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THE REACTIONS OF THE PHOTOINDUCTIVE DARK PERIOD[1,2]

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Since the demonstration by Hamner and Bonner (7) of the importance of the dark period for photoperiodic induction in the short-day plant Xanthium, there have been many attempts to elucidate the reactions which take place within the plant during this period (2, 11, 13, 18). The dark period reactions appear to be concerned directly with the act of induction—the persistent change of the plant from the vegetative to the flowering condition. In Xanthium the flowering condition persists after a single completed act of induction even though the photoperiod during floral development is too long for induction to occur in vegetative plants. The condition of the plant after induction has taken place will be termed the induced state to distinguish it from the act of induction.

The induced state in Xanthium is a quantitative one. Rate of development of the floral bud is dependent upon the intensity of the original act of induction. A measure of the rate of development based upon a series of floral stages has been previously described (21). By the application of this system the quantitative nature of induction can be demonstrated by the relationship between length of the inductive dark period and subsequent rate of floral development, as measured by floral stage a number of days after induction (this is illustrated by the control points of the experiment shown in fig 4). The longer the dark period, the more rapidly the buds develop. This may be interpreted on the supposition that the rate of bud development is dependent upon the amount of flowering hormone produced, and that longer dark periods result in the production of more flowering hormone. This view is supported by the fact that buds develop at different rates when the leaves are removed from Xanthium plants at different times after the beginning of induction (9, 21, 22, 23). If leaves are removed immediately following induction the plants seldom flower. If leaves are removed after a sufficient time, however, floral buds develop at a rate almost as great as that attained by control plants with leaves. Intermediate rates of floral development characterize plants whose leaves are removed at intermediate times (this is illustrated by the points labeled "leaf removed" in fig 5). These results are in accord with the hypothesis that rate of floral development is determined by the amount of flowering hormone which reaches the growing point. The longer the leaves remain on the plant, the more hormone is translocated from them to the growing point.

The experiments below concern kinetic studies on the reactions of the dark period. *Xanthium pensylvanicum* Wall.[4] plants were treated at various times by red light interruption of the dark period and/or the application of auxin (which inhibits the act of induction, 21). The effects of these treatments on floral induction were measured in terms of rate of subsequent floral development, which is assumed to measure the amount of flowering hormone exported from the leaf.

METHODS

Plants were grown as previously described (3, 20, 21) from seed and maintained in a vegetative condition by daylight supplemented with incandescent irradiation of approximately 100 fc to make up a total day length of approximately 20 hours. To facilitate auxin treatment and to insure controlled light intensity during interruption of the dark period, plants were defoliated to a single leaf after first being classified according to the size of the most rapidly expanding leaf, the one most sensitive to induction. In the experiments reported below, the plants were defoliated to the most rapidly expanding leaf and given a single dark period, unless stated otherwise in the figure headings. A weak green light (ca 2 μw/cm^2 for 10 minutes at 2-hour intervals) was used to facilitate treatment during the dark period.

The growing points of treated plants were examined under a dissecting microscope (36 diameters magnification) approximately 9 days after induction. Rate of floral development as measured by the pres-

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[4] Synonymous with *X. saccharatum*, the name used by various other workers in photoperiodism. Specimens of the type of plants used in these studies have been filed by K. C. Hamner at the herbarium of the University of California at Los Angeles.

ent stage system is usually constant with time over this period (20, 21). The appropriate time for dissection was determined by examining controls at various times after induction.

Red light interruption of the dark period was obtained by placing a group of plants under an air-cooled box containing 6 incandescent 300-watt bulbs placed 6 inches above red filters. The intensity at the leaf level (approximately 10 inches below the filters) was 1,800 $\mu w/cm^2$ of red light. The plants were adjusted under the lights so that the level of the leaves would be approximately the same.

Naphthaleneacetic acid (NAA) was used as the auxin unless stated otherwise. When leaves were dipped in auxin solution (NAA dissolved by addition of KOH and then adjusted to pH 7.0; containing 2 drops of Tween 80 wetting agent per liter) at the beginning of induction and allowed to dry, it was found that a 2.0×10^{-4} M NAA solution inhibited flowering by about 50 % (induction by a single 16-hr night). A second method of auxin application consisted in dipping plants in auxin solution and then rinsing them after a measured interval of time. A rinse with tap water was followed by a rinse with distilled water. In this case a 30.0×10^{-4} M NAA solution left on the leaf 10 minutes inhibited flowering after a 16-hour night by about 50 %. The second method is the most desirable since it defines exactly the time of auxin penetration into the leaf (20).

