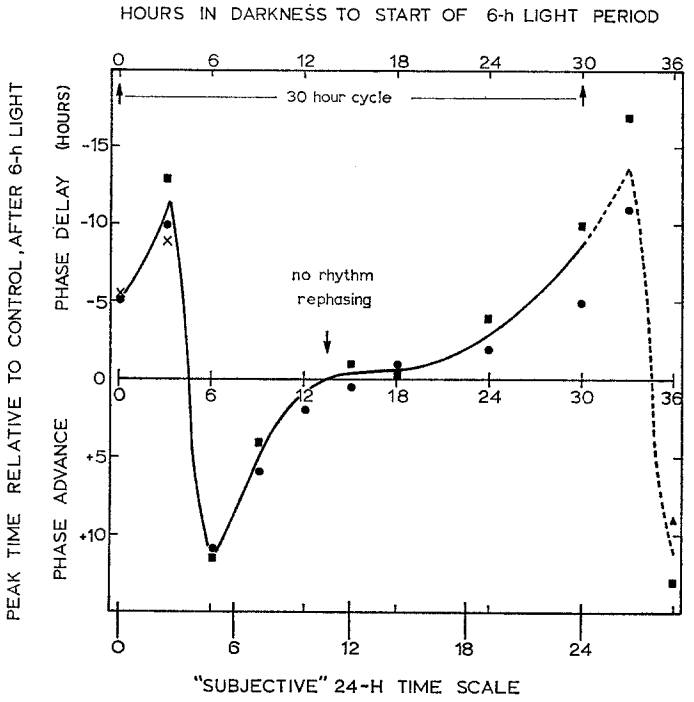

Fig. 6. Rhythm peak timing: hours phase delay or advance relative to the peak timing of the uninterrupted control rhythm, as affected by the time of exposure during darkness to a 6-h light period. Rephasing of the first (×), second (●), third (■) or fourth (▲) peak of the free-running rhythm. Values derived from Fig. 5

of 24 hours, *i.e.*, $30 \times \frac{24}{30}$ or "subjective" 24-h time. Then, when a steady-state condition of rephasing and entrainment has been achieved, the peak time of the rhythm will coincide with the beginning of each daily 6-h photoperiod, *i.e.*, 18 h after the dusk signal of the last photoperiod.

Rephasing of the Rhythm by a 12-h Light Interruption

In the same manner as elaborated for a 6-h light period, 12-h interruptions with red or fluorescent light were given at different times in darkness. To assess rephasing of the rhythm, the length of the dark period following the 12-h light period was varied by 3-h increments. Irrespective of the phase of the pre-existing (*i.e.*, control) oscillation, upon rephasing the next peak of the rhythm of flowering occurred at about 13 h in darkness and the following peak about 32 h later (Figs. 7 and 8).

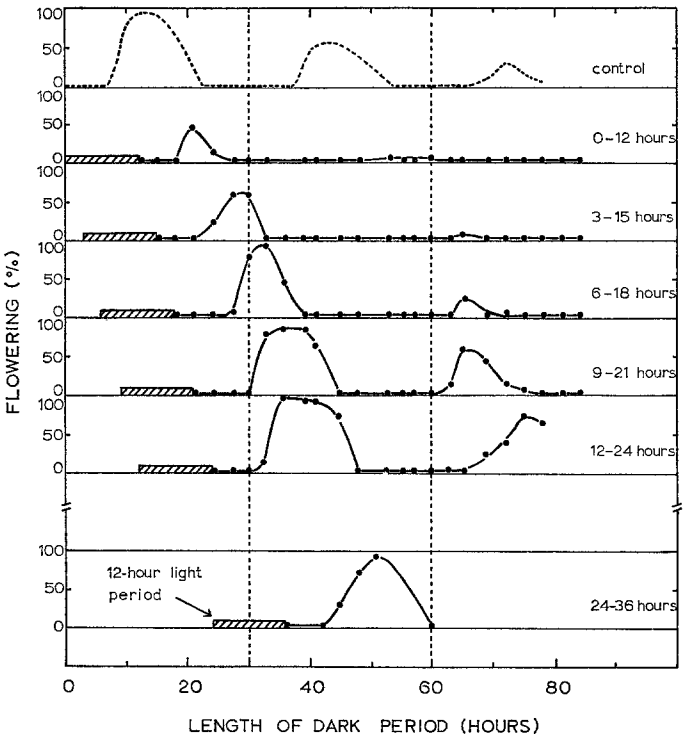

Fig. 7. Rephasing of the rhythm of flowering of *C. rubrum* by exposure to 12-h red light (115 μw/cm^2) administered at different phases of the control rhythm. Vertical lines, 30-h segments of the time scale. Upper curve: control rhythm redrawn from Fig. 2

