Studies on the Involvement of an Endogenous Rhythm in the Photoperiodic Response of Hyoscyamus niger

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Summary. An attempt was made to determine the involvement of an endogenous circadian rhythm in the flowering response of the long-day plant Hyoscyamus niger L. grown in a modified White’s medium. Both variable-cycle-length and light interruption experiments were employed in this attempt. In the variable-cycle experiments, plants were subjected to light periods of 6, 12, or 18 hours followed by varying lengths of darkness. The total lengths of the cycles varied from 12 to 72 hours. In experiments utilizing a 6-hour photoperiod, a high level of flowering occurred in cycle lengths of 12, 36, and 60 hours. Flowering was suppressed in the 24-, 48-, and 72-hour cycles. When a 12-hour photoperiod was used the flowering response was low between 24 and 36 hours and flowering did not indicate a rhythmic response. When an 18-hour photoperiod was used, the flowering response was suppressed in the 36- and 60-hour cycles.

Light-break experiments were conducted to study further the flowering response in Hyoscyamus. These experiments consisted of a 6-hour main photoperiod followed by varying lengths of darkness to make cycles of 24, 48, and 72 hours. At given intervals the dark period was interrupted by 2-hour light breaks. In a 24-hour cycle, flowering was promoted when a light break was given at either the twelfth or eighteenth hour of the cycle. In a 48-hour cycle, flowering was strongly promoted by light breaks given near the beginning or at the end of the dark period. In a 72-hour cycle, light breaks given at the eighteenth, forty-second, and sixty-sixth hour of the cycle stimulated flowering as compared with light breaks given at the thirtieth and fifty-fourth hour. These results are indicative of the involvement of an endogenous rhythm in the flowering response of Hyoscyamus niger.

In 1936 Bunning (2) proposed that the photoperiodic induction of flowering may be controlled by an endogenous circadian rhythm. In the absence of substantial supporting evidence his theory was not immediately accepted. However, recent studies of the short-day plants Glycine max (5,11), Kalanchoe blossfeldiana (7), Chenopodium rubrum (6), and Pharbitis nil (14,15) strongly support Bunning’s hypothesis. The reports of rhythm experiments with long-day plants have not been conclusive. However, some evidence has been reported which indicates that 1 long-day plant, Hyoscyamus niger, may have a rhythmic flowering response. In 1947, Claes and Lang (3) subjected Hyoscyamus to a light break experiment in which 2-hour light interruptions were applied at various times during the 41-hour dark period of a 48-hour cycle. Their results indicated that flowering levels increased when light interruptions were given either near the beginning or near the end of the dark period.

Clauss and Rau (4) reported that 2-hour light interruptions given at the twenty-second and sixty-fourth hour of a 72-hour cycle strongly promoted the flowering response of Hyoscyamus. Flowering was also promoted by light interruptions given at the forty-second and forty-sixth hours, however, this promotion was not statistically significant.

In 1960, Finn (8) subjected Hyoscyamus to several light-dark cycles of different duration. A pronounced inhibition of the flowering response occurred in the plants which received cycle lengths of 24 or 30 hours. Cycles either shorter or longer resulted in a much higher flowering response. Finn also indicated that the plants which received repeated cycles of 30 or more hours lost vigor. Therefore, he suggested that if there was a rhythm, its expression might have been obscured by this weakening of the plant. In the present experiments, plants were grown in a modified White’s medium (13) so that they could tolerate the treat-
ments with long dark periods without an excessive loss of vigor and studies were made of the possible involvement of an endogenous rhythm in the flowering response of *Hyoscyamus niger*.

**Materials and Methods**

Seeds of the annual variety of *Hyoscyamus niger* L. were originally obtained from Dr. Anton Lang. The seeds were soaked in 90% alcohol for 5 minutes to dewax the seed coat. They were then sterilized in a 10% calcium hypochlorite solution for 30 minutes and rinsed 3 times with sterilized distilled water. Under aseptic conditions, 1 seed was planted in each 18 × 150 mm culture tube containing 10 ml of a modified White's medium. This medium consisted of: 200 mg Ca(NO₃)₂, 360 mg MgSO₄, 200 mg Na₂SO₄, 80 mg KNO₃, 65 mg KCl, 16.5 mg NaH₂PO₄, 4.5 mg MnSO₄, 1.5 mg ZnSO₄, 0.75 mg KI, 4 mg Fe-citrate, 50 g sucrose, 10 g agar and 1 liter distilled water.