The application of auxin by any method increases by a considerable factor the variation of floral stages within a given group of treated plants. This appears in the data as a poor fit of points to the smooth curves drawn in the figures presented below as compared to the data of experiments or treatments in which auxin was not applied.

Results

Effect of Red Light Interruption during the Dark Period: Although previous workers have investigated the kinetics of the low intensity light reaction (4, 5, 8, 15, 19, 24, 25), the data available record only the presence or absence of flower buds and hence cannot be considered quantitatively. In order to obtain quantitative data on the effectiveness of various red light interruption treatments, groups of plants were irradiated as described above for brief intervals (from 10 sec to 10 min in different experiments) at various times during an inductive dark period. Figure 1 gives the results of an experiment in which the inductive dark period was either 12 or 16 hours and the light interruption was for 60 seconds. The data of figure 1 show the following: 1) Red light interruption 8 hours after beginning of the dark period completely inhibits induction regardless of the length of the dark period. 2) Red light interruption after 8 hours of darkness, in this experiment is equivalent to returning the plants to continuous light. The second halves of both curves of figure 1 are identical with the curves which are obtained by plotting length of inductive dark period against floral stage. 3) The

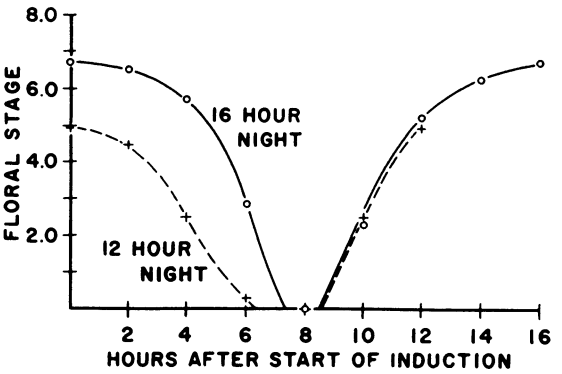

Fig. 1. The effect of red light interruption (60 sec) upon floral induction as a function of time of application during a 12- or 16-hr dark period. May 4, 1954—14 plants per treatment, dissected after 9 days.

first part of each curve is approximately that expected on the basis that the period of uninterrupted darkness following the light interruption alone functions as the dark period. The longer the period of remaining darkness, the higher the floral stage (the more flowering hormone produced in the leaf). 4) The symmetry of the two halves of each curve is, however, not perfect. Darkness following a light interruption is somewhat more effective in induction than darkness preceding a light interruption. This effect is lessened as the 8-hour point is approached.

Observations 1, 3 and 4 have been confirmed in 5 separate experiments. In certain experiments, however, observation 2 does not hold. Darkness near the end of an extended dark period may inhibit induction to some extent. This effect may be especially pronounced when the darkness follows a light interruption. This is illustrated in figure 2. Plants were treated as in the experiment of figure 1 with brief light interruptions (12 sec) during dark periods of

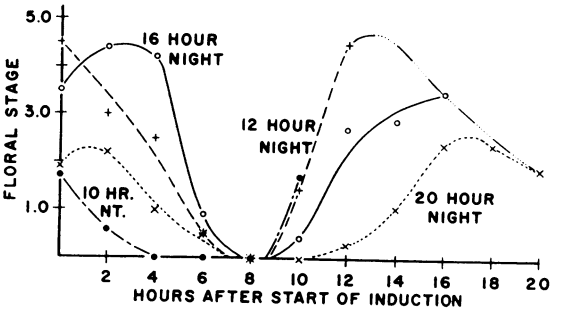

Fig. 2. The effect of red light interruption (12 sec) upon floral induction as a function of time of application during 10-, 12-, 16- or 20-hr dark periods. July 28, 1954—10 plants per treatment, dissected after 9 days. The line connecting the control points of the 12-, 16- and 20-hr nights shows the relationship between floral stage and length of the dark period, and shows the destruction of flowering hormone which takes place near the end of extended dark periods even without light interruption.

