COLORADO STATE UNIVERSITY

FORT COLLINS, COLORADO 80521

DEPARTMENT OF BOTANY AND PLANT PATHOLOGY

January 27, 1966

Answered 5 Feb 66

Mr. Grote Reber
CSIRO
Tasmanian Regional Laboratory
"Stowell"
Stowell Avenue
Hobart, Tasmania

Dear Mr. Reber:

Thanks for your interesting reprints relating to your experiments about reversing bean vines. I'm afraid that I can't be of much help to you. I had a little experience with the measuring of cocklebur leaves (we hope to relate growth to flowering response) in which a daily handling of only a few seconds caused a distinct inhibitory effect upon the growth of the leaves. This seems to come the closest, but my responses were opposite to yours in that you got a higher yield. There is a book entitled Plant Morphogenesis by Edmond W. Sinnott published by ~~Macmillan Co~~ McGraw Hill. 1960, I believe. Mr. Sinnott discusses in more detail than anyone else I know the experiments which have been done relating to plant growth. A number of these involve manipulations similar to yours, although I don't remember seeing anything exactly similar.

I wish that I could be of more help, and I will certainly keep my eyes open in the future, having now become familiar with your work. Thanks again for sending the reprints.

Sincerely,

Frank B. Salisbury

Frank B. Salisbury
Professor of Plant Physiology

FBS:ch

asked librarian for book on 7/2/66

The initiation of flowering

Frank B. Salisbury

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The initiation of flowering

Frank B. Salisbury

Many plants flower as a result of exposure to a particular length of day. It now seems clear that the length of day is measured by a light-absorbing pigment in the leaf, and that this releases a hormone that initiates the flowering. This article describes the work that has led to these conclusions, and to the separation of the pigment. It also suggests the direction of future work.

Perhaps the most fundamental problem of contemporary biology is that of the origin of form. We know that the genes control the ultimate form of the living organism, but since all the cells of an organism have the full complement of genes, we are perplexed by the manner in which certain genes are active only at certain times. In the growth of a plant, cells of the tip are continually dividing and enlarging and becoming specialized in such a way as to form stems and leaves. At a certain point in the plant's life cycle, however, other genes apparently become active so that these same cells develop, but into a highly modified version of the stem and leaves, the flower (figure 1). This redirection in growth apparently occurs in response to a chemical substance that is not synthesized in the dividing cells at the stem tip, but in the maturing leaves. This hormone, as yet uncharacterized, arises in many species in response to some external change in the environment. The manner in which morphogenesis may occur as directed by a flowering hormone, which in turn arises in response to environmental change, is the topic of this article [1–5]. We will consider four basic questions.

The environmental factors that cause flowering

The first clear answers regarding the light environment were put forth in a series of papers beginning in 1920, when the United States Department of Agriculture scientists, W. W. Garner and H. A. Allard [6], published a detailed investigation of factors that might cause soybean and Maryland Mammoth tobacco to flower under winter greenhouse conditions in Beltsville, Maryland. Temperature, nutrition, humidity, and light intensity were each carefully studied and eliminated, but it was found that both of the species would flower when the days were shorter than some maximum length, regardless of other conditions. Other species such as spinach (*Spinacia oleracea*), dill (*Anethum graveolens*), and henbane (*Hyoscyamus niger*) would flower only when the days were longer than some minimum. The first group of plants were called short-day plants and the second group long-day plants. The phenomenon itself was termed photoperiodism. Plants in a third category, which flowered under virtually any daylength condition, were called day-neutral plants (figures 2 and 3).

The picture has now become much more complicated [3]. For example, there are 'short-long-day' plants, which flower only when they are exposed to short days followed by longer days (as might occur in the spring), and there are also 'long-short-day' plants which flower in the autumn. There are even intermediate-day plants which flower only when the daylength is intermediate, and conversely there are plants which fail to flower when the daylength is longer than some value but shorter than another. Some plants flower in response to low (or rarely high) temperatures, instead of in response to daylength; yet others flower only as a consequence of a combination of temperature and daylength.

There are many interactions with other environmental factors, such as temperature, light intensity, and even factors such as soil nutrients and atmospheric humidity. Because of these many complications, most of the work has been concentrated on a few fairly straightforward species.

The way that the plant 'measures' daylength

This is quite clearly a physiological question. The initial clue came from recognition of the importance of the dark period. A few short-day plants (for example, the cocklebur *Xanthium pennsylvanicum*, and the Japanese morning glory *Pharbitis nil*) can be grown vegetatively under continuous light and then be induced to flower by exposure to only one dark period of sufficient length. Furthermore, a very brief interruption of moderately intense light applied at the right time completely nullifies the effect of this dark period (figure 3).

These and other experiments led workers in the period 1930–1950 to suggest that we should speak of long-night, rather than short-day plants. The long-day plants also proved to be short-night plants: if the dark period were interrupted in this case, flowering was promoted instead of being inhibited. However, the terminology was not changed, possibly because of the inertia usual in such matters.

