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Time Measurement In The Photoperiodic Control Of Flowering

Roderick Whitfield King

TIME MEASUREMENT

IN THE

PHOTOPERIODIC CONTROL OF FLOWERING

by

Roderick Whitfield King

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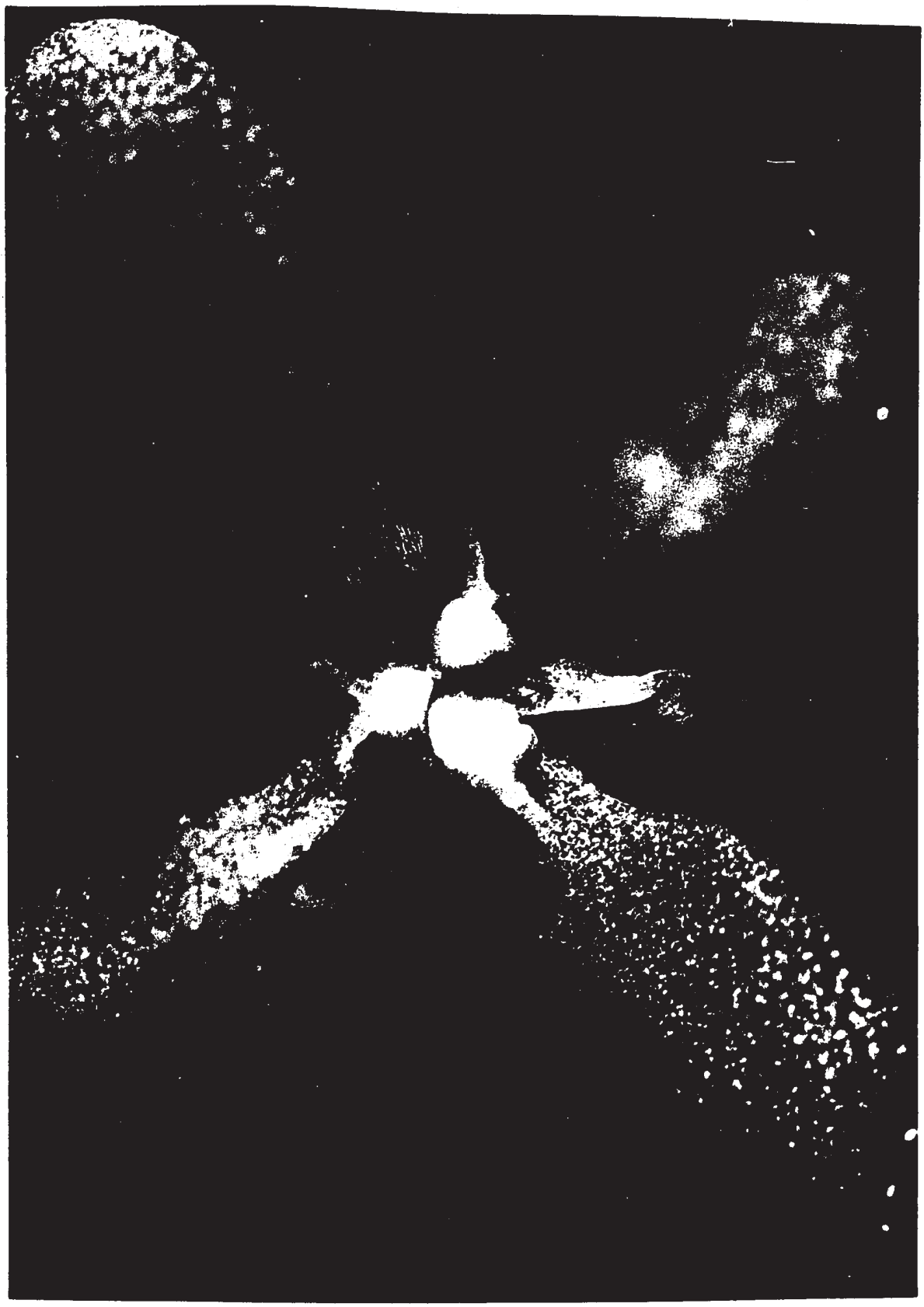


Why this absurd concern with
clocks, my friend?
Walter de la Mare
The Winged Chariot

ABSTRACT

The experiments presented in this thesis relate to the action of dual "clocks" - rhythmic and an hourglass timer - that are shown to be involved in photoperiodic time measurement in the control of flowering of Chenopodium rubrum.

A most important determinant of the photoperiodic response of C. rubrum is the regular oscillation this plant undergoes in darkness in its capacity to flower (i.e. sensitivity to light terminating darkness). The period length of this rhythm was 30 hours (Chapter II). Daily photoperiodic cycles control the phasing and possibly the period length of this rhythm so that in photoperiodic cycles the time of greatest capacity to flower may occur at different times of the day. Only photoperiods of 6, 12 or 18 hours were studied extensively and two methods were applied in assessing rhythm phasing (Chapter III). Phasing and entrainment of the rhythm in repeated photoperiodic cycles was calculated from the phase response curve of the rhythm to a single photoperiodic interruption. Also, rhythm phasing was determined by progressively increasing the number of photoperiodic cycles given prior to a single dark period.

For a daily 6 hour photoperiod rhythm phase was controlled by an interaction with the timing of both the light-off and light-on signals of the photoperiod. Daily rhythm phase advance and delay reached an equilibrium in relation to the timing of the daily photoperiodic cycle after 3 to 4 cycles. The calculated or observed rhythm peak times fell 18 to 23 hours after the last light-off signal of the photoperiod. For 12 or 18 hour photoperiods only the light-off signal of the photoperiod determined

rhythm phasing. Thus the rhythm peak fell 10 to 13 hours after the daily light-off signal of the photoperiod. The relationship established between photoperiod duration and rhythm phasing accounted for the photoperiodic flowering response of this plant. Hence, rhythms are effective in photoperiodic time measurement in the control of flowering.

Phytochrome is probably the photoreceptor pigment controlling phasing of the rhythm of flowering by daily photoperiodic cycles. This point was established by examining the influence of light quality on rephasing of the rhythm of flowering by a 6 or 12 hour light period (Chapter IV). Quite low intensities of red irradiation (115 uw/cm^2) gave rephasing identical to that obtained with a higher intensity of fluorescent light (1037 uw/cm^2). Furthermore, provided they were spread evenly over the 6 or 12 hour period, 5 minute pulses of red light could induce rephasing equivalent to that following 6 or 12 hours of continuous red irradiation. However, far-red light if given immediately following the red light pulse reduced its effectiveness for phase shifting of the rhythm. These responses are characteristic of phytochrome action.

Less direct effects of the environment on floral development must also be considered. In part, the supply of photosynthates could limit flowering in very short daily photoperiods. This conclusion is supported by the observation that the amount of flowering increased with higher light intensities given after photoperiodic induction. Flowering was also enhanced by supplying sugars before or after induction. This requirement for photosynthate was related to limitations imposed at the apex during floral development (Chapters II and VI) and hence did not reflect a direct relationship to the action of a photoperiodic clock.