various lengths (10, 12, 16 or 20 hrs). In this experiment dark periods of longer than 13 hours were less effective than shorter dark periods. If a light interruption is given after 13 hours of darkness, the resultant inhibition of flowering is proportional to the remaining period of darkness (except for the 16- and 20-hr interruptions of the 20-hr night). Furthermore, interruptions given near the beginning of the 16- or 20-hour dark periods actually increased the degree of flowering over that of the non-light interrupted controls. It would appear that these early interruptions increase hormone production by shortening the effective period of darkness and thus reducing the excess over the critical 13-hour period.

The inhibitory effect of prolonged darkness appears to be identical with that reported by Lockhart and Hamner (16). Thus the curves of figure 2 may be interpreted by the hypothesis that a destruction of flowering hormone may take place in an excessively long inductive dark period. Why this effect may be noted in some experiments and not in others is not clear. The destructive effect may, however, be demonstrated most easily in experiments in which the single remaining leaf is younger than the most rapidly expanding one (20). The plants used in the experiment shown in figure 2 were somewhat younger than those used in the other experiments reported herein, but the physiological age of the one leaf left on each plant was the same in all experiments.

The 16- and 20-hour curves of figure 2 have a somewhat cyclic form similar to those obtained by Wareing (24) and by Carr (5). It is possible that the above supposition relating to a destruction of flowering hormone in prolonged darkness may also apply to the results of these workers. A critical test of this possibility would require the study of floral development after dark periods which are multiples of 24 hours.

The same general relations between time of red light interruption and subsequent rate of floral development were obtained regardless of the length of the red light interruption. To measure the amount of light energy required to produce the maximum interruption effect, groups of plants were irradiated for varying lengths of time (2, 4, 6, 12 and 60 sec) at various times after the beginning of a 16-hour dark period (3.25, 5.75, 8 and 12 hrs). The results are given in figure 3. It is evident that the maximum inhibition of floral induction elicited by light interruption is strongly dependent upon when the interruption is given, as has already been shown by the data of figures 1 and 2. The amount of light required to produce this maximum effect appears, however, to be constant regardless of when the interruption is given. Parker et al (19) report that the product of light intensity and period of exposure to light needed to produce a given amount of inhibition of flowering in Xanthium is constant. The amount of light energy required to produce a given amount of inhibition at any given time may therefore be calculated from the present data since the intensity remains constant. The data of figure 3 indicate that maximum response

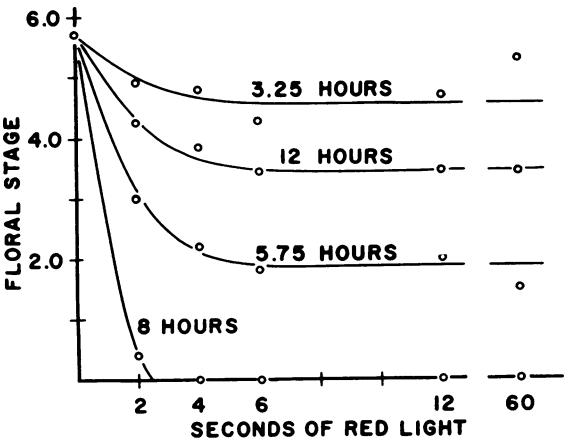

FIG. 3. Relation of floral stage to the duration of a red light interruption given at various times during a 16-hour dark period. July 26, 1954—10 plants per treatment, dissected after 9 days.

was obtained by exposing the plants to periods of more than 6 seconds of red light. Since the intensity of the red light was approximately 1,800 $\mu w/cm^2$, it follows that approximately 10,000 $\mu w/cm^2$ of red light are sufficient to produce maximum response at any time more than 3.25 hours after the beginning of the dark period.