This basic light-interruption observation opened up a world of possibilities. To begin with, it was possible to determine when the light interruption was most effective and how much light was required to bring about the response. It was also possible to find out which wavelengths of light were most effective in interruption of the dark period. Determination of this action spectrum, in which relative response is plotted as a function of wavelength, proved to be the most valuable experiment of all. It immediately became apparent that the resulting curve (figure 4), with its peak in the red region, closely resembled that already determined for other light-controlled systems, such as the germination of some light-sensitive seeds. This implied that the pigment system acting in photoperiodism was the same as that acting in other photo responses. There are also a number of blue-light responses, such as phototropism, which are apparently under the control of at least one other pigment system.

An extremely important discovery was reported in 1952 [7]. It was found that the effects of red light in promoting lettuce-seed germination could be completely

F. B. Salisbury, *B.Sc., M.A., Ph.D.*

Was born in Provo, Utah, in 1926, and educated at the University of Utah and the California Institute of Technology. He became Assistant Professor of Botany at Pomona College in California in 1954, and moved to the corresponding post in the Colorado State University in 1955. Since 1961, he has been Professor of Plant Physiology there. His particular research interests are the physiology of flowering, physiological ecology, and the biological problems of outer space.

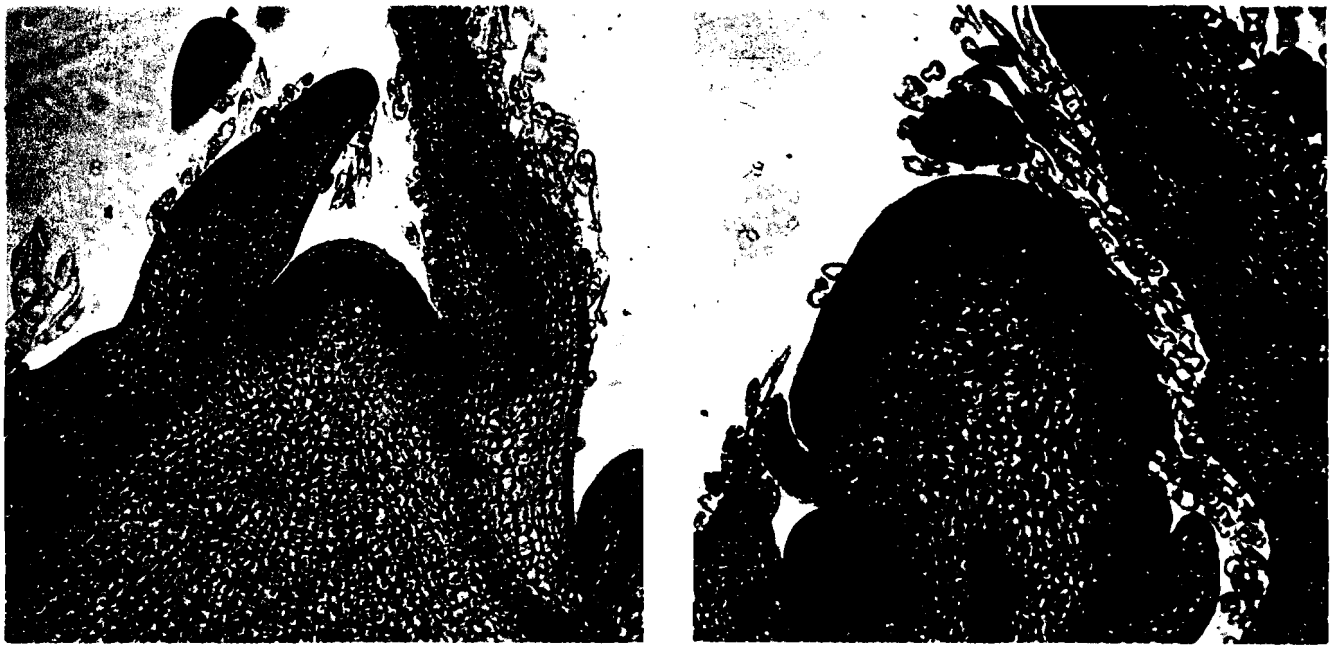
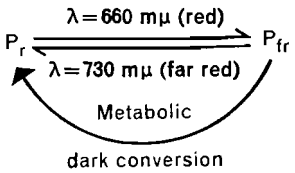


Figure 1 Longitudinal median sections of (left) a vegetative apical meristem of *Xanthium pennsylvanicum* Wall, and (right) an apical meristem which has begun to develop into a flower. Different stains were used in preparation of the two sections. (Both × 140)

reversed by subsequently exposing the seeds to light of longer wavelengths, in the far red. This reversal was also found to apply to photoperiodism and many other systems [8]. These results suggested that the pigment involved was converted from one form to another by absorption of red light and then back to the original form by absorption of far-red light. The reversal could occur a number of times, although in some systems other factors might limit the reversibility. Since a short-day plant is exposed to sunlight which is relatively rich in red before it is subjected to the extended dark period, and since this same light is effective in interruption of the dark period, it seems that the pigment must be converted in the dark from the far-red absorbing form, P_{fr}, to the red-absorbing form, P_r. These facts of conversion by red or far-red light and conversion in the dark are summarized by the following equation:



It soon became apparent that the far-red absorbing form of the pigment was the biologically active one, although the red-absorbing form could sometimes also play a role. For example, if a red-light interruption is given in the middle of the dark period, only a limited period of time (approximately 30 minutes in the case of the cocklebur) can be allowed to elapse before subsequent far-red illumination is completely unable to reverse the effect [9]. Apparently the pigment in the far-red absorbing form completes its inhibition during this time.