Early responses to an inductive dark period include the production and export from the cotyledons of a stimulus to flowering. If the

cotyledons were removed at any time during the 7 hours after a 13 hour dark period, little or no flowering resulted. Delaying cotyledon removal a further 9 hours resulted in almost maximal flowering. By this time the cotyledons had fulfilled their essential role for flowering . . . the production and export of the floral stimulus. Soon after export of the stimulus the mitotic index at the apex reached a value double that of vegetative plants held in continuous light or given a non-inductive dark period (Chapter VI).

Thermo- and photoperiodic treatments prior to an inductive dark period induced rhythmicity in both cell division at the apex and in the capacity of the seedlings to respond to an inductive dark period. The period lengths of the rhythm of mitosis and in capacity to flower were similar (20 to 24 hours). As cell division is an integral and essential part of floral development the correlations between rhythmic cell division and the capacity to flower suggested a causal relationship. A rhythmic limitation to flowering by cell division would therefore provide a timing function that is superimposed at the apex and limits the expression of floral induction.

Further evidence for rhythmicity at the apex during floral development was obtained by varying the time of application to seedlings of solutions of glucose, gibberellic acid or alcohol. The sensitivity of flowering to the time of application of the solutions changed rhythmically. The period length of the rhythm was about 20 hours. For glucose solutions at least, rhythmic sensitivity was maintained long after export of the floral stimulus from the cotyledons and was observed even if the cotyledons had been removed following export of the floral stimulus.

Rhythmic responses at the apex were distinct from the rhythm of floral induction. The latter rhythm maintained a period length of 30

hours and was present whether or not seedlings had been grown previously in constant or fluctuating temperature conditions. It appears therefore that an heirarchy of independent but interacting rhythms must be accepted in any model of the photoperiodic control of flowering.

While not in conflict with the evidence of a rhythmic photoperiodic timer, the results presented in Chapter V indicated phytochrome dark reversion could influence time measurement during a daily dark period. In these experiments the proportion of phytochrome P_{fr} present in the cotyledons at different times in darkness was estimated by irradiating different groups of plants once with light of various spectral compositions. The proportions of red radiation in the interruption which had no effect on the flowering response was taken as an indication of the proportion of phytochrome in the P_{fr} form at the time of the interruption.

These experiments showed that the proportion of P_{fr} remained high over the first hours of darkness but, P_{fr} reverted rapidly by the fourth hour. Reintroduction of P_{fr} after its reversion partially delayed the timing of the first peak of the rhythm of flowering but had no influence on the second and third rhythm peaks. After 8 to 10 hours of darkness P_{fr} may have begun to reappear.

If the level of P_{fr} was set high at the start of darkness more P_{fr} could be tolerated later in darkness. When most of the phytochrome was initially set in the P_r form P_{fr} reappeared gradually until reversion occurred. The tolerance to P_{fr} introduced later in darkness was now reduced.

Not only is P_{fr} normally present during the light period but it is also acting over prolonged periods during the photoperiod. If a subsequent dark period was to be maximally inductive the presence of P_{fr} had to be maintained during the light period. Far-red radiation replacing

red radiation during the photoperiod reduced flowering. Brief red irradiations reversed the effect of brief far-red light pulses but a far-red irradiation of an hour or more was only reversed by 2 to 3 hours of red radiation. Because the presence of P_{fr} was required over prolonged periods of time and immediately prior to darkness it is suggested that the light period P_{fr} requiring process interacted with dark period phytochrome action. This suggestion is substantiated by the observation that the tolerance to P_{fr} introduced during darkness was greater if high levels of P_{fr} were maintained during the photoperiod.

Rhythm phasing was not influenced by any of the treatments that changed the levels of P_{fr} during the light period or during darkness. Thus interactions between the high P_{fr} reaction of the light period and a low P_{fr} reaction associated with P_{fr} reversion in darkness could provide time measuring limitations to the photoperiodic response of C. rubrum.

It is suggested on the basis of timing responses to phytochrome and to the rhythmic "clock" that a rhythm is the dominant photoperiodic timer in C. rubrum. On the other hand, in Pharbitis nil an hourglass timer, alone, can be demonstrated or rhythms can also be introduced into its flowering response. It appears therefore that the dominance of rhythmic or hourglass clocks might vary with the species and its preconditioning. The possible interactions between these dual timers is discussed in Chapter VII.

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CHAPTER I

REVIEW OF THE LITERATURE.

I. INTRODUCTION.

Many, if not all, animals and plants can accurately measure time on a daily and seasonal basis. Some organisms also show period measurement with tidal and lunar frequencies, and celestial navigation of animals appears to involve some sort of clock or timer mechanism. As an adaptation for survival it is clearly important that plants regulate their developmental responses, such as flowering or dormancy, according to the season. Likewise, insects have synchronized the timing of their growth and diapause to coincide with environmental factors such as the availability of host plants or the seasonal cycle of temperature. For birds, survival often depends on migration well in advance of the onset of unfavourable climatic conditions. It appears, therefore, that during their evolution organisms have developed timers that accurately utilize the available environmental information. But, what are these "clocks" and how do they operate?

a) Historical aspects.

Before 1920 investigators of biological periodism were unable to establish any clear relationships between developmental responses and variation of environmental factors such as nutrition, temperature or light intensity. In the control of flowering, Klebs (1918) and Kraus and Kraybill (1918) emphasized the importance of the carbohydrate / nitrogen ratio in the plant. Gassner (1918) established the importance of a period of low temperature - later called vernalization - in controlling

the reproductive development of some winter annual plants. Neither of these factors, however, provided the accuracy required for the seasonal control of development, nor were they generally involved in the control of flowering of all plant species.

Meanwhile, others had begun to realize the importance of daylength in the control of flowering. In 1914 Tournois, in a rather neglected paper, first suggested that daylength might be the controlling factor in precocious flowering of hops. Later, in a more thorough analysis using a wide array of plants Garner and Allard (1920) clearly demonstrated daylength control of flowering. Their work stemmed from two earlier observations that they made. Firstly, an odd strain of tobacco (Maryland Mammoth) grew to an extraordinary height (5 - 10 feet) without flowering when grown in a glasshouse at Washington D.C. during the summer; however, it flowered, produced seed and showed no signs of gigantism when grown during the winter. Secondly, it was observed that after serial sowings of soybean in the field during the summer, all plants produced blossum at about the same date irrespective of the date of sowing.

To explain these observations Garner and Allard reasoned that the controlling factor could be the length of the daily photoperiod. They examined this proposition by using a dark chamber to shorten the length of the daily period of exposure to the sun. Maryland Mammoth tobacco, soybean and a number of other species flowered with the shorter daily photoperiod while plants held in the long days of summer did not flower. Those plants which flowered in shorter photoperiods were designated short-day plants (SDP) while those which flowered only in the longer photoperiods they designated as long-day plants (LDP). In discussing these results they introduced the terms "photoperiodism"

for the response of an organism to the relative length of day and night and "photoperiod" for the length of the daily light period; their definition of photoperiod is adhered to in this thesis and the term "photoperiodic cycles" is used to denote combinations of light and darkness of any total duration. If the duration of a photoperiodic cycle is 24 hours it may be referred to as a daily photoperiodic cycle and the term daily photoperiod necessarily infers a photoperiod given in combination with a dark period, so that the total duration of light and darkness is 24 hours.