The amplitude of the rhythm of flowering was often reduced when a 6- or 12-h light interruption was given during the early hours of darkness (Figs. 3, 7). As a partial explanation of this response, relatively more flowering could be expected when the effects of two inductive dark periods were summated, *e.g.*, 12 h darkness; 12 h light; darkness. However, an additional factor, seedling age, was also found to be important in determining the amplitude of the flowering response. If darkness was begun after 6 days in fluorescent light rather than on day 5.5 or 6.5, flowering was reduced in its amplitude. As might be expected, a similar situation therefore arose when a 12-h light period was imposed from 0 to 12 of a dark period that began on day 5.5. Likewise, a 6-h light period beginning at the sixth hour in darkness is terminated on day 6 and hence a reduced amplitude of the rhythm (Fig. 3) might be explained as a similar "age" effect.

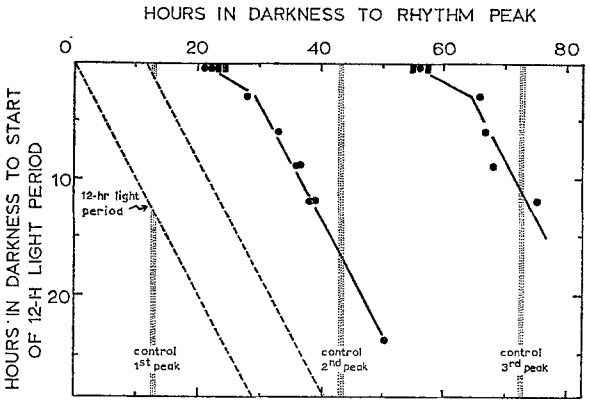

Fig. 8. Relationship between the time in darkness when a 12-h light exposure was given and the time in a subsequent dark period of maximum flowering at the peaks of the rhythm. Red light, 115 μw/cm² (●); white fluorescent light, 1037 μw/cm² (▪). Control peak times, stippled vertical lines

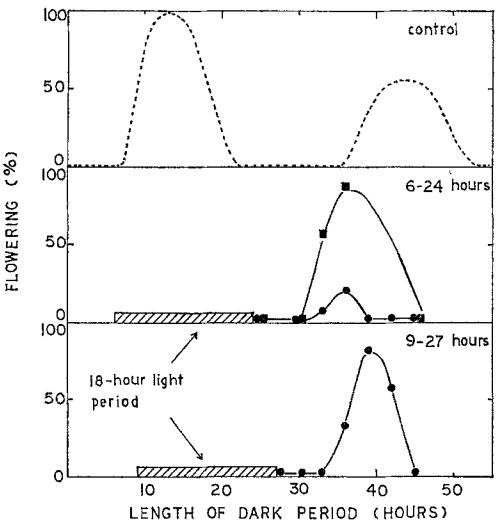

Fig. 9. Rephasing of the rhythm of flowering of *C. rubrum* by exposure to 18-h red (● 115 μw/cm²) or fluorescent light (▪ 1037 μw/cm²) administered from hour 6 to 24 or hour 9 to 27 in darkness

Rephasing of the Rhythm by an 18-h Light Interruption

As with a 12-h light period, when darkness was interrupted with 18 h of light the next peak of the rhythm occurred at about hour 13 in the subsequent dark period. Fig. 9 illustrates the response to an 18-h