During the initial growing period illumination was provided by a combination of cool-white fluorescent and incandescent lamps. This source provided approximately 700 ft-c at leaf surface. Temperature ranged between 23° during the light period and 20° during the dark period. When the plants were approximately 1 month old, they were carefully graded by size. All treatment groups thus consisted of plants ranging from 2.0 to 2.5 cm tall (from culture medium to tip of stem).

All experiments were conducted in temperature controlled rooms where the temperature was maintained at 21 ± 0.5°. The light source used in all experiments was similar to that used for growing the stock plants. During the experimental period plants were maintained in photocyclers (12). The desired photoperiods were obtained by the automatic opening and closing of the photocyclers with electric time clocks. In the variable-cycle-length experiments plants were subjected to a photoperiod of constant length followed by dark periods of various durations. In the light-break experiments the plants were exposed to 2-hour light interruptions during the dark periods of 24-, 48-, and 72-hour cycles. In all experiments 24 to 28 plants were used for each specific treatment.

Various methods have been used by previous workers (3, 4, 8) to determine the degree of flowering response in *Hyoscyamus niger*. Among these are leaf count, number of flower primordia or flowers, height of the floral stalk, and the number of days to first appearance of bolting. In general, these methods have proven to be comparable (8). Plants were examined daily for stem elongation until the termination of an experiment. Following the experimental treatments the plants were returned to short-day conditions. In some cases, plants bolted before the treatment had elapsed and therefore were scored at that time. Plants which had not bolted 50 days after the beginning of an experiment were scored as vegetative. The results are presented both on the basis of percentage of plants bolting at the end of 50 days and as the number of days to bolting of those plants that actually bolted. In interpreting the results, both measurements were used in making a judgement of the response.

**Experimental Results**

*Experiment 1.* Lang and Melchers (9) determined the critical day length of *Hyoscyamus* to be 10.75 hours at 22°. In subsequent experiments they found that the critical day length varied with temperature (10). An experiment was conducted to determine the approximate critical day length under the present experimental conditions. Plants were divided into 5 groups of 24 plants each. Each group was subjected to photoperiods of either 10, 12, 14, 16, or 24 hours each day for 40 days. The flowering responses are shown in figure 1. Plants given 10 hours of light daily were vegetative, remaining in the rosette stage at the termination of the experiment. Bolting occurred in plants which received 12 or more hours of light daily, therefore, the critical daylength under the present experimental conditions is between 10 and 12 hours.

*Experiment 2.* In the previous experiment the flowering response of *Hyoscyamus* increased with an increase in the duration of the photoperiod beyond the critical daylength. Continuous light was most effective for floral induction. The following experiment was designed to determine the number of days of continuous light required to induce a maximum flowering response. Ten groups of 24 plants each were exposed to continuous light for 6 to 15 days respectively and were then returned to short day chambers. The flowering response increased almost linearly with the increasing days of continuous light and reached a high level after receiving 12 or more days of continuous light (fig 2). Thus it was decided that 14 repeated cycle treatments should give a satisfactory flowering response in both variable-cycle-length and light-break experiments.

*Experiment 3.* Blaney and Hamner (1) demonstrated that the flowering response of *Glycine max* was dependent on the length of the experimental light period as well as the length of the associated dark period. Nanda and Hamner (11) subjected *Glycine* to treatment with an 8-hour photoperiod followed by dark periods of varying lengths and observed that the level of flowering fluctuated in a rhythmic pattern with an increasing cycle length. Flowering was strongly promoted in cycles of 24, 48, and 72 hours but was suppressed in 36- and 60-hour cycles.