Although Garner and Allard suggested that "the biologist who attempts to draw sweeping generalizations regarding responses of plants or animals as a whole to conditions of the environment is in serious danger of going astray," they predicted with remarkable perception that photoperiodism would be important in the geographic distribution of plants, in crop yields, the seasonal behaviour of algae and the migration of birds. In fact, it was only three years after Garner and Allard announced their discovery that Marcovitch (1923) showed daylength control of sexuality in aphids and, in 1925 Rowan (1926) commenced a series of experiments which were ultimately to demonstrate that bird migration was a photoperiodic phenomenon.

In plants photoperiodic responses have been observed in such diverse phenomena as flowering, tuberization, bulb formation, bolting, senescence and autumn leaf fall, stomatal movement, seed germination, leaf movement and growth (see Garner and Allard, 1923).

b. Basic requirements for biological time measurement.

Every major group of organisms has evolved the ability to use the daily progression of daylength as a source of environmental information - see articles in Withrow (1959) and Aschoff (1965)

and reviews by Bunning (1967), Cumming and Wagner (1968) and Sweeney (1969a) Furthermore, not only is biological time measurement universal, it is also extremely accurate. Withrow (1959) computed that organisms growing in temperate zones must be able to measure daylength with a precision of 1-3% if they are to measure seasonal time to one week and with an accuracy of 4-12% for measuring seasonal time to one month. At lower latitudes or during mid-summer the smaller absolute changes and the slower rates of change of daylength make precision of time measurement even more crucial. Such a degree of accuracy is evident in the photoperiodic flowering response of many temperate zone plants which may respond to differences in daylength of as little as 15 - 30 minutes per day (cf. review by Lang, 1965).

On the other hand, it might be expected that in the tropics where daylength changes so little, evolutionary selection would be for photoperiod insensitivity. However, evidence of seasonal alternations in the capacity of rice to flower (Dore, 1959) and in leaf fall of Plumeria acuminata (Murashige, 1966) suggest that these species may be able to detect seasonal changes of daylength of less than 15 minutes. Certainly, changing the daily photoperiod from 11 hours 50 minutes to 12 hours delayed flowering of rice by 30 to 50 days (Dore, 1959). Thus, some tropical plants may be extremely sensitive to photoperiod.

Photoperiodic time measurement may also require some sort of photoreceptor to act as a couple between the environment and the timer in the organism. Preferably there should be a low saturation energy for the photoreceptor so that daily and seasonal fluctuations in light intensity will be of little importance. Then massive energy transfer via the photoreceptor will be quite unnecessary.

Finally, as a corollary to the need for accurate measurement

of photoperiod the timer should be rather insensitive to other environmental factors. In both plants and animals, photoperiodic response is often little affected by temperatures between $10^{\circ}C$ - $30^{\circ}C$ or by nutrition (see Bunning, 1967).

c. Concepts of the timer.

Historically, the tenor of arguments concerning the type of clock measuring daily time in plants can be seen in the pendulum-like swing of emphasis from Garner and Allard's original definition of photoperiodism which stressed the effect of the light period (Garner and Allard, 1923), to evidence that the length of the dark period may be more important (Hamner and Bonner, 1938), and more recently to an emphasis on the separate nature of the "photo" and "periodic" aspects of photoperiodism. In association with these changes of viewpoint two supposedly opposed concepts of time measurement have developed. Borthwick, Hendricks and their associates (see Hendricks, 1960) suggested that the plant pigment phytochrome acted in time measurement in a manner that can be compared to the action of an hourglass. As if inverting an hourglass, the daily transition to darkness initiated phytochrome reversion from the active to the inactive form in darkness. Then on the completion of dark reversion and hence of time measurement, biochemical processes that led to flowering could commence. The second explanation of time measurement in the photoperiodic control of flowering was advanced by Bunning (1936) and has been supported by a number of others more recently. These workers have separated the "photo" and "periodic" aspects of the daily light : dark cycle and have related timing to the phasing of an innate, free running, circadian oscillation.

d. A general outline of the approach taken to the question of photoperiodic time measurement.

The following discussion of the literature attempts to analyse time measurement in the photoperiodic control of flowering with respect to the concepts of an hourglass and a rhythmic timer and each chapter of experimental results in turn is concerned with one aspect of this problem. In Chapter II a general description is given of the photoperiodic control of flowering of Chenopodium rubrum and some data are introduced to show the effect on flowering of light : dark cycles that diverge from a 24 hour period. Such results highlight the difficulties associated with relating observations of a rhythm to the specific question of rhythmic time measurement under photoperiodic treatments. In order to approach this problem in another way, the actual phasing by photoperiodic treatment, of the rhythm of flowering of C. rubrum is illustrated in Chapter III. Sensitivity of rhythm phase to the timing and duration of the photoperiod also indicates the involvement of a photoreceptor and this question is examined in Chapter IV. While not in conflict with the evidence of a rhythmic "clock" the results presented in Chapter V indicate timing might sometimes be limited by phytochrome dark reversion and, even under conditions in which phytochrome appears to have no influence on timing, it is shown to have significant effects on the amount of flowering. It is suggested in the general discussion that the concepts of rhythmic and hourglass control of the photoperiodic response might not be mutually exclusive.

Probably the least clearly defined question concerns the site of localization of "clocks" in a plant. Is the "clock" a property of the plant as a whole or of specific organs and tissues acting separately?

The results outlined in Chapter VIindicate that, for flowering, the photoperiodic sensor is possibly the leaf although the responding organ, the apex, may also function rhythmically and hence the involvement of an heirarchy of "clocks" may be required in models of the photoperiodic induction of flowering.

II. CIRCADIAN RHYTHMS AS BIOLOGICAL CLOCKS.

a. Nature of a rhythm and some definitions.

The term circadian rhythm is used to refer to regular oscillations which show a period length of about (circa) a day (diem). A circadian rhythm is distinct from diurnal environmentally induced changes since the latter do not persist in the absence of environmental fluctuations whereas circadian rhythms continue under constant conditions, e.g. continuous light (LL) or darkness (DD) at a constant temperature. Under constant conditions the rhythm is not forced (entrained) by external synchronization and hence it is said to be free running. In fluctuating environmental conditions rhythm period length may be entrained, for instance by light :dark cycles (L : $\overline{D}$) and rhythm phase is also controlled by L : $\overline{D}$ cycles. The phase of the rhythm refers to the instantaneous state of the oscillation within a period. Phase shifting or resetting refers to displacement of an oscillation along the time axis. Further definitions are given by Aschoff et al (1965).

Table 1 lists some of the rhythms observed in plants and animals. No attempt has been made to be all-inclusive in this Table nor has historical priority for an observation been considered. Cumming and Wagner (1968) have presented a detailed and up to date survey of the occurrence of circadian rhythms in plants.

It is clear (Table 1) that many properties of cells and

TABLE 1.

Some circadian rhythmic phenomena in plants.