Borthwick et al (4) present evidence that the low intensity light reaction consists of a conversion of a red-receptive form of a photo-receptor pigment to a far-red-receptive form of this pigment. They suggest further that flowering hormone production does not take place unless the pigment is in the red-receptive form; that this pigment is in the far-red-receptive form at the beginning of the dark period (due to exposure to red wave lengths during the day); and that the pigment undergoes a spontaneous conversion to the red-receptive form in the dark. This suggests the possibility that the critical night length is controlled by the time required for the spontaneous conversion of far-red-receptive to red-receptive pigment to take place. It is possible that the amount of light required to produce a maximum red light interruption effect may be a measure of the amount of photoreceptor pigment present in the red-receptive form. The data of figure 3 interpreted on this basis indicate that conversion of far-red-receptive to red-receptive pigment takes place before 3.25 hours after the beginning of induction. Borthwick et al (4) found it possible to shorten the critical night length about 2 hours by irradiating plants with far-red light just before the beginning of the dark period. This finding is in agreement with the above conclusion, and with the conclusion of Withrow and Withrow (25) based upon experiments of a completely different nature. It follows that far-red light should have no effect upon the critical night length when given more than about two hours after the beginning of induction.

EFFECTS OF APPLIED AUXIN: It will now be shown that the effect of applied auxin upon the photoperi-

odic response is not due to any effect upon the critical night length but consists in a net reduction in the amount of flowering substance transported from the leaf. Groups of plants were treated with various concentrations of auxin and then with dark periods of various lengths. The results of 3 such experiments are given in figure 4. Applied auxin has little or no effect upon the critical night length, but the degree of flowering (net amount of flowering hormone produced) of plants given longer than the critical dark period is reduced in proportion to the concentration of applied auxin.

To test the effect of the time of auxin application upon flowering, groups of plants were treated as described above with various concentrations of auxin at various times before, during and after an inductive dark period. The data of figure 5 are typical of 10

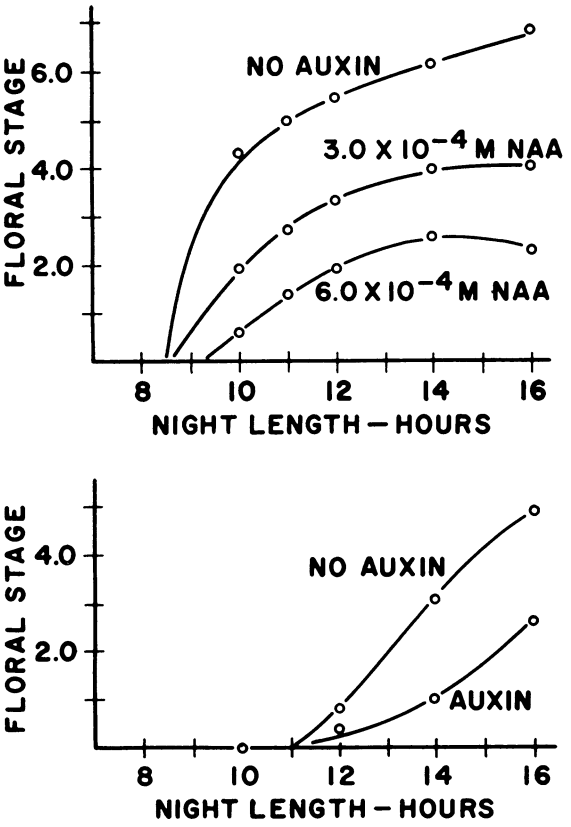

FIG. 4. The relation of flowering to length of the dark period and to applied auxin. *Upper figure.* For plants with leaves dipped in 3.0×10^{-4} M NAA. March 11, 1954—20 plants per treatment, dissected after 13 days. For plants receiving 6.0×10^{-4} M NAA: March 1, 1954—20 plants per treatment, dissected after 10 days. The stages were adjusted so that controls matched the controls of the 3.0×10^{-4} M treatment. *Lower figure.* May 26, 1953—20 plants per treatment, dissected after 17 days, 1.5×10^{-4} M NAA sprayed on the leaves; plants defoliated to the next leaf older than the most rapidly expanding one.