A similar experiment was designed to detect a flowering rhythm in *Hyoscyamus*. One hundred and ninety two plants were divided into 8 groups. Seven groups were subjected to 14 repeated light-
dark cycles consisting of an 18-hour photoperiod in conjunction with dark periods of varying lengths. At the end of the experimental treatments, the plants were returned to the short day chambers. One group of plants, which served as controls, received 14 days of continuous light. The results are shown in figure 3. Flowering was nearly as high on 24- and 30-hour cycles as in continuous light. On a 36-hour cycle, in contrast, there was a definite suppression of flowering based both upon percentage of flowering and days to bolting (of those plants which bolted). On daylengths longer than 36 hours, there were only slight differences in number of days to bolting. On the other hand, on a 42-hour cycle the percentage of bolting was 100% and gradually decreased as the cycle length was increased. This fluctuation of the flowering response, with low levels of flowering resulting from both 36- and 60-hour cycles, indicates the possible involvement of a rhythm in the flowering response.

Experiment 4. In experiments 1 and 3, 16-hour and 18-hour photoperiods were very stimulatory in initiating flowering of *Hyoscyamus*. In the fol-

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**Fig. 1.** (top, left). Effect of day length on floral initiation. Plants were exposed for 40 days to various photoperiod lengths in a 24-hour light-dark cycle.

**Fig. 2.** (top, right). Effect of continuous light on floral initiation. Groups of plants were exposed to continuous light for 6 to 15 days and thereafter to short days.

**Fig. 3.** (bottom, left). Floral response of plants to various lengths of darkness. Plants were exposed to 14 repeated light-dark cycles consisting of an 18-hour photoperiod in conjunction with dark periods of various lengths.

**Fig. 4.** (bottom, right). Floral response of plants to various lengths of darkness. Plants were exposed to 14 repeated light-dark cycles consisting of a 12-hour photoperiod in conjunction with dark periods of various lengths. In each figure the solid line indicates the mean number of days to first appearance of bolting of all plants that bolted in each treatment. The broken line indicates the percent of plants bolted in each treatment. The vertical bars indicate 1 standard error.
ollowing experiment a 12-hour photoperiod, which is near the critical day length, was employed to determine if a rhythmicity in the flowering response could be detected. Plants were subjected to 14 consecutive cycles consisting of a 12-hour photoperiod followed by dark periods of varying lengths. The plants were then returned to short day chambers. The results (fig 4) show that 24-hour and 36-hour cycle lengths were less effective in promoting flowering than were cycles either shorter or longer. No distinct rhythmic fluctuation of flower induction occurred.

Experiment 5. The results of experiments 3 and 4 indicate that not only the cycle duration but also the length of the photoperiod affects the level of floral induction. A 6-hour photoperiod was employed in the present experiment. This photoperiod is shorter than the critical day length required for floral induction. Eleven groups of plants received treatment cycles consisting of a 6-hour photoperiod followed by dark periods of different lengths. An attempt was made to induce a high level of flowering by repeating the treatment for 42 days. The results (fig 5) show that the flowering response to different cycle lengths varied in a rhythmic pattern. The treatments with cycle lengths of 24, 48, and 72 hours resulted in little or no flowering while cycle lengths other than the above were relatively stimulatory. These results strongly suggest that an endogenous rhythm may be involved in the photoperiodic flowering response of Hyoscyamus.

Experiment 6. Bunning’s theory postulates that the endogenous rhythm consists of 2 12-hour phases which are differentially sensitive to light. Therefore light-break experiments have been widely employed to detect flowering rhythms. If there is a rhythm in the flowering response of Hyoscyamus, it also should be detectable by a light-break experiment. The following experiment was conducted to determine the effectiveness of 2-hour light interruptions on the floral induction of Hyoscyamus.

Two groups of plants were given a light interruption either at the twelfth or eighteenth hour of a 24-hour cycle (6L:18D). A third group of plants, serving as controls, received no light interruption. All plants were given 14 consecutive cycles of treatments and then returned to short day conditions (8L:16D). The results are given in table I. Although the combined length of the main light period and the light interruption is shorter than the critical daylength, Hyoscyamus flowered when light interruptions were applied at the twelfth or eighteenth hour. The control plants which received 6-hour photoperiods and no supplemental light breaks did not flower.