PHENOMENON	SPECIES	REFERENCE
Bioluminescence	*Gonyaulax polyedra*	Hastings and Sweeney (1959)
Cell division	*Gonyaulax polyedra*	Hastings and Sweeney (1959)
Flower induction		
SDP	*Chenopodium rubrum*	Cumming *et al* (1965)
	Glycine max	Hamner (1960)
	Kalanchoe blossfeldiana	Carr (1952), Melchers (1956), Engelmann (1960)
	Lemna perpusilla	Hillman (1964a)
	Pharbitis nil	Takimoto and Hamner (1964)
	Xanthium pensylvanicum	Mitchell (1964)
		Chorney *et al* (1970)
LDP	*Arabidopsis thaliana*	Clauss and Rau (1956)
	Hyoscyamus niger	Claes and Lang (1947)
		Hsu and Hamner (1967)
	Lemna gibba	Nakashima (1966, 1968)
	Silene armeria	Hamner (1960)
Fungal zonation	*Neurospora crassa*	Sargent and Briggs (1967)
Gas exchange		
Light: O_2 evolution	*Gonyaulax polyedra*	Sweeney (1960)
	Acetabularia mediterranea	Schweiger *et al* (1964)
Light: CO_2 fixation	*Bryophyllum* sp.	Jones and Mansfield (1970)
Dark: CO_2 evolution	*Kalanchoe fedtschenkoi*	Wilkins (1960)
Leaf movement	*Phaseolus multiflorus*	Bunning (1967)
	Phaseolus vulgaris	Hoshizaki and Hamner (1964)
	Albizzia nana compacta	Pfeffer (1915)
	Coleus sp.	Halaban (1968a)
Petal movement	*Kalanchoe blossfeldiana*	Bunsow (1960)
Phototaxis	*Euglena gracilis*	Pittendrigh and Bruce (1959)
Pigment accumulation		
chlorophyll	*Chenopodium rubrum*	Wagner and Cumming (1970)
beta cyanin	ecotype 50°10'N 105°35'W	

organisms show persistent oscillations under constant conditions. The period lengths of these rhythms generally fall between 20 and 30 hours and the oscillations will often be sustained for many cycles in constant conditions. For instance, leaf movements in Phaseolus vulgaris have been observed to continue for at least 28 days in LL of 950 ft-c with no damping of the rhythm (Hoshizaki and Hamner, 1964); rhythmic changes in oxygen evolution of Acetabularia mediterranea persist for at least 40 days in LL of 250 ft-c (Schweiger et al, 1964) and, in the alga Gonyaulax polyedra, the rhythm of stimulated luminescence persists in cells kept in LL of low intensity for three months (Sweeney, 1969a). That rhythms persist and that their period remains constant, despite a lack of evident environmental synchronization provides strong support for their innately endogenous nature.

In plants, probably the most widely studied rhythm has been that of leaf and petal movement and period lengths in the more than 40 species studied (see Cumming and Wagner, 1968; Sweeney, 1969a) are all circadian. The results of a genetic cross between two strains of Phaseolus multiflorus that differed in the length of the period of their leaf movement rhythm suggested inheritance of period length though not on a simple Mendelian basis (Bunning, 1935). Temperature has only a slight influence on rhythm period length (cf. Bunning, 1967) and period length may also vary with the intensity of constant illumination (see Aschoff, 1960). Lorcher (1958) reported that light quality was also important; the period length of the leaf movement rhythm of Phaseolus multiflorus varied from 26.3 hours in constant darkness to 28.1 hours in red radiation (610 -690 nm) to 24.7 hours in far-red radiation (690 -850 nm).

As a general rule the period of a rhythm may be forced or entrained to match exactly the period of some external forcing

oscillation (Bunning, 1967). In most cases, on termination of the entraining cycle the period reverts to its natural circadian value. Light and temperature are probably the most effective environmental factors controlling rhythm entrainment and, under entrainment by daily light : dark cycles, a circadian rhythm will adopt a period of exactly 24 hours.

The failure of an oscillation to persist under constant conditions was advanced earlier as a distinction between exogenous and truly endogenous oscillations (see Pittendrigh, 1960). However, some workers have questioned whether truly constant conditions have ever been achieved experimentally (see Brown, 1965) as daily fluctuations have been observed in subtle geophysical factors such as electrostatic fields, magnetic and gamma radiation and the solar radio flux. Therefore, an apparently endogenous rhythm may in reality reflect forcing by exogenous oscillations. This theory has met considerable opposition in the past (see discussion by Sweeney, 1969a) but is currently supported by experiments performed by a number of workers including Brown et al, (1966) and Dowse and Palmer (1969). Whatever the answer to this question of the nature of the rhythm there remains no doubt about the importance and effectiveness of light in rhythm phasing and entrainment. Thus an understanding of the effect of a photoperiod on a circadian rhythm is not prejudiced by the position adopted regarding its endogenous or exogenous nature.

b. Biochemical aspects of rhythms.

From the diversity of rhythmic display in plants and animals, it would seem that a biochemical understanding of the "clock" will reveal processes fundamental to cellular physiology and biochemistry. Descriptions of processes which show oscillations - e.g. Table 1 - have

not, to date, answered the question of what mechanism underlies circadian rhythmicity. An alternate approach depends on blocking or interfering with metabolism in the hope of finding a compound showing specific effects on the clock alone. However, many metabolites, plant and animal hormones, inhibitors and antimetabolites have been applied with little effect on the phase and hence timing of the rhythm. In fact of more than 50 reports in the literature of different compounds tested, only a few have had any effect. Application of D_2O as concentrated as 50% may lengthen the period in Euglena (Bruce and Pittendrigh, 1960), in Phaseolus (Bunning and Baltes, 1963) and in mice (Suter and Rawson, 1968). Ethanol (2.5%) also lengthened the period of the leaf movement rhythm in Phaseolus (Bunning and Baltes, 1962). Potassium cyanide, an inhibitor of respiratory metabolism induced phase shifts in the rhythm of petal opening in Kalanchoe (Steinheil, 1970) and anaerobic conditions may induce phase shifts of a rhythm (Wilkins, 1967). Finally, cycloheximide, an inhibitor of protein synthesis lengthened the period of the rhythm of phototaxis in Euglena (Feldman, 1967). Apart from the experiments with D_2O, for each response to a compound there has been at least one negative experiment with another organism and, even within one organism, closely related inhibitors may fail to show similar control. Therefore, to theorize on this basis is premature and it is not surprising that the theory which attributed circadian oscillations to periodic transcription of the nuclear genetic message (e.g. Ehret and Trucco, 1967) cannot simply explain the observation of Schweiger et al. (1964) of rhythmic photosynthesis continuing for at least 40 days in enucleated Acetabularia mediterranea.

An early suggestion of Bunning (e.g. 1952a) of distinct catabolic and anabolic phases has also proved to be too simple "as have

all other analogous explanations based on simple metabolic processes" (Bunning, 1967). In a recent comprehensive review, Sweeney (1969a) likewise concluded that the solution to the problem of the mechanism of the cellular clock was not yet apparent. Both Sweeney (1969a) and Bunning (1967) have suggested the possibility of membrane control and Wagner and Cumming (1970) enlarged this concept to include energy transformations in a membrane-bound system, but the validity of these concepts still awaits experimental confirmation.

The foregoing discussion also raises the enigma that detection of a rhythm does not constitute a demonstration of its "clock" function and many rhythmic attributes, including biochemical changes, may themselves be controlled rather than controlling. Even the period length of the controlled rhythm need not be identical to that of the basic "clock" since beat phenomena with a particular period may develop from the interaction of rhythms of different periods.