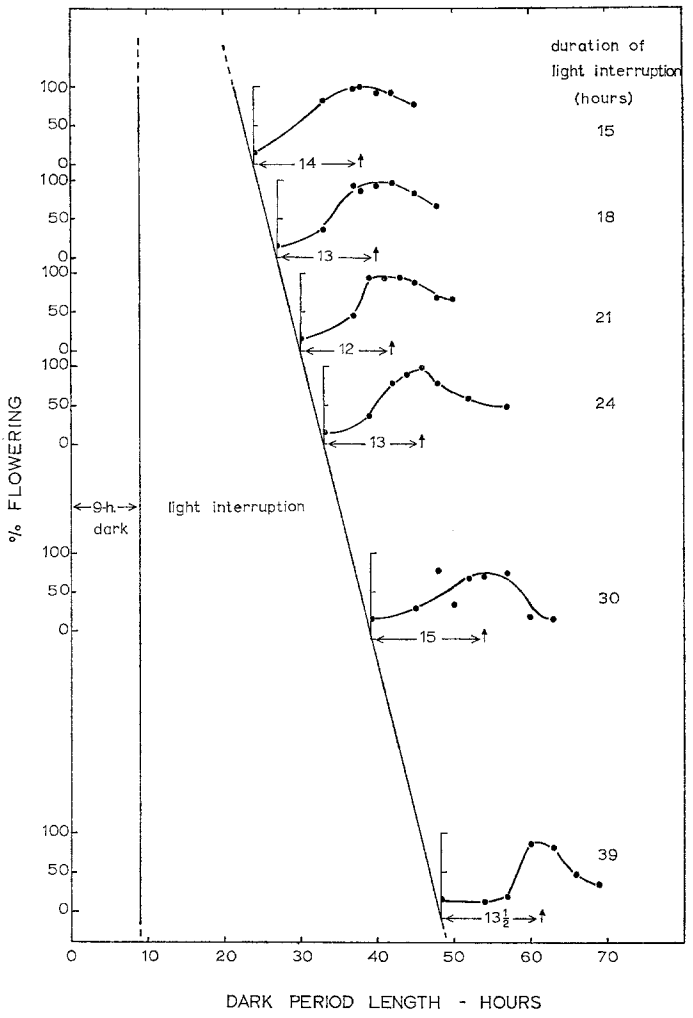

Fig. 10. Time in darkness to maximum response at the first peak of the rhythm of flowering of *C. rubrum* given a single dark period interrupted with white fluorescent light (1037 μw/cm^2) for various durations and commencing at the ninth h of darkness

interruption from hour 6 to 24 or 9 to 27 in darkness. The position of the first peak from the end of the light period, *ca.* 12 to 15 h, was identical whether fluorescent or red light exposures were given, although more flowering resulted with the higher intensity fluorescent light. Entrainment of rhythm period to 24 h has not been incorporated into discussion of responses to daily photoperiodic cycles of 12 or 18 h. At the most,

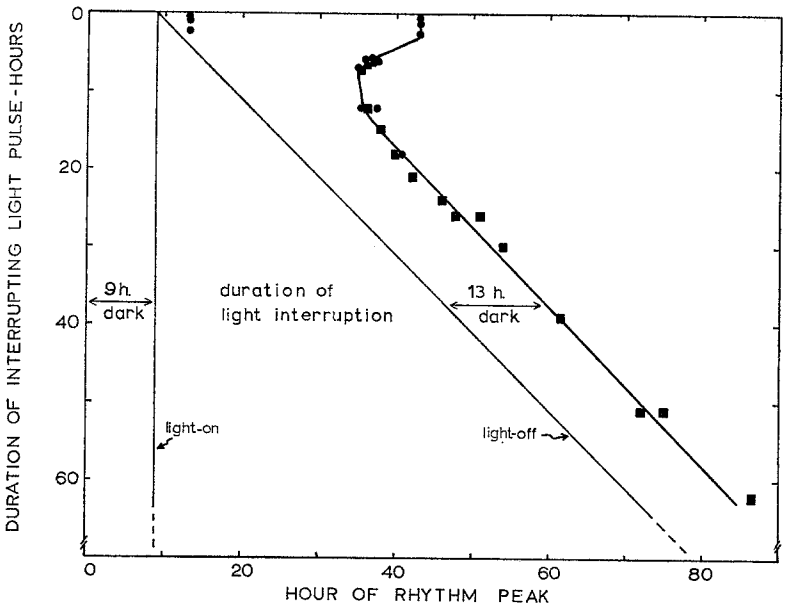

Fig. 11. Time in darkness to maximum response at the first peak of the rhythm of flowering of *C. rubrum* given a single dark period interrupted with white fluorescent (■) or red (●) irradiation for various durations commencing at the ninth h of darkness. Fluorescent light 600 ft-c, 1037 μw/cm²; red light, 115 μw/cm²

the peak time of the rhythm would shift from 13 h to 10.4 h in darkness ($13 \times \frac{24}{30}$).