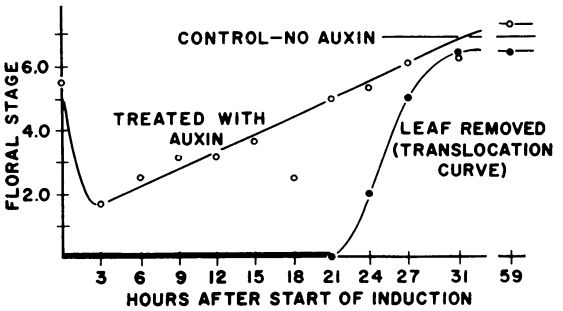

FIG. 5. Effect of auxin in inhibiting floral induction as a function of time of application. April 10, 1954—16 plants per treatment, dissected after 13 days, leaves dipped in water, then after 5 min in 20.0×10^{-4} M NAA, and then after 10 min rinsed in tap water followed by distilled water.

such experiments. It is apparent that auxin becomes increasingly more effective in floral inhibition as it is applied later and later during the first two or three hours after the beginning of the dark period. Auxin applied after this time becomes less and less effective until finally it is completely ineffective. Auxin applied still later increases rate of bud development slightly (21). It is evident also that the slope of the curve relating rate of floral development to time of auxin application shows no sharp change at the end of the dark period. The data show considerable scatter, but no consistent deviation has been detected in different experiments,[5] and a smooth curve appears to best describe the actual kinetics of the auxin reaction. Finally, the time at which auxin becomes ineffective in floral inhibition is essentially the time at which the concentration of the flowering substance has been built up to maximum level outside the leaf, as shown by defoliation experiments (explained above). From experiments with defoliation at varied times after various night lengths (not shown in fig 5), night length seems to be relatively unimportant in determining time of translocation of the flowering stimulus from the leaf and hence in determining the time at which applied auxin becomes ineffective in floral inhibition. Time of translocation is, however, affected strongly by time of year due perhaps to differences in light intensity. Translocation was found to be half completed in 25 hours after the beginning of induction in July but only in 40 hours in December.

The relationship between inhibition of flowering by red light interruption and inhibition by applied auxin was studied with plants which were irradiated with red light or treated with auxin or both at various times during a 16-hour inductive dark period.

[5] In some experiments, auxin seems to be less effective when applied in the heat of the day. This is probably due to low humidity which results in rapid drying of the auxin solution on the leaves so that there is insufficient time for auxin penetration. This effect may be overcome by raising the humidity and/or using the method in which the auxin solution is rinsed off the leaves after a brief, measured interval.

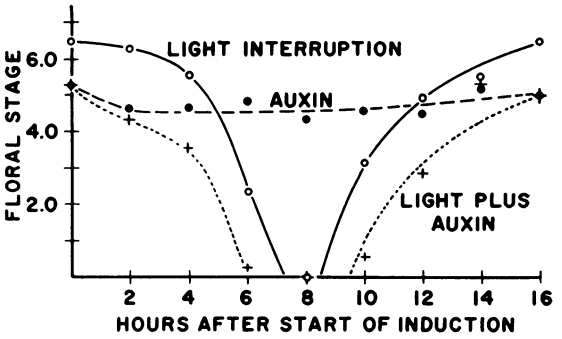

FIG. 6. Inhibition of floral induction by a red light interruption (60 sec), by applied auxin, or by both together, as a function of time of application during the dark period. May 19, 1954—15 plants per treatment, dissected after 9 days. Leaves of auxin-treated plants were dipped first in water, then in 20.0×10^{-4} M NAA, and then rinsed after 5 min as explained in figure 5. The dotted line is theoretical and represents the sum of the inhibitions of the other two curves.

The experiment was repeated three times. In two cases NAA was used as the auxin while in the third case indoleacetic acid (IAA) was used. Figure 6 gives the results of an experiment with NAA. Light or auxin alone acted as in the experiments described above. The inhibition produced by the two factors together is very nearly the sum of the inhibition produced by each factor applied alone.

DISCUSSION AND CONCLUSIONS

The experimental results of this work may be summarized as follows: 1) A red light interruption of sufficient intensity, given 8 hours after the beginning of induction inhibits flowering completely. 2) Later interruptions are equivalent to returning the plants to full light, although in certain cases darkness following the light interruption inhibits subsequent flowering. 3) The effectiveness of a red light interruption given earlier than 8 hours after the beginning of the dark period depends upon the length of darkness which follows the interruption. 4) The quantity of light energy required to bring about the maximum inhibitory effect of an interruption of the inductive dark period is constant after the first 2 or 3 hours of the dark period. 5) Application of auxin decreases the amount of flowering hormone which reaches the growing point, but has little effect upon the critical night length. 6) Inhibition of flowering is maximal when auxin is applied shortly after the beginning of the dark period. As auxin is applied later, its effectiveness decreases at a nearly constant rate and it is ineffective when the full concentration of flowering hormone has been built up outside the leaf. There is no sharp change in effectiveness of auxin in floral inhibition at the end of the dark period. 7) The inhibitory effect of applied auxin and of red light interruption applied simultaneously are approximately equal to the sum of the inhibitory effects of these agents applied separately.