Despite the absence of a precise "biochemistry" of circadian rhythms, physiological parameters such as flowering, leaf movement or photosynthesis can be used in approaching the question of the involvement of circadian rhythms as clocks in photoperiodic time measurement. The use of overt rhythms as indicators of the clock has shown that the timer is temperature compensated (Q_{10}=0.8 - 1.2), operates continuously and accurately, is very sensitive to photoperiod and is present in a wide variety of plants and animals (cf. Bunning, 1967). Thus circadian rhythms do meet all the requirements of a clock measuring photoperiod duration.

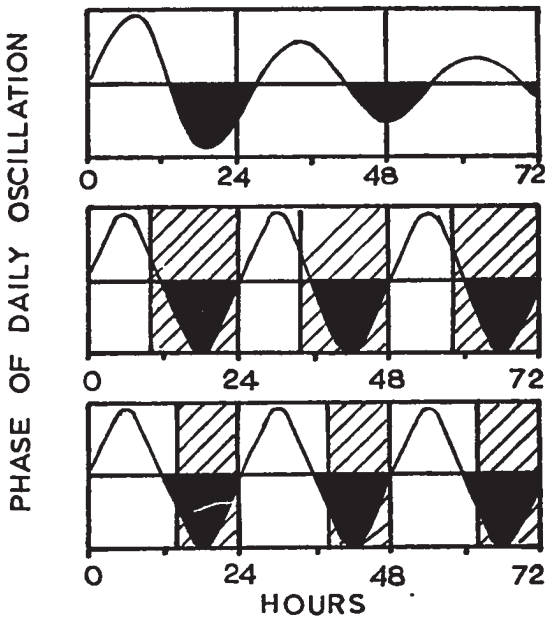
Additional support for rhythms as photoperiod timers comes from the observation that light : dark cycles entrain and phase some

circadian rhythms. Indeed, of all the attributes of an oscillating timer, period entrainment and phase control are most essential for photoperiodic time measurement. Rhythm phasing and entrainment are, in fact, the chief components of Bunning's hypothesis of an endogenous rhythm acting as the timer measuring daylength. In the subsequent discussion, following an introduction to Bunning's hypothesis, the concepts of rhythm phasing are developed.

III. TIME MEASUREMENT IN PHOTOPERIODISM - RHYTHMS AS THE TIMER.

When Bunning first proposed in 1936 that a daily oscillation was causally involved as a timer in photoperiodism, he envisaged an oscillation with a period of about 24 hours that underwent a regular alternation of two half-cycles of about 12 hours duration. In one phase of the cycle - the photophile or light-liking phase - light was not inhibitory but if light impinged on the scotophile or dark-liking phase it was inhibitory. It was proposed that variation in cellular activities of the two half-cycles conferred different light sensitivity on the phases (see Bunning, 1952a). The term "coincidence effect of light" is used subsequently in discussing the effect of light impinging on a rhythm at its different phases.

Under natural conditions Bunning suggested that the scotophile phase commenced 12 hours after the light-on signal of sunrise. Therefore light would coincide with the scotophile phase in the long days of summer but not in the short days of winter. The diagram presented below summarizes the relationship suggested by Bunning (1960) for rhythm phase and the effect on flowering of the timing of coincidence of light on the different phases of the oscillation.



Action as a clock measuring photoperiod duration (hatched areas - darkness) of a circadian rhythm that undergoes cyclical changes in sensitivity to light (darkened portions of the rhythm dark-liking or scotophile phases). After Bunning (1960).

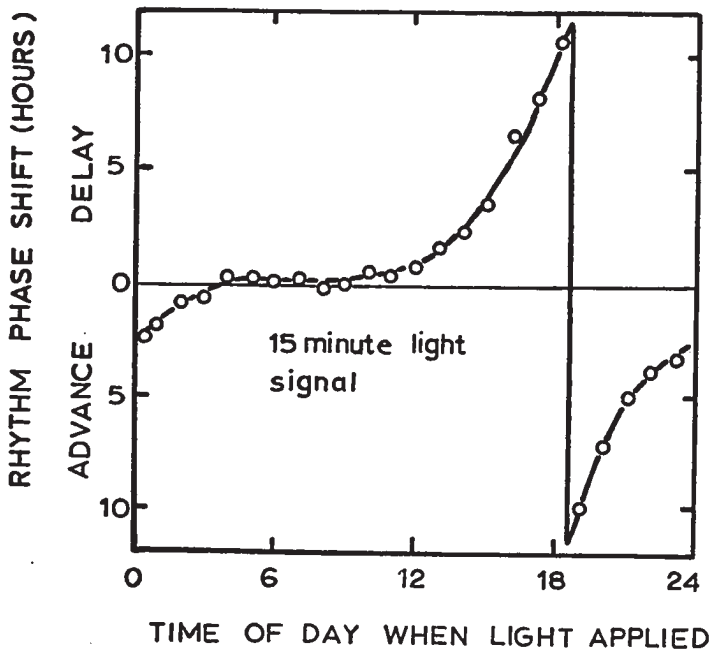
For short-day plants coincidence of light on the scotophile phase would prevent flowering as shown first by Hamner and Bonner (1938). In their experiments a one minute light interruption in the middle of a 9 hour dark period completely annulled the inductive effect of that dark period for the flowering of the short-day plant Xanthium pensylvanicum. As also predicted by Bunning's hypothesis the same treatment involving a short light interruption during a long dark period had the opposite effect i.e. it induced flowering in long-day plants (Claes and Lang, 1947; Borthwick et al, 1948). Currently there is an abundance of evidence indicating opposite responses of flowering of long-day and short-day plants to light interruptions of darkness (see Hillman, 1962; Lang, 1965). However, this approach generally has not resolved whether the differences between photoperiodic response types depends on rhythm phasing or on the type of metabolic coupling.

Originally to explain how light promoted rather than inhibited flowering in long-day plants, Bunning postulated that the two half cycles of the oscillation were 12 hours out of phase for long-day plants as compared to short-day plants. However, at least by 1959, and as shown in the diagram, Bunning maintained that phasing need not be different in the two response types, but that "light offered during the light sensitive phase may be involved in two different reactions . . . the light sensitivity of these two reactions being different in long-day and short-day plants." Recently, experiments of Cumming et al (1965) provide substantial support for the latter concept . . . that the sensitivity to light changes rhythmically.

In addition to the coincidence effect of light Bunning's hypothesis requires that the photoperiod controls rhythm phasing. Therefore to understand how a rhythm measures the duration of a photoperiodic cycle it is important to know how the photoperiod controls the phase of the rhythmic sensitivity to light. In answering this question studies of rhythm rephasing by a single light exposure have been of considerable significance. For example, it has been found that the amount and direction of phase displacement induced by a single light stimulus depends on the phase of the oscillation at which the stimulus was applied. At one time a delay of rhythm phase may result and at another, an advance. How rhythm phase responds to a single light period can be shown graphically by plotting the time of the light exposure as a function of the time in the cycle at which the stimulus was given; such curves are called phase response curves.