Rhythm Phasing by Light-On and Light-Off Signals

After either a 12- or 18-h light period the next peak of the rhythm occurred at about 13 hours in darkness. Since it appeared that the light-off signal alone controlled rhythm phasing in these instances, in further experiments light periods from 12 up to 62 h in duration were examined for their control of rhythm phasing. The timing of the light-on signal was held constant at the ninth h in darkness and thus only the timing of the light-off signal was varied as the duration of the light interruption increased. Representative results (Fig. 10) indicate that the peak of the rhythm of flowering occurred at a fixed time in darkness (12 to 15 h) after the end of a fluorescent light interruption. Fig. 11 combines the data for the peak times from Fig. 10 with that from other similar experiments but in which either red of fluorescent light were used. As established earlier (Figs. 4, 5, 9), rephasing was identical following red (115 μw/cm²) or fluorescent light (1037 μw/cm²). Sensitivity to low in-

tensity red light suggests the involvement of a photoreceptor such as phytochrome. It is also clear that light interruptions of 12 h or longer rephased the rhythm to the same degree. The peak always fell at a fixed time after the light-off signal. Moreover, not just the next peak but subsequent peaks were rephased to an equivalent degree (Fig. 8).

The lower limit for effective rephasing by a single light period must lie between 2.5 and 6 h (Fig. 11). The precise critical value has not yet been determined.

Table 1. Flowering response of *C. rubrum* to different combinations of light and dark periods tested in photoperiodic cycles of varying total lengths (4 cycles in total). First dark period commenced 5.5 days after sowing. Hoagland's solution supplied at 20% normal strength. White fluorescent light 600 ft-c (1037 μw/cm^2)

Light period length (h)	Flowering %						
	Dark period length (h)						
	0	6	12	18	23	24	36
0						0	
1					24		
6		0		84			
12			100			82	84
18		0					
24	0		100			0	
36			100				

Rephasing of the Rhythm in Repeated Photoperiodic Cycles

As was established above for a single light period, in repeated photoperiodic cycles of 12 h or longer the light-off signal remains dominant for rhythm rephasing. This dominance of the light-off signal was also demonstrated by giving seedlings four cycles of darkness and light that commenced 5.5 days after sowing of the seeds. The total length of a cycle was varied between 6 and 72 h. In each cycle the proportions of light and darkness were varied independently between 0 and 36 h. Maximum flowering resulted in light:dark cycles (L:$\overline{\text{D}}$) of 12:$\overline{12}$, 24:$\overline{12}$ and 36:$\overline{12}$ h (Table 1). Such results agree with the suggestion that phase was determined by a dominant light-off signal following prolonged irradiations. As might also have been expected, there was still, apparently, a rhythmic response to the duration of darkness. However, the period of this rhythm may be close to 30 h which suggests that photocycles of 36 or 48 h could not entrain the period of the rhythm.

Dominant phase control by the light-off signal was also evident in a preliminary experiment involving four cycles of an 18-h photo-