The pigment was finally isolated in the spring of 1959 [10]. This was a considerable achievement, firstly because it is present only in extremely small quantities, and secondly because measuring its optical absorption at one wavelength converts it to the form that absorbs at the other wavelength. These problems were solved, and the pigment has been directly demonstrated in many species of plants. The importance of this achievement for photobiology should not be underestimated.

The discovery of the reversible pigment system, named by its extractors phytochrome, seemed to solve many of the problems of photoperiodism. For example, the manner in which the plant might measure the length of the dark period might simply be a function of the dark-conversion times for the pigment system. If the critical dark period for cocklebur is 8 hours and 30 minutes, then one might say that 8 hours and 30 minutes are required for the inhibitory P_{fr} to be removed from the plant by dark conversion [11].

The role of phytochrome in photoperiodism is still considered to be paramount, but the picture has become very complicated. The idea of phytochrome conversion as a measure of time proves to be an insufficient one. Firstly, time measurement under threshold light conditions—light of an intensity such that it inhibits flowering slightly but not completely—is studied by determining the minimum time required for the first perceptible initiation of flowers. This time is called the critical dark period or the critical night. Threshold light does not extend this time (figure 5), although under these conditions the inhibition of flowering implies that phytochrome is not converted completely to the P_r form.

Secondly, there are many analogies in the flowering process to other rhythmic processes in both plants and animals. These correlations are impressive, and the idea that time measurement in the flowering process may be performed by the same biological clock that controls other rhythms seems quite reasonable. The results of a representative experiment, performed by Karl Hamner [12] and his colleagues at the University of California in Los Angeles, are illustrated in figure 6.

Thirdly, time measurement in the flowering process, as well as in the other rhythms, is very insensitive to temperature changes, although many aspects of the