Detailed phase response curves for the dozen or so organisms - both plants and animals - studied to date are presented in articles by Aschoff (1965), Pittendrigh (1966) and Winfree (1970). As an example,

the figure below presents a phase response curve for the adult emergence rhythm of Drosophila pseudoobscura. The degree and direction of the phase resetting (ordinate) varied with the phase of the cycle when a single 15 minute light exposure commenced (abscissa). Greatest sensitivity for phase advance and delay was observed at the eighteenth



The phase response for Drosophila pseudoobscura adult emergence rhythm. 15 minute signals of 100ft-c fluorescent light given at different times and the steady-state of the phase-shifted rhythm plotted as an advance or delay of rhythm phase. From Pittendrigh and Minis (1964).

hour after the dawn signal and hence in the middle of the scotophile phase of the rhythm. Because sensitivity to phase resetting is not constant over the period of an oscillation, it can be seen that rephasing will only occur when photoperiodic treatments impinge on specific portions of the oscillation. Furthermore, over this period, sensitivity to phase resetting is constantly changing and hence a phase response curve predicts that an equilibrium will be reached at some particular point where successive phase advances and delays balance

each other. These concepts were later substantiated by Pittendrigh and Minis (1964) again from a study of the adult emergence rhythm of Drosophila pseudoobscura. Their results, as discussed below, provide an elegant demonstration of an integrated analysis of light-on, light-off and durational effects of photoperiodic cycles on rhythm phasing and period entrainment.

From the phase response curve of Drosophila, Pittendrigh and Minis (1964) reasoned that a brief (15 minute) light pulse must have affected the phase of the rhythm almost instantaneously and could be used to simulate either a light-on or light-off signal. Therefore, two brief (15 minute) light interruptions that were, for example, 6 hours apart should act as a skeleton 6 hour photoperiod and mimic the light-on, light-off and duration effect of a complete 6 hour photoperiod. Knowing the phase response to each individual light signal and assuming independent action of each new signal on the reset phase of the rhythm, they calculated the expected steady state phasing of the rhythm under repeated skeleton photoperiodic cycles. The calculated and experimentally determined phases of the rhythm in response to skeleton and complete photoperiods showed remarkably good agreement between observation and theory. Skeleton photoperiods of two 15 minute light pulses as much as 12 hours apart simulated a complete photoperiod of 12 hours.

Under the conditions of experimentation used by Pittendrigh and Minis the free running period of the emergence rhythm was 24 hours and 10 minutes. Therefore, entrainment of this rhythm to a 24 hour period by a daily skeleton photoperiod would have had less significance for rhythm phasing than the effect of the photoperiod to repetitively reset rhythm phase. This is not to say that photoperiod cannot entrain rhythm period as Pittendrigh and Minis also demonstrated that skeleton

photoperiods entrained rhythm period to values ranging at least from 21 to 25 hours. Again, the computed net phase shifts described by the phase response curve agreed completely with the observed response to skeleton photoperiods. Thus, for Drosophila at least, these experiments of Pittendrigh and Minis (1964) verified the usefulness of phase response curves. Taking the example of a rhythm with a period of 30 hours as found in C. rubrum, daily photoperiods will clearly act both to entrain rhythm period and to repetitively reset rhythm phase.

While not invalidating the interpretation of Pittendrigh and Minis a rather novel feature arose with skeleton photoperiods longer than 12 hours. The phase of the rhythm jumped to a new position so that a skeleton cycle of $16:\overline{8}$ appeared to mimic a skeleton or complete photoperiod of $8:\overline{16}$. Regardless of which signal had started first (e.g. $8:\overline{16}$ or $16:\overline{8}$) rhythm phasing was determined by the shorter of the two skeleton periods between successive light signals.

Simultaneously with the publication of Pittendrigh's and Minis' s results, Hillman (1964a) presented comparable data for photoperiodic control of flowering of Lemna perpusilla. Under skeleton photoperiods greater than 12 hours in duration, the flowering response showed a phase jump identical to that obtained in Drosophila. Hillman's experiments leave no doubt that photoperiod controls flowering through its effects on phasing and period entrainment of an endogenous rhythm.

b. Phase control of light inhibited rhythms.

The essential feature of the clock in Bunning's model is how the timing of the photoperiod coincides with the scotophile phase of the daily oscillation. When responses are essentially insensitive to light impinging on the scotophile phase, photoperiodic control of rhythm phasing can be easily established as has been the case for the Drosophila

emergence rhythm (Pittendrigh, 1960), leaf and petal movement (Bunsow, 1960; Halaban, 1968a) or Euglena phototaxis (Bruce, 1960). On the other hand without knowledge of how rhythm phase, and hence the timing of the scotophile phase are determined, general evidence of rhythmicity in a response such as flowering (see Table 1) is insufficient to show how a rhythm acts as the clock measuring photoperiod duration. Unfortunately, when a response is inhibited by light impinging on the scotophile phase, as flowering is, it becomes almost impossible to measure rhythm phase.

Some resolution of this question may be achieved by utilizing photoperiods that are not quite sufficient to inhibit the response completely i.e. at the critical dark period. This was essentially the approach adopted by Pittendrigh and Minis (1964) to assess coincidence effects of a photoperiod on the degree of oviposition in Pectinophora gossypiella. There appeared to be a relationship between the phasing of the rhythm of oviposition and the degree of response to photoperiod but they could not establish the statistical validity of this trend.

Another approach has been to measure the phasing of an overt rhythm whose expression is not inhibited by light. Such a method assumes that photoperiod controls phasing of the overt rhythm and the light inhibited rhythm in the same manner. This, however, may not be true. An alternative approach, as developed in Chapter III, necessitates the measurement of a phase response curve and it will be shown that once the phase response curve is known, rhythm entrainment to a 24 hour period can be evaluated and the effect of photoperiod on rhythm phasing assessed.

IV. TIME MEASUREMENT IN PHOTOPERIODISM - AN HOURGLASS.

In 1936, while Garner and Allard were continuing their experiments, a new unit of the United States Department of Agriculture was established at Beltsville with the purpose of examing physiological changes in plants exposed to different daylengths. Borthwick and Parker commenced this work and in 1944 began, in collaboration with S. B. Hendricks, to unravel the puzzle of the pigment that acted as the photoreceptor perceiving the daily photoperiod. The main point of departure for this work was the recognition by Hamner and Bonner (1938) that in the short-day plants soybean and Xanthium pensylvanicum flowering could be prevented by a brief illumination given near the middle of an otherwise inductive dark period. Subsequently, Parker et al (1945 and 1946) found that red light of short duration and very low energy (20 kerg/cm^2 at 660 nm) was most effective for inhibiting flowering of these plants. Not only did such red light breaks block flowering of short-day plants but they promoted flowering in long-day plants such as Wintex barley (Borthwick et al, 1948) and Hyoscyamus (Parker et al, 1950) held under non-inductive conditions. A similar action spectrum was found for the low energy reaction controlling lettuce seed germination (Borthwick et al, 1952a) and as will be discussed later (part V) the same low energy reaction controls many other plant growth responses.

A most important observation was that promotion of lettuce seed germination or inhibition of flowering in Xanthium could be reversed by terminal far-red radiation of low energy (Borthwick et al, 1952a,b). On the basis of these experiments it was concluded that the pigment (named "phytochrome" in 1959) existed in two photointerconvertible forms.