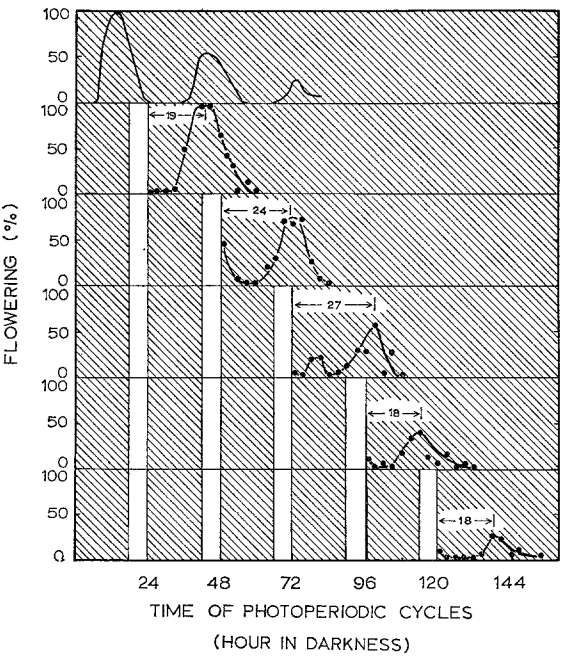

Fig. 12. Rephasing of the rhythmic flowering response of *C. rubrum* subjected to 1 to 5 daily photoperiodic cycles; 18 h darkness, 6 h red light (115 μw/cm²). Phasing has been estimated as the time from the end of the last light period to the immediately following peak of the rhythm of flowering

period. The peak of the rhythm occurred between 10 and 12 h in a dark period that followed the fourth photocycle.

To determine, experimentally, the phase relationship at its steady-state in 6-h photocycles, the number of photoperiodic cycles was increased progressively from one to five photocycles. Phasing was measured on the basis of the timing of the peak of the rhythm of flowering in a subsequent dark period.

In the experiment shown in Fig. 12, and also in a repeat experiment, after four daily 6-h photoperiodic cycles the time to the peak of the rhythm reached a fixed value, at the 18th h in darkness. In this experiment there was little flowering when plants were returned immediately to fluorescent light at the termination of a particular number of photoperiodic cycles (24, 48, 72, 96 or 120 h dark, Fig. 12). This result is in contrast to the response shown in Fig. 1 in which a 100% flowering response resulted after six daily photoperiodic cycles. The difference between these two experiments probably arose from the use of low rather

than high intensity light for each photoperiod in the experiments reported in Fig. 12.

Discussion

Photoperiodic Time Measurement and the Involvement of a Rhythm

The experiments reported here establish the relationship between photoperiod duration and the phasing of an endogenous rhythm in the capacity of *C. rubrum* to flower. The phasing of this oscillation coupled with its daily alternation in sensitivity to light is important for photoperiodic time measurement.

In a 6-h photoperiod, phasing of the rhythm of flowering depended on the timing of the dawn and dusk signals relative to the position of the rhythm at which the light was administered (Fig. 6). As a result, after a sufficient number of cycles, daily rephasing could force the oscillation to adopt a fixed or steady-state relationship to the timing of the daily photoperiod (Fig. 12). Thus, under conditions of steady-state rephasing of the oscillation, the peak of the rhythm of flowering occurred 18 h in darkness after the preceding light period. It will be recalled that a similar value was calculated from the phase response curve (Fig. 6) but only when entrainment of the period, from 30 to 24 h, was incorporated into the calculation. Comparable findings for rephasing of rhythms by single light interruptions have been reported previously (see summaries in Pittendrigh, 1966, and Winfree, 1970).

In contrast to the phase response to 6-h light periods, when 12-h or more light was administered to *C. rubrum*, on rephasing, there was no longer any interaction between the timing of the light period and the position of the oscillation at that time. Phasing of the rhythm now depended solely on the timing of the daily dusk signal (Figs. 8, 10).

From these observations, and without making any inferences about the nature of the rhythm, it is possible to predict how daily photoperiodic cycles control flowering. In a daily 6-h photoperiodic cycle the peak of the rhythm occurs after 18 h of darkness (Fig. 12) and, therefore, flowering should be close to maximal. In longer photoperiods, such as 12 h or 18 h, the rhythm is rephased each day so that peak capacity to flower results after 13 h of darkness (Figs. 8, 10). As a consequence, maximal flowering should result in a photoperiod of about 12 h with its associated 12-h dark period. However, in longer photoperiods and, hence, shorter dark periods, flowering should decrease. Little or no flowering would be expected in an 18-h photoperiod since the critical dark period for flowering (7 to 8 h, Fig. 2) is not exceeded. These predictions based on rhythm phasing account, completely, for the observed photoperiodic response of *C. rubrum* (Fig. 1 and Cumming, 1963). It is clear, therefore, that, as first postulated by Bünning (1936), an endo-

genous rhythm that determines capacity to flower functions as a photoperiodic timekeeper.