These results and those of other workers (1, 2, 3, 4, 5, 6, 9, 10, 12, 14, 15, 16, 17, 19, 25) suggest that during the dark period 3 consecutive reactions take place:

a. The spontaneous conversion, in the dark, of the photo-receptor pigment from a far-red-receptive to a red-receptive form. (Pigment Conversion.)
b. Reaction(s) preparatory to hormone synthesis. (Preparatory Reaction.)
c. Synthesis in the leaf of flowering hormone. (Hormone Synthesis.)

The following features are characteristic of these reactions and at the same time help to define them: Critical night length is determined by the first two reactions. For the Preparatory Reaction to proceed to completion at least some of the pigment must be in the red-receptive form, as this reaction is stopped by red light interruption. Since induction can take place under very weak red light (1, 12), however, Pigment Conversion may not have to be complete for the Preparatory Reaction to begin.

When Pigment Conversion is reversed by a red light interruption, the Preparatory Reaction is also reversed and must begin again, although Hormone Synthesis is only halted but not reversed. If the Preparatory Reaction is stopped (by light interruption) just before the beginning of Hormone Synthesis (near to the critical night length), it may not be resumed regardless of the length of the dark period which follows (5, 6, 24). It seems probable that this may be due to a depletion of energy substrates built up by photosynthesis in the previous light period. Indeed, Liverman and Bonner (14) obtained flowering in plants exposed to a long night interrupted at 3-hour intervals by a flash of light and then given a long period of uninterrupted darkness, providing sucrose or other energy substrates were supplied to the leaves.

The first two reactions seem to be rather insensitive to applied auxin since auxin affects the critical night length only slightly if at all. Net rate of Hormone Synthesis is, however, strongly depressed by applied auxin.

Rate of flowering Hormone Synthesis is a function of the number of hours by which the dark period exceeds the critical length. This rate is rapid for the first 2 hours or so, then tapers off and finally reaches zero after 4 to 8 hours (12 to 16 hrs after the beginning of the dark period).

Under some conditions a destruction of flowering hormone in the leaf appears to take place during the later hours of a prolonged dark period. This produces an apparent cycle of sensitivity to red light interruption.

Applied auxin has been shown to be without effect on critical night length. The data presented here also cannot be interpreted on the basis that auxin inhibits the translocation of flowering hormone from the leaf. Auxin is particularly effective during the period of Hormone Synthesis and translocation seldom begins until the plants are returned to light (9, 20, 21, 23).

In addition, antiauxins which, if applied before induction, overcome the inhibitory effect of auxin, do not counteract the inhibitory effect of auxin if applied after induction as might be expected if the effect were only upon translocation (20). It seems more probable at present that auxin brings about a destruction of flowering hormone in the leaf (13, 22). This idea is compatible, for instance, with the fact that there is no sharp change in effectiveness of applied auxin at the end of the dark period. Further, the reduction in net rate of Hormone Synthesis by applied auxin may be a destruction of a fraction of the flowering hormone normally produced during Hormone Synthesis.

We do not know, of course, how auxin could result in the destruction of a hormone which has been synthesized in the leaf. It appears possible that auxin might initiate other energy-using processes (e.g. related to growth), and that these processes might utilize energy or materials which would otherwise have been used to maintain the flowering hormone in the leaf. These matters remain to be elucidated.

It appears possible that the native auxin of Xanthium is relatively unimportant in the reactions of the dark period. Experiments with applied antiauxin usually show only a slight promotive effect upon flowering, and often this effect may be detected only when the plants receive an inductive dark period under conditions close to the critical (1, 12). Thus application of antiauxin may spare a certain amount of flowering hormone destruction, but since this amount is slight, the amount of auxin normally present may be slight compared to the amount applied in the above experiments.