The form that absorbed maximally in the red region (665 nm) - labelled P_r in equation 1 below - was converted by red irradiations to the form absorbing in the far-red region at 725 nm - termed P_{fr} in equation 1. Far-red radiation converted P_{fr} the presumed active form, into P_r, the inactive form. Subsequently, the accuracy of these concepts was verified both by *in vivo* and *in vitro* spectrophotometric assays and these results are further discussed in part V of this chapter.

$$P_r \underset{\text{far-red radiation}}{\overset{\text{red radiation}}{\rightleftharpoons}} P_{fr} \qquad \text{Equation 1.}$$

Short of obtaining an action spectrum, red/far-red photoreversibility has become a decisive test for phytochrome and such tests have indicated that phytochrome may play an important role in controlling flowering of many other short-day and long-day plants including *Lemna perpusilla* (Purves, 1961); *Chenopodium rubrum* (Kasperbauer *et al*, 1963); *Pharbitis nil* (Fredericq, 1964); *Lolium temulentum* (Vince, 1965); *Hyoscyamus niger* (Schneider *et al*, 1967) and *Lemna gibba* (Cleland and Briggs, 1967).

Taken alone, the sensitivity of flowering to P_{fr} could suggest that the role of phytochrome in photoperiodic control of flowering involves a direct action of P_{fr} to prevent the expression of the timing system. However, at the outset of their investigations, Borthwick and coworkers had performed two crucial experiments that suggested that phytochrome participated in time measurement itself. Firstly, from experiments on seed germination (Borthwick *et al*, 1952a and 1954) it was deduced that P_{fr} reverted slowly in darkness to the inactive P_r form

and at a temperature of 35°C the half-life for reversion was about 10 hours and at 30°C about 18 hours. They therefore reasoned that, since daylight was known to convert most of the pigment to the P_{fr} form, its subsequent reconversion in darkness controlled photoperiodic time measurement. Thus terminating the dark period with far-red radiation should hasten P_{fr} dark reversion and shorten the critical dark period. In their experiments using Xanthium (Borthwick et al, 1952b) this proved to be true. The critical dark period for flowering was shortened by several hours when plants were exposed to a brief far-red irradiation terminating the photoperiod and comparable results have since been obtained with other short-day plants including Setaria (Downs, 1959); Pharbitis nil (Takimoto and Naito, 1962a; Fredericq, 1964; Evans and King, 1969) and Chenopodium rubrum (Cumming, 1963). On the other hand, in some experiments, terminating the light period with a far-red irradiation failed to shorten the critical dark in Pharbitis nil (Nakayama, 1958) and Xanthium (Eshashi and Oda, 1964; Borthwick and Downs, 1964).

The concept of phytochrome reversion from P_{fr} to P_r in darkness was later confirmed with the development of spectrophotometric techniques for measuring phytochrome in vivo in non-green tissue (Butler et al, 1959; Hendricks, 1960). While Hendricks and Borthwick (1963) claimed only that "dark conversion to P_{660} (P_r) is the central factor in time measurement in photoperiodism," there was a clear resemblance between phytochrome reversion and the action of an hourglass clock. Because of excessive screening by chlorophyll it has not been possible to provide spectrophotometric verification of phytochrome reversion in green tissue. Also the application of techniques such as cyclic lighting treatments (Borthwick and Cathey, 1962) or prolonged irradiations with red

or far-red during darkness (Kasperbauer *et al*, 1964) may not provide a valid estimate of phytochrome reversion (see Borthwick *et al*, 1969; Cumming, 1969b). Cumming *et al* (1965) have developed a technique that is not open to the criticisms of the approaches cited above. They used brief irradiations with various mixtures of red and far-red radiation, so that the composition of light having no net effect on flowering, i.e. which gave a null response, indicated the proportion of phytochrome in the P_{fr} form at that time. Cumming and coworkers had difficulty measuring a null response value over the early hours of darkness but found phytochrome was predominantly in the P_{fr} form at the eleventh hour of darkness and the proportion of P_{fr} gradually declined until the fifty-ninth hour of darkness. Evans and King (1969) used this technique during a single dark period of little more than critical length given to *Pharbitis nil* and followed the reversion of P_{fr} to P_r during the early hours of darkness. By contrast to the early concept of an hourglass in which phytochrome reversion began as soon as plants were placed in darkness, Evans and King found that phytochrome appently remained mostly in the P_{fr} form until the sixth hour of darkness and reversion when it occurred was rapid and almost complete within an hour. Thus it would seem that spectrophotometric measurements of phytochrome reversion in aetiolated tissue may have little relevance to photoperiodism. Likewise, the paradoxes and discrepancies observed in the past for phytochrome transformations (see Hillman, 1967a and part V of this chapter) cannot be advanced as serious arguments against action of phytochrome in photoperiodic time measurement.

Observations of phytochrome reversion do not, however, show whether phytochrome reversion influences photoperiodic time measurement, but two types of experiments suggest that reversion may account for some

but not necessarily all time measurement. For instance, as discussed earlier, terminating the light period with far-red irradiation, a treatment which would be expected to hasten P_{fr} reversion, may shorten the critical dark period. Furthermore, in Pharbitis nil this shortening of the critical dark period length was found to be associated with an earlier P_{fr} reversion (Evans and King, 1969). In the second type of experiment brief light interruptions of darkness were used to introduce P_{fr} before or after its normal time of reversion. Thus in Pharbitis the presence of P_{fr} before its reversion at the sixth hour had no effect on time measurement but, interruptions with red light given after P_{fr} reversion caused a delay in time measurement (Takimoto and Hamner, 1964; Evans and King, 1969). Papenfuss and Salisbury (1967) had earlier obtained a similar response in another short-day plant Xanthium strumarium.

There are still some inadequacies to the concept of phytochrome reversion acting as a timer (see Salisbury, 1963; Sweeney, 1969a) and foremost is the evidence that circadian rhythms may play a role in time measurement. Also, measurements of changes in the null value of phytochrome need further verification. However, evidence supporting the concept of phytochrome reversion acting as a timer as developed here and expanded in Chapters V and VII indicates that phytochrome plays an important role in photoperiodic time measurement.

V. PHYTOCHROME.

a. Nature and action.

Following the realization that a red/far-red photoreversible pigment existed in plants (Borthwick et al, 1952a, b) further major contributions to our understanding of the pigment included its spectrophotometric detection (Butler et al, 1959); identification of the

chromophore of phytochrome (Siegelman *et al*, 1966) and the discovery of rapid responses to phytochrome - 10 to 30 seconds - (Fondeville *et al*, 1966; Tanada, 1968) suggesting its action on cell permeability.

Phytochrome is spectrophotometrically detectable in a wide variety of dark-grown plant tissues; in the colourless florets of cauliflower; in light-grown achlorophyllous fronds of *Lemna perpusilla* (Rombach, 1965) or carrot tissue culture (Wetherell, 1969); and is extractable from green tissue (see Hillman, 1967a). It is present in species of the plant kingdom as diverse as algae and angiosperms and controls many aspects of morphogenesis including seed germination, hypocotyl elongation, leaf unrolling and enlargement, plumular hook opening, pigment formation, geotropic response and nyctinastic leaflet closure (see review by Hendricks and Borthwick, 1965).