Experiment 7. In the previous experiment a 2-hour light interruption given during the dark period of a non-inductive (6L:18D) 24-hour cycle was sufficient to evoke a long-day effect. Similar light interruptions were therefore employed in an effort to determine the flowering response during a 48-hour cycle. Each group of plants was given a single 2-hour light interruption during the dark is

![Graph](https://via.placeholder.com/150)

**Fig. 5. (top).** Floral responses of plants exposed to various lengths of darkness. Plants were exposed for 42 days to treatments consisting of a 6-hour photoperiod in conjunction with dark periods of various lengths.

**Fig. 6. (middle).** Floral response of plants exposed to a 2-hour light interruption during the dark period of 48-hour cycles. The duration of the main photoperiod was 6 hours. Plants received 14 consecutive cycles of treatment and then were returned to short-day conditions.

**Fig. 7. (bottom).** Floral response of plants exposed to a 2-hour light interruption during the dark period of 72-hour cycles. The duration of the main photoperiod was 6 hours. Plants received 14 consecutive cycles of treatment and then were returned to short-day conditions. In each figure the solid line indicates the mean number of days to first appearance of bolting of all plants that bolted in each treatment. The broken line indicates the percent of plants bolted in each treatment. The vertical bars indicate 1 standard error.
A 6-hour photoperiod is less than the critical day length for floral induction of *Hyoscyamus*. Nevertheless flowering was promoted when a 6-hour photoperiod was given in cycles of 12, 36, and 60 hours. No flowering occurred in a 24-hour cycle and flowering was low in 48- and 72-hour cycles. This demonstrates that the length of the treatment cycle has a pronounced effect on floral induction and the alternation of promotion and suppression of flowering with a periodicity of approximately 24 hours strongly suggests that an endogenous circadian rhythm is involved.

The rhythm in the flowering response of *Glycine* (1, 11) has peaks of maximum flowering approximately 12 hours out of phase from those of *Hyoscyamus*. If the basic rhythms are the same in both the long-day plant *Hyoscyamus* and the short-day plant *Glycine*, the photoperiodic reaction in these 2 plants must be different in relation to the rhythms. This is in accord with the Bunning theory (2).

Additional evidence of the involvement of an endogenous rhythm was demonstrated by light-break experiments. A 2-hour light interruption applied during the dark period of an otherwise non-inductive 24-hour cycle (6L:18D) induced flowering. Such an effect is also in agreement with the rhythm theory which states that light given in the second phase of the 24-hour oscillation promotes flowering of long-day plants (2). In a 48-hour cycle flowering was stimulated by light interruptions applied near the beginning or near the end of the dark periods. Light given in the middle of the dark period was slightly inhibitory or ineffective. On the basis of a similar experiment with *Hyoscyamus*, Claes and Lang (3) theorized that a long-day effect was elicited by an interaction between a light interruption and the previous or subsequent main light period of repeated treatments. One can test for such light interaction by treating plants during a 72-hour cycle (6L:66D). The results from such an experiment (fig 7) indicate a clear rhythmic response in the time to bolting (of those whichbolted). The percentage of bolting substantiates this finding in all treatments except at 42 hours. These results tend to exclude a simple interaction of the light interruption with the nearest main light period and provide evidence for the rhythm hypothesis.

It seems that flowering in long-day plants occurs when the light falls out of phase with the time measuring rhythm. It may be that each main light period initiates a circadian rhythm and that flowering results when successive rhythms are out of phase with one another. This hypothesis is supported by an experiment in which a 6-hour photoperiod was employed. A strong flowering response resulted in those plants which received 12-hour cycles. If we assume that each 6-hour photoperiod in the repeated treatment cycles initiates a new rhythm, then the basic rhythm is disturbed every

### Table I. Floral Response of *Hyoscyamus niger* Exposed to a 2-Hour Light Interruption During the Dark Period of 24-Hour Cycles

<table>
<thead>
<tr>
<th>Hour light break given</th>
<th>Days to bolting</th>
<th>Standard error</th>
<th>% Bolting</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>24.9</td>
<td>0.80</td>
<td>91.6</td>
</tr>
<tr>
<td>18</td>
<td>27.5</td>
<td>0.60</td>
<td>87.5</td>
</tr>
<tr>
<td>24-hour control</td>
<td>...</td>
<td>...</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The duration of the main photoperiod was 6 hours. Plants received 14 consecutive cycles of treatment and then were returned to short-day conditions.