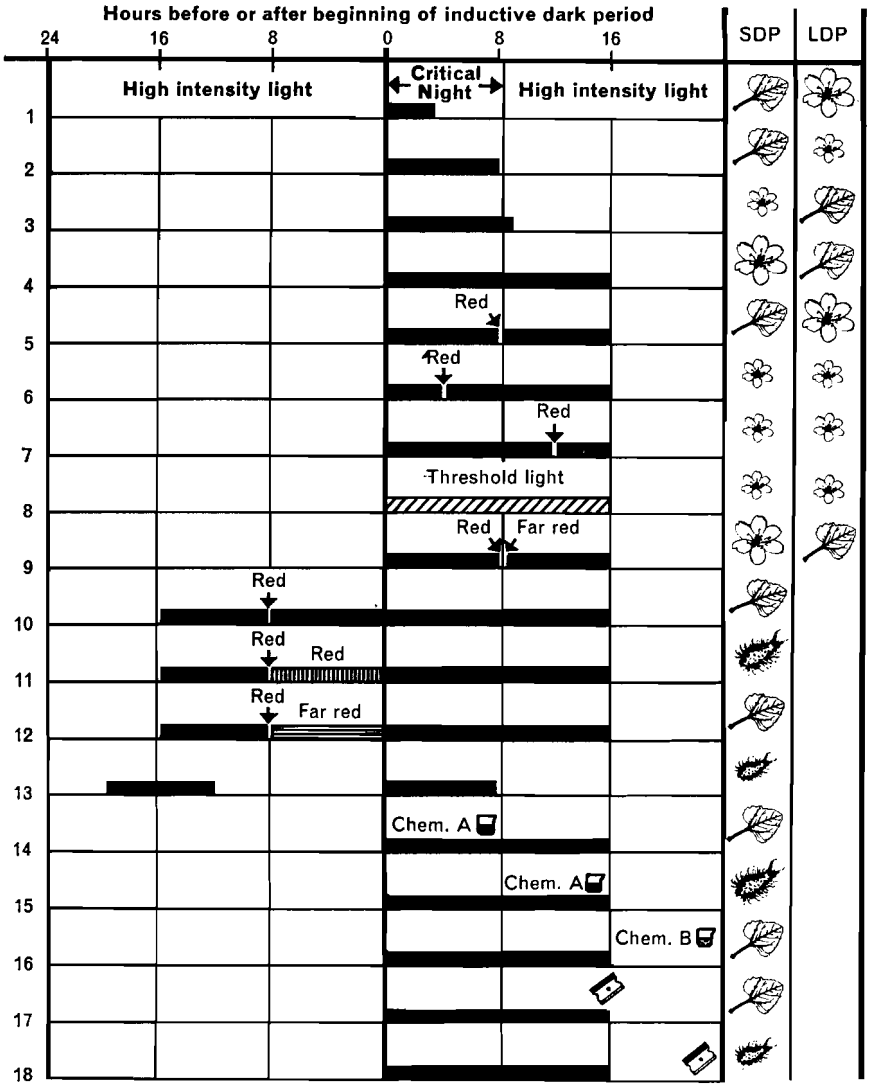


Figure 3 A summary of a number of photoperiodism experiments. (1) A dark period shorter than the critical night induces flowering in long-day plants (LDP) but keeps short-day plants (SDP) vegetative. (2 and 3) As the dark period just approaches the critical night-length, long-day plants flower slightly and as it just exceeds the critical night-length, short-day plants flower slightly. (4) An extended dark period causes profuse flowering of short-day plants but keeps long-day plants vegetative. (5) If the dark period is interrupted with red light near the critical night, short-day plants are inhibited in their flowering and long-day plants are promoted. (6 and 7) If the interruption comes at some other time, flowering may be inhibited or promoted in both response types but to a lesser degree. (8) Under so-called threshold light conditions, flowering of short-day plants is quantitatively inhibited and that of long-day plants is quantitatively promoted. (9) The effects of a red interruption are overcome by subsequent exposure to far-red light. (10) This and the following experiments all refer to cocklebur (and, in specific instances, a few other plants). If a light flash is given after 7½ hours (close to the critical night), a subsequent dark period is ineffective even though it is very long (as the light interruption becomes shorter and shorter, the dark period required for a minimum of flowering becomes longer and longer). (11) If this initial dark period is followed by red light, then a subsequent dark period is very effective. (12) If far-red light is used instead, the dark period is not effective. (13) If the light period following the first dark period is 12 hours long, conditions are apparently so optimal that a subsequent dark period shorter than the critical night will still induce flowering. (14 and 15) Some chemicals when applied to the plant (by dipping the leaves in a solution of the chemical), will inhibit flowering when they are applied before the end of the critical night, but not when they are applied after the end of the dark period. (16) Other chemicals will inhibit flowering even when they are applied some time after the dark period. (17 and 18) If the leaves are removed immediately following the end of the dark period, plants remain vegetative, but if some 8–20 hours are allowed to elapse, plants flower.

flowering process, including phytochrome conversion, are highly temperature-sensitive. Figure 7 shows the critical dark period determined for the cocklebur and the Japanese morning glory at various temperatures. The effect of temperature upon time measurement is very slight with the cocklebur, but appears to be quite large for the Japanese morning glory. Yet when time measurement is determined not by measurement of the critical dark period, which could be influenced by other factors, but by the time of maximum sensitivity to a light interruption, then time measurement is unaffected by temperature in Japanese morning glory also (figure 8).

Fourthly, a number of recent experiments from various laboratories, each carried out in a different way, seems to indicate that there are two phases to the flowering process, one of which is inhibited by red light and the other of which is promoted by red light [13, 14, 15; but see 16]. This would imply, if the effects are always *via* the phytochrome system as all the evidence indicates, that P_{fr} is essential for flowering at one time during the cycle and inhibitory to flowering at another. This is another close analogy to the other rhythms; light may inhibit a rhythm at one time and promote it at another. This is true of both plants and animals, including many organisms in which phytochrome is clearly absent.

Figure 3 illustrates the basic approach that we have taken at Colorado State University. Plants are left in continuous light for a few days and then given a dark period of 7½ hours—too short for the initiation of flowering. Following this dark period, they are given a brief interruption of light, normally quite inhibitory to the flowering process, and then after special experimental treatment, they are given a dark period of length sufficient to initiate flowering. The special treatments may consist of light periods of various durations, intensities, or qualities. The idea is to try to discover the conditions that will overcome the inhibitory effects of the 7½ hour dark period and the short-light interruption. Our results so far indicate that a light period of approximately 8 hours works well; that 12 hours is optimal and so effective that less than 8 hours of darkness following this will initiate flowering; that intensity during this 8 hours is of