Interaction of Light with the Rhythm

Previous models of photoperiodism such as those of Bünning (1960) and of Pittendrigh and Minis (1964) have not made allowance for "durational" (quantitative) effects of photoperiod on rephasing of rhythms. As a result, various authors have placed an emphasis on phase control by the dawn signal alone (Bünning, 1960; Hammer, 1960); the dusk signal (Halaban, 1968b; Cumming and Wagner, 1968); dawn and dusk signals together (Pittendrigh and Minis, 1964); or on two separate rhythms controlled separately by a dawn and a dusk signal (Takimoto and Hamner, 1964). However, our results (Figs. 5, 8, 11) show that, as the duration of the photoperiod increases, there can be a change in effectiveness of dawn and dusk signals for control of the phase of the rhythm. Similar responses to those in *C. rubrum* have been reported by Pittendrigh (1960, 1966) for *Drosophila*, and experiments of Wilkins (1960) on the rhythm of CO_2 output in *Bryophyllum fedtschenkoi* also provide support for the more widespread occurrence of this phenomenon.

It is not known how, in prolonged photoperiods, light overrides the action of the rhythm so that it is reinitiated at a fixed phase in a subsequent dark period. Cumming *et al.* (1965), in commenting on the dominance of the light-off signal following four days of continuous light given to *C. rubrum*, suggested that the rhythm was either suspended (*cf.* Wilkins, 1960) or that it continued in light but was reset to a fixed phase by the light to dark transfer. On the basis of the phase response curve (Fig. 6) the latter explanation appears unlikely. The dusk signal would not be expected to reset different phases of a continuing oscillation to one fixed phase. Alternatively, the rhythm may be suspended in continuous light. Suspension of the rhythm in continuous light could result from the generation of a state of arhythmicity (see Winfree, 1970) or the rhythm might be uncoupled from the action of a "master rhythm" [see Brown's (1965) concept of autophasing].

Photoperiodic Time Measurement and the Involvement of an Hourglass

Bünning (1960) considered it central to his hypothesis that "the time-measuring processes in photoperiodic reactions are not carried out by the hourglass principle but, rather, by means of endodiurnal oscillations". The action of an endogenous rhythm is clearly important to time measurement in *C. rubrum*, however, the evidence presented here does not exclude the additional action of a timer operating on an "hourglass" principle. In fact, the involvement of two types of photoperiodic timers—an "hourglass" and rhythms—was postulated by

Takimoto and Hamner (1964) as an explanation of their findings on the photoperiodic control of flowering in *Pharbitis nil*. In *C. rubrum*, also, the fact that a prior period of darkness was always required for expression of the rhythm (Figs. 3, 7, 9, 10) is suggestive of the operation of an "hourglass" reaction during the early hours of darkness. This proposal is also more plausible in view of recent measurements in *Pharbitis* and *C. rubrum* which show that the P_{fr} form of phytochrome disappears only after several hours of darkness (Evans and King, 1969; King, 1971). Moreover, delaying the time of P_{fr} disappearance delayed time measurement in *Pharbitis* (Evans and King, 1969) and suppressed the expression of the rhythmic component of time measurement in *C. rubrum* (King, 1971).

On the other hand, not all rhythmic responses need be regulated by additional "hourglass" processes. For instance, on rephasing by light, rhythms such as leaf or petal movement (Zimmer, 1962; Halaban, 1968a) show none of the displacement in darkness of the next peak of the rhythm that was evident on rephasing of the rhythm of flowering in *C. rubrum* (Figs. 3, 5). However, although photoperiodically controlled, leaf and petal movement rhythms are not photoperiodically induced in the sense that flowering is induced by short or long days. Thus, the action of dual photoperiodic timers may be uniquely associated with photoperiodic induction of a response such as flowering.

At present it remains uncertain how rhythmic and P_{fr}-dependent components of time measurement might interact. However, many of the effects on flowering of brief red light interruptions of darkness (see Takimoto and Hamner, 1964, 1965; Papenfuss and Salisbury, 1967; Halaban, 1968b) might be explained in terms of changes in the timing of P_{fr} disappearance over the early hours of darkness. Certainly, as has been established for *Xanthium* (Denney and Salisbury, 1970) and *C. rubrum* (Cumming *et al.*, 1965; and see Fig. 11), brief interruptions of darkness with red light can prevent flowering but need have no influence on phasing of a rhythm.

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