Period of each 48-hour cycle (6L:42D). This treatment was repeated for 14 cycles after which the plants were maintained in short-day conditions. The results (fig 6) indicate a possible rhythmic flowering response with 2 maxima occurring at an interval of approximately 24 hours. Flowering was not stimulated by light interruptions given during the middle of the dark period.

*Experiment 8*. A possible flowering rhythm was indicated by giving light interruptions during a 48-hour cycle. However, this might have been caused by an interaction between the light interruptions and the nearest main light period (3). Therefore an experiment was conducted with 2-hour light interruptions applied at different times during the dark period of a 72-hour cycle (6L:66D). Each group of plants was given 14 consecutive treatments and then returned to short-day conditions. The results (fig 7) indicate that the flowering response of *Hyoscyamus* was both promoted and suppressed by exposure to 2-hour light interruptions during the dark period with respect to time to bolting (of those which did bolt). The percentage of bolting substantiates the above with the exception of the 42-hour treatment. Except for this single discrepancy these results clearly indicate a rhythmic flowering response.

### Discussion

Since the critical daylength of *Hyoscyamus* is between 10 and 12 hours, one would expect 12-hour and 18-hour photoperiods to be inductive regardless of cycle length. This is a logical assumption since partial induction is cumulative in *Hyoscyamus* (9). Such photoperiods did induce flowering in all treatments. In all cycle lengths longer than 24 hours, however, the flowering response was below the level of the continuous light treatment. If the dark period is deleterious rather than innocuous to flowering one would expect the response to progressively decrease as the dark period is lengthened. However, cycle lengths of 24 hours with 12-hour photoperiods and cycle lengths of 36 hours with 18-hour photoperiods inhibited flowering more than shorter or longer cycles. It appeared possible therefore that an endogenous rhythm was involved.
12 hours and flowering results because the rhythm is disturbed. The same argument holds for treatments with 18-, 30-, 36-, 42-, 54-, 60-, and 66-hour cycles. In these treatments the photoperiods of repeated cycles are out of phase with each other in relation to the period of an endogenous circadian rhythm. On the other hand, one would expect the endogenous rhythm to be reinforced at those cycle lengths where little or no flowering resulted, i.e., 24, 48, and 72 hours. The magnitude of the flowering response may depend on the degree of interaction between the newly initiated rhythm and the basic rhythm as well as the strength of the basic rhythm itself. In a similar experiment, maximum flowering of *Glycine max* occurred in 24-, 48-, and 72-hour cycles (11). It seems possible that the short-day plant *Glycine max* flowers best when the rhythm is reinforced in each cycle whereas the long-day plant *Hyoscyamus niger* flowers best when the endogenous rhythm is suppressed. Light periods longer than 12 hours would extend into the second 12-hour phase of the endogenous rhythm thereby such a treatment would suppress the rhythm and thus stimulate flowering as was found.

The rhythmic response curves of the light interruption experiments are 6 hours out of phase with those of the variable-cycle-length experiments. The 6-hour main light period of each cycle should establish the basic endogenous rhythm and with cycle lengths in some multiple of 24 hours, each main light period should reinforce this rhythm. Unless a 2-hour light interruption of the dark period is sufficient to establish a new rhythm, one must assume that the effects of the interruptions are a more direct interaction with the basic rhythm. Our 2-hour light interruption experiments do indicate that flowering occurred in a rhythmical pattern. Whether the 6-hour phase difference indicates the presence of 2 rhythms as was found in *Pharbitis* (14) can not be answered at this time. The question as to how these interruptions affect the rhythm must await further experimentation.

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**Literature Cited**