Summary

1. The reactions of the photoinductive dark period in Xanthium may be studied kinetically by assuming that rate of development of the floral bud (measured by floral stage) is a function of the amount of flowering hormone produced during induction. Evidence in support of this assumption is based upon the varying rate of floral development of plants and induced by various night lengths, or after defoliation at various times following induction (during translocation of the flowering hormone).

2. Red light interruption of the dark period is most effective approximately 8 hours after the beginning of induction. The effectiveness of light interruption before this time depends primarily upon the length of the remaining dark period. After 8 hours, light interruption of the dark period may be equivalent to returning the plants to full light, although in some experiments a destruction of flowering hormone takes place in the darkness which follows this interruption.

3. A constant amount of red light is required to bring about maximum inhibition of flowering for any given time after the first 2 or 3 hours of darkness. This may indicate that a red-receptive, photo-receptor pigment has reached its maximum concentration within the first 2 or 3 hours of darkness.

4. Auxin applied at the beginning of induction may strongly affect the amount of flowering hormone subsequently produced, but has little effect upon the critical night length.

5. Applied auxin is most effective in inhibiting floral induction when applied 2 or 3 hours after the beginning of the dark period. After this time its effectiveness decreases gradually until it becomes ineffective some hours after the plants have been returned to light. This is also the time required for a maximum concentration of flowering hormone to be built up outside the leaf by translocation. The slope of the curve relating floral stage to time of auxin application shows no sharp change at the end of the dark period.

6. The inhibitory effects of light and of auxin applied together equals the sum of the inhibitions caused by each agent applied alone.

7. It is suggested that three reactions take place during the dark period. a. Conversion of photoreceptor pigment from a far-red to a red-receptive form (complete in 2 or 3 hours), b. Preparatory Reaction(s), which together with Pigment Conversion determine the critical night length, c. Actual Hormone Synthesis.

8. It is suggested that applied auxin may bring about a destruction of flowering hormone in the leaf. The native auxin of the leaf may participate in the flowering response in a similar way, although it is ordinarily present in concentrations too small to be of great importance.

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INDUCTION OF FLOWERING IN LONG DAY PLANTS BY APPLIED INDOLEACETIC ACID[1,2]

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In short day plants the promotion of flowering by photoinductive cycles may be nullified by the application of auxin (2) and by interruption of the dark period with red light (17). Both of these inhibitory effects can be reversed by the application of an anti-auxin (1, 2, 12). On the other hand light interruption of the dark portion of the photoinductive cycle promotes floral induction in long day plants (3, 8). On the basis of these results we might expect either that the flowering of long day plants would be inhibited by auxin applied during photoinduction, or that auxin would replace or supplement low intensity light and actually cause flowering of plants kept in a short day regime. The experiments reported in this paper were undertaken to try to discover the role of auxin in the flowering of long day plants. The results demonstrate for the first time that auxin given in addition to low intensity light may cause flowering of long day plants under conditions in which the non-auxin treated controls remain vegetative.

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Materials and Methods

Two long day plant species, *Hyoscyamus niger* (annual variety) and *Silene Armeria*, were used for the experiments reported here. Experiments with a third long day plant, *Crepis capillaris*, also indicate a flower promoting effect of auxin applied to plants grown under threshold conditions. This material, however, proved so variable, that the experiments, taken by themselves, would not be conclusive and are not presented here. The photoperiodic response of Hyoscyamus was studied by Lang and Melchers (9). The action spectrum for the light flash reaction in this species was determined by Parker and co-workers and was found to be identical with the photoperiodic action spectrum of other day-length-dependent plants of both the long day and short day types (16). The strain used in our experiments is the same as that used by earlier workers (annual yellow flowered variety). The photoperiodic behavior of Silene, a species not previously used for photoperiodic work, was studied by Liverman and Lang (12, 14) and was found to be typical for a long day plant. This strain[5] was collected in the yard of one of us (J. L.) in San Gabriel, California where it had grown spontaneously for a number of years. A majority of the experiments reported below were carried out in the con-

[5] Seed of this species may be obtained from the Vaughan Seed Company, 601 W. Jackson Blvd., Chicago, Illinois.