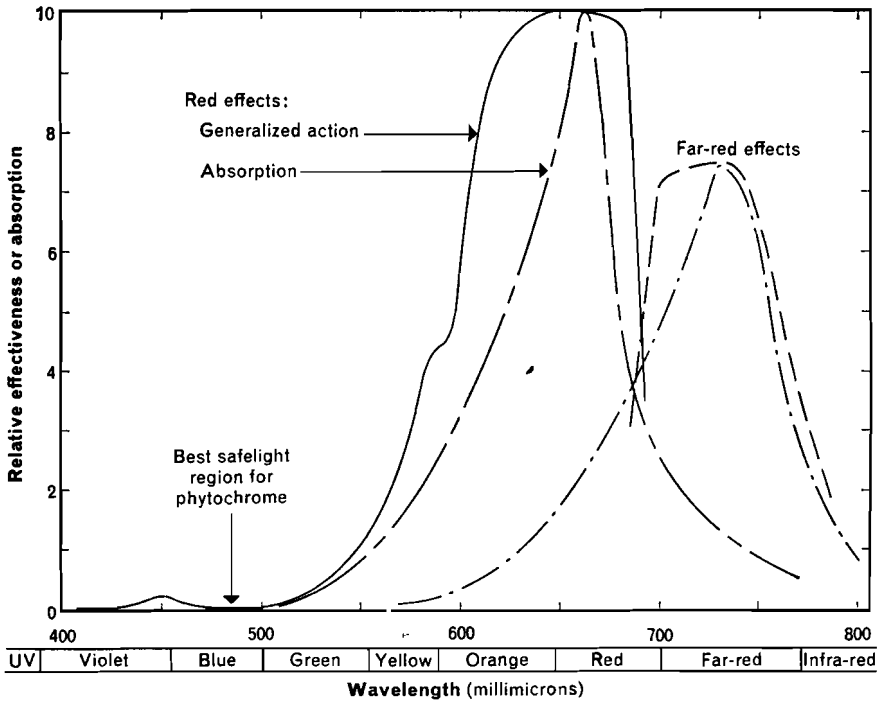


Figure 4 Action spectra for the red and far-red responses controlled by phytochrome [11].

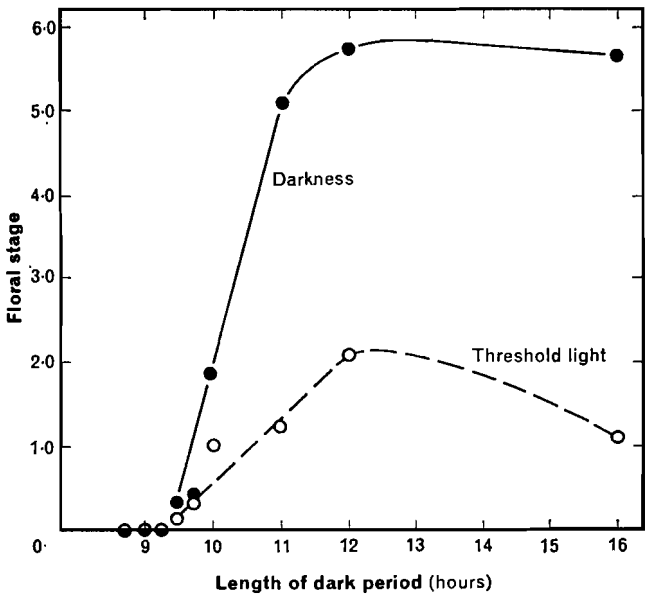


Figure 5 The critical night (as determined by exposing plants to dark periods of different lengths) is not changed when plants are maintained under threshold light, although flowering at longer night lengths is strongly inhibited [3].

secondary importance at best; and that red light is by far the most effective during this period and far-red light is inhibitory.

All of this clearly demonstrates the importance of the light period as well as of the dark period. Some plants, such as the cocklebur and the Japanese morning glory, appear to be brought to a state during the light period that they will remain in during many subsequent hours or even days of continuous light. When plants are finally placed in the dark, they will complete the cycle and thus respond to a single dark period. Yet both parts of the cycle are equally indispensable to the ultimate flowering response, and with many other species (such as the soybean) both phases of the cycle must match a cycling, light-dark environment tuned approximately to a 24-hour period. It turns out, thus, that photoperiodism is exactly what the name implies, a response to a periodic cycling of light and darkness. It is fortunate that the 'short-day' terminology was retained.

A biological clock [17] is clearly implicated in the flowering process. Furthermore, the clock seems to be coupled to the environment *via* the phytochrome system in higher plants. The question of how the same pigment system can inhibit or promote depending upon the cycling of the clock, must be connected with the question of how long-day plants and short-day plants can be so diametrically opposite in their responses and yet both ultimately produce the reproductive floral structure.

The immediate initiation of flowering

This proves to be a biochemical question. Recent work on phytochrome chemistry is essential to our ultimate solution of this problem, tying this question into the last one. But there is much to do beyond studying the nature of phytochrome.

It was shown towards the end of the 1930s that in flowering it is the leaf that responds to the 'photoenvironment'. If the leaf of a cocklebur plant is placed in a dark bag for the requisite number of hours, the plant will subsequently flower, but if only the tip, the part of the plant that ultimately becomes a flower, is darkened, no response occurs; nor is there any response if only the stem is darkened. This suggested that a chemical stimulus was produced in the leaf and translocated to the bud

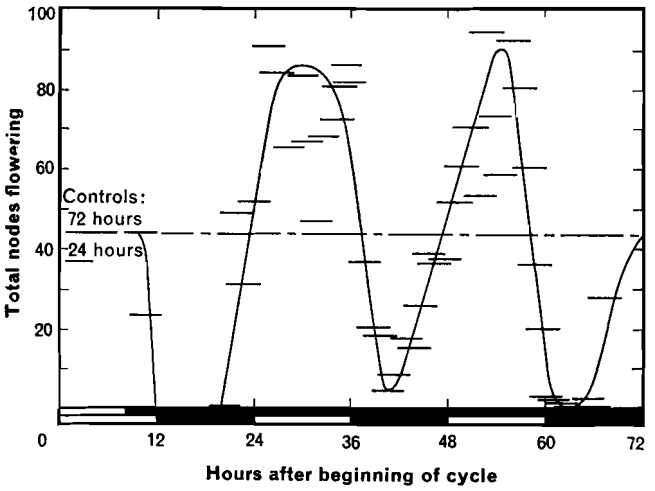


Figure 6 Responses of Biloxi soybean plants to 4-hour light interruptions given at various times during a 72-hour cycle. The lines indicate the 4-hour interruption periods. The upper bar on the bottom of the graph indicates the 8 hours of light and the 64 hours of darkness given in each of the 7 cycles to which the plants were exposed. The lower bar indicates the daily cycle of light and darkness under normal conditions [12].

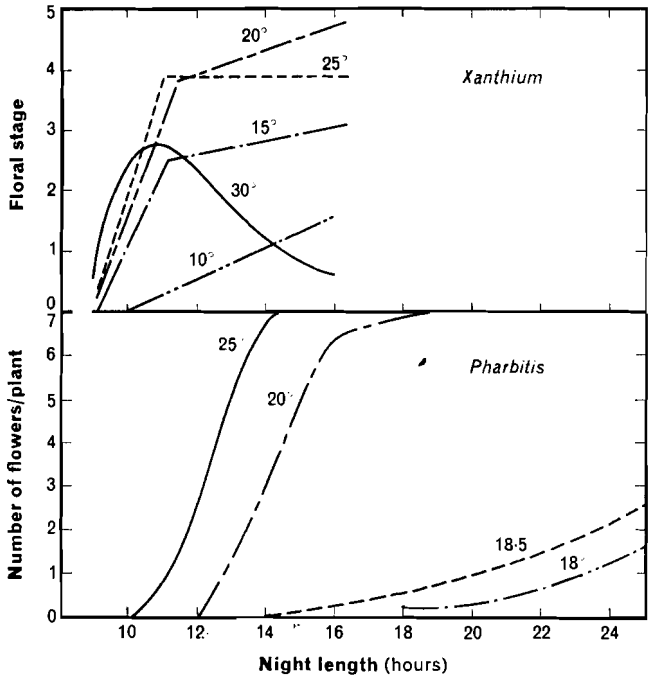


Figure 7 Responses of *Xanthium* (cocklebur) and *Pharbitis* (Japanese morning glory) to nights of different lengths applied at different temperatures [3]. (*Pharbitis* data from Hamner and Takimoto, personal communication.)

to initiate flowering there. In conjunction with these experiments by K. C. Hamner and J. Bonner [18], and by M. K. Chailakhyan [19], who called the hypothetical hormone florigen, it was also shown that the flowering stimulus could be translocated from one plant to another across a graft union. More recently, experiments have been performed in which the rate of movement of the flowering hormone from the leaves of certain plants can be measured, by removing these leaves at various times after a single inductive dark period.

This situation has, however, also become somewhat more complicated. Inhibiting compounds have been implicated in many cases rather than a hormone promoting flowering, and the interaction of these compounds with promoting compounds may well be the rule [5].

The most direct way to study the flowering hormone would be to extract it from the plant, analyse its chemical structure, and study its synthesis. To do this, however, we must be able to detect it. The only method available is that of applying a suspected substance to a vegetative plant, and causing it to flower. So far success in this kind of experiment has been meagre.

In 1961, R. G. Lincoln, D. L. Mayfield, and A. Cunningham [20] claimed success. They extracted cocklebur plants with alcohol at very low temperatures. Their results are not spectacular, in that the extracts produce only very early stages of flower development, but have been substantiated by D. J. Carr [21] in Belfast. The active fraction of these extracts is acidic, and Lincoln named his active substance florigenic acid [22].

It was shown by A. Lang [23] that certain plants that normally require low temperatures or long days for flowering would flower at high temperatures or short days when they were treated with gibberellins. How this relates to the overall problem of the physiology of flowering remains to be seen. In one very real sense, these compounds are the nearest to flowering hormones that have been extracted from a higher plant. They can be extracted from flowering long-day plants, or cold-requiring plants, in quantities sufficient to cause similar plants to flower under non-inductive conditions.

In spite of this, the gibberellins are probably not the postulated florigen [24]. For example, they will not remove the need for a short day. However, an applied compound which mimics the effect of the flowering hormone in some special way, or even leads to its synthesis in the plant, could be of tremendous commercial importance even if it did not solve the basic questions.

Because of the difficulties involved in extraction, we have taken a different approach [25]. We have searched for compounds that, when applied to the intact plant, inhibit the flowering process. Knowing how these compounds work in other biological systems, we have drawn some conclusions about the biochemistry of flowering. Our approach has involved three essential steps. In the first, a survey is made by applying potential inhibitors over a range of concentrations. Compounds which are found to be effective are then studied in a series of experiments designed to pinpoint the stage of the flowering process that they inhibit. For example, they are applied at various times before, during, and after a single inductive cycle (figure 3). Finally, if the compound which we are studying is an antimetabolite, then we attempt to reverse its inhibitory effect by simultaneously applying the suspected metabolite in solutions of increasing strength (figure 9). The results of these studies have also become increasingly complex over the years, but a few conclusions [3, 26] may be of interest.

Biosynthesis of flowering hormone. Dinitrophenol inhibits the formation of the flowering hormone. This suggests that respiration, and its product ATP, are required, as might be expected. At least two anti-amino acids (ethionine and *p*-fluorophenylalanine) inhibit hormone synthesis in cocklebur, and both are reversed by their corresponding metabolites (methionine and phenylalanine). A number of other anti-amino acids fail to have any inhibitory effects. Certain compounds that inhibit the formation of nucleic acids have also been effective in these experiments [27–29]. Some of these are at least partially reversed by their corresponding metabolites, but there is a very perplexing complication. Some are

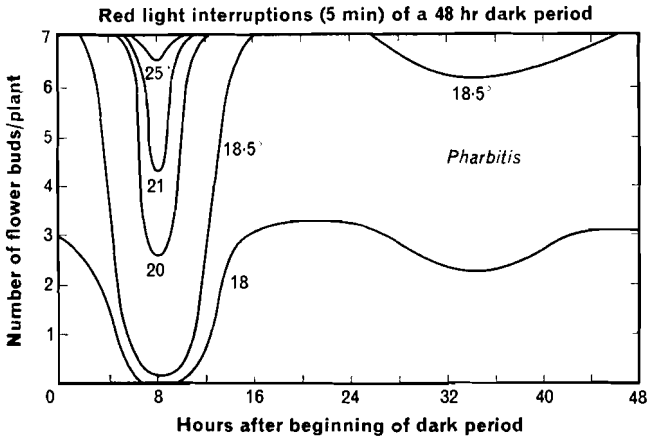


Figure 8 In spite of the strong effects of temperature upon critical dark period in *Pharbitis* (Japanese morning glory), time measurement is still seen to be independent of temperature. (Data from Hamner and Takimoto, personal communication.)

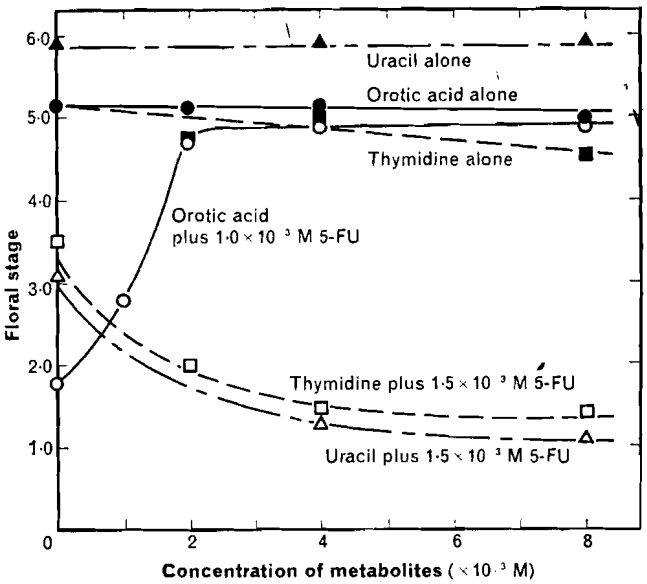


Figure 9 Reversal of 5-fluorouracil (5-FU) inhibition by application of orotic acid. Thymidine and uracil fail to reverse (they appear to enhance somewhat) the inhibition due to 5-fluorouracil [3].

much more powerful inhibitors of flowering when they are applied to the bud than when they are applied to the leaf. Their effectiveness in the leaf however, can also be demonstrated.

Tris (2-diethylaminoethyl) phosphate, an antisteroid compound, also inhibits flowering [28]. It would be exciting to think that the flowering hormone might be a steroid, since many animal hormones are steroids. Unfortunately, attempts to reverse this inhibition have failed, and the inhibition itself occurs only very early in the dark period, some hours before hormone synthesis has begun. Its effect could be upon some early precursor, rather than upon the final steps of hormone synthesis.

Translocation of the hormone from the leaf to the bud. Plant stem-growth hormones, auxins, as well as certain compounds inhibiting the formation of nucleic acids, inhibit the flowering process when they are applied after hormone synthesis is complete, but before the hormone has arrived at the bud. The meaning of these observations eludes us.

Transformation and development of the bud. Certain herbicides will stop the development of the bud at any time. In addition, it has been shown that a nucleic-acid inhibiting compound (5-fluorodeoxyuraidine) that inhibits cell division (as demonstrated for the Japanese morning glory) also inhibits flowering, provided that it is allowed to act during the time when the flowering hormone arrives at the meristematic cells in the bud [29]. This shows that the nucleic acid synthesis of cell division is required if the flowering hormone is to act.

The response of the meristematic cells to the hormone responsible for flowering

Morphologically, the transition cells just below the central mother cell zone are the first ones to become active upon arrival of the flowering hormone (figure 1). Perhaps the best morphological description comes from investigators in Paris, and in Liège [30]. They recognize, for example, a series of phases from the developing embryo in the seed to the final developing flower.

At the histochemical level, it can be shown [31] that protein and ribonucleic acid synthesis increases sharply upon arrival of the flowering hormone, as though certain genes in control of this synthesis were being activated. Certain basic proteins called histones are especially prevalent in the cytoplasm during transformation, and this could be related to the gene activation. Starch grains disappear shortly after transformation. The electron microscope [32] reveals a striking increase in the number of ribosomes, concurrently with transformation. The activity of the Golgi apparatus also increases.

References

[1] Hillman, W. S. 'The Physiology of Flowering'. Holt, Rinehart, and Winston, New York. 1962.
[2] Lockhart, J. A. *Handb. PflPhysiol.*, **16**, 390, 1961.
[3] Salisbury, F. B. 'The Flowering Process'. Pergamon Press, Oxford. 1963.
[4] 'Symposium on Flower Induction'. *Abstr. 10th Int. Bot. Congr.*, 187, 1964.
[5] Zeevaart, J. A. D. *Science*, **137**, 723, 1962.
[6] Garner, W. W. and Allard, H. A. *J. agric. Res.*, **18**, 553, 1920.
[7] Borthwick, H. A., Hendricks, S. B., Parker, M. W., Toole, E. H., and Toole, V. K. *Proc. nat. Acad. Sci., Wash.*, **38**, 662, 1952.
[8] Borthwick, H. A., Hendricks, S. B., and Parker, M. W. *Ibid.*, **38**, 929, 1952.
[9] Downs, R. J. *Plant Physiol.*, **31**, 279, 1956.
[10] Butler, W. L., Norris, K. H., Siegelman, H. W., and Hendricks, S. B. *Proc. nat. Acad. Sci., Wash.*, **45**, 1703, 1959.
[11] Hendricks, S. B. and Borthwick, H. A. 'Environmental Control of Plant Growth' (edited by L. T. Evans), p. 233. Academic Press, New York. 1963.
[12] Hamner, K. C. *Ibid.*, p. 215.
[13] Borthwick, H. A. and Downs, R. J. *Bot. Gaz.* (In press.)
[14] Meijer, G. *Acta bot. néerl.*, **8**, 189, 1959.
[15] Könitz, W. *Planta*, **51**, 1, 1958.
[16] Kofranek, Anton M. and Sachs, R. M. *Amer. J. Bot.*, **51**, (5),520, 1964.
[17] Bünning, E. 'Die physiologische Uhr'. Springer-Verlag, Berlin. 1963.
[18] Hamner, K. C. and Bonner, J. *Bot. Gaz.*, **100**, 388, 1938.
[19] Chailakhyan, M. K. *Dokl. Akad. Nauk, SSSR*, **3**, 442, 1936.
[20] Lincoln, R. G., Mayfield, D. L., and Cunningham, A. *Science*, **133**, 756, 1961.
[21] Carr, D. J. *Commun. 10th Int. Bot. Congr.* 1964.
[22] Lincoln, R. G. *Abstr. 10th Int. Bot. Congr.*, 189, 1964.
[23] Lang, A. *Proc. nat. Acad. Sci., Wash.*, **43**, 709, 1957.
[24] Lang, A. and Reinhard, E. *Adv. Chem. Ser.*, **28**, 71, 1961.
[25] Salisbury, F. B. 'Recent Advances in Botany', p. 1294. University of Toronto Press, Toronto. 1961.
[26] Collins, W. T., Salisbury, F. B., and Ross, C. *Planta*, **60**, 131, 1963.
[27] Salisbury, F. B. and Eichhorn, G. L. *Ibid.*, **60**, 145, 1963.
[28] Bonner, J., Heftmann, E., and Zeevaart, J. A. D. *Plant Physiol.*, **38**, 81, 1962.
[29] Zeevaart, J. A. D. *Ibid.*, **37**, 296, 1962.
[30] Bernier, G., Nougarède, A., Bronchart, R., and Rondet, P. *Rev. gén. Bot.* (In press.)
[31] Gifford, E. M. and Lance-Nougarède, A. *Abstr. 10th Int. Bot. Congr.*, 293, 1964.
[32] Jensen, W. and Healey, P. *Commun. 10th Int. Bot. Congr.*, 1964.