Only partial purification of phytochrome has been achieved to date and its molecular weight is thought to be about 60,000 (Mumford and Jenner, 1966). The chromophore is a bilitriene (Siegelman *et al*, 1966) associated with protein (Mumford and Jenner, 1966) and, from measurements of extinction coefficients of both extracted phytochrome and other bilitriene chromophores, Siegelman (1969) proposed that the number of chromophores per molecule was probably three or four, suggesting an enzymatic rather than an energy transforming function of phytochrome. The molecular changes on photoconversion of P_r to P_{fr} may involve isomerization of the chromophore, and Rudiger and Correll (1969) have postulated conformational changes of the protein that depend on its covalent bonding to the chromophore.

Absorption spectra show that as well as absorbing in the red and far-red regions both forms of phytochrome have substantial absorption in the blue and long-ultraviolet regions (Butler *et al*, 1964). *In vivo*

spectrophotometric measurements of the action spectra for the phototransformation of phytochrome (Pratt and Briggs, 1966; Hartmann and Spruit, reported by Mohr, 1970) are similar to action spectra of extracts. Blue light sets effectively 30-40% P_{fr} of the total phytochrome ($P_{tot.}$) *in vitro* but may be considerably less effective *in vivo* (Everett and Briggs, 1970).

b. *Phytochrome and morphogenesis.*

In general, morphogenesis requires the synthesis of new protein and nucleic acid and hence there is usually a dependence on DNA directed RNA synthesis. It is therefore not surprising to find that many aspects of phytochrome mediated photomorphogenesis require nucleic acid and protein synthesis. This point is most elegantly demonstrated in the explorations of Mohr and his colleagues with the cruciferous plant *Sinapis alba*. For example, studying the photocontrol of anthocyanin synthesis they found that phytochrome also controlled the activity of the enzyme phenylalanine ammonia-lyase (PAL) (Durst and Mohr, 1966). This enzyme controls phenol and flavonoid biosynthesis and therefore is rate limiting for anthocyanin biosynthesis. Furthermore, actinomycin D, an inhibitor of DNA directed RNA synthesis, could prevent PAL synthesis and anthocyanin formation (Durst and Mohr, 1966) as could the inhibitors of protein synthesis, chloramphenicol and cycloheximide (Rissland and Mohr, 1967). Recently Zucker (1970) reported that synthesis of PAL may not be the only step controlled by phytochrome but that it might also control the synthesis of proteolytic enzymes which determine the rate of PAL breakdown. Nevertheless the generalization appears valid that phytochrome may control photomorphogenesis in plants by controlling gene activation.

A number of other enzymes show increased activity in response to phytochrome and these reports involve enzymes localized in the

chloroplasts (Graham *et al*, 1968; Butler and Bennet, 1969), the cytoplasm (Butler and Bennet, 1969; Van Poucke *et al*, 1969), etioplasts (Bottomley, 1970), microbodies (Van Poucke *et al*, 1970) and the nucleus (Bottomley, 1970). Some of these responses are probably DNA dependent involving *de novo* enzyme synthesis, but this has been proven unequivocally only for the enzyme isocitric lyase (Hock, 1970).

The diverse control by phytochrome of morphological and biochemical sequences leaves little doubt that regulation by P_{fr} involves a basic metabolic reaction. However, gene repression and activation as proposed by Mohr (1966) does not account for all responses. For instance, ascorbic acid production in *Sinapis alba* shows phytochrome control dependent on activation of the enzyme ascorbic acid oxidase, but, this response is not sensitive to inhibitors of DNA directed RNA synthesis nor to inhibitors of protein synthesis (Bienger and Schopfer, 1970). Tezuka and Yamamoto (1969) have also obtained evidence suggesting that phytochrome may be capable of direct activation of the enzyme NADP-dehydrogenase. Therefore, whether by increasing the levels of cofactors for a reaction or by activating the enzyme itself, not all actions of phytochrome require gene action. In this context it seems probable that the primary action of phytochrome is on cell permeability (Hendricks and Borthwick, 1967) and there is considerable evidence that phytochrome may be membrane bound (Galston, 1968; Haupt, 1968), may be organized in a dichroic structure on the membrane, and that it can control ion fluxes (Galston *et al*, 1969) and bioelectric potentials (Jaffe, 1968).

c. *Spectrophotometric transformations of phytochrome.*

Spectrophotometric assay of phytochrome has been possible *in vivo* only in aetiolated seedlings, in tissues free of chlorophyll or *in vitro* using isolated phytochrome. In dark-grown seedlings phytochrome

is entirely in the P_r form but is converted by red irradiation into the P_{fr} form with about 81 % as P_{fr} and 19 % as P_r (Butler *et al*, 1964). In darkness, detectable P_{fr} may disappear gradually by reverting to P_{fr} and this was first shown by Butler *et al* (1963) in cauliflower floret tissue. Other workers subsequently reported similar responses in parsnip and artichoke receptacles (Hillman, 1964b) and parsnip roots (Koukkari and Hillman, 1967). Two investigations using partially purified phytochrome solutions have also indicated dark reversion in extracts from dark-grown oats (Mumford. 1966) and in extracts from a number of dark-grown green tissues (Taylor, 1968).

Often P_{fr} undergoes rapid and complete decay or destruction in darkness in aetiolated seedlings (Butler *et al*, 1963; Hopkins and Hillman, 1965; Pratt and Briggs, 1966; Kendrick and Frankland, 1968), in brussel sprout receptacles (Hillman, 1964b) and gladiolus corms (Koukkari and Hillman, 1966). Decay of P_{fr} may be more rapid in monocotyledonous than in dicotyledonous tissue: half-life for decay 0.8 to 1.0 hours compared to 2.0 to 4.0 hours (Hopkins and Hillman, 1965). Hence in some dicotyledonous tissues reversion, which occurs simultaneously with decay, may be relatively more significant. These experiments are often cited as disproof of the original proposition of phytochrome "reversion-as-a-timer" in photoperiodic control of flowering, but as discussed in part IV , reversion in aetiolated tissue may differ from that in green tissue.

A significant development in attempting to understand phytochrome transformations was the attempt to relate physiological responses to spectrophotometrically detectable levels and proportions of phytochrome. A number of workers have shown a close correlation between the initial state of optically detectable phytochrome *in vivo* and, for example, the photocontrol of pea stem elongation (Hillman, 1965),

frond production in Lemna perpusilla (Rombach, 1965), growth of oat coleoptiles (Hopkins and Hillman, 1966) and mesocotyls (Loercher, 1966) and bean-hook opening (Klein et al, 1967). In most instances a sigmoidal relationship was found between the physiological response and the percentage conversion of P_{fr} to P_r, suggesting a first order reaction dependent on the absolute levels of phytochrome P_{fr} present. In at least one instance reported recently a very low threshold level of P_{fr} (less than 2.5% P_{fr}) controlled the formation of the enzyme lipoxygenase in Sinapis alba (Oelze-Karrow et al, 1970). But, again, the absolute level of P_{fr} was found to be the important factor, not the P_{fr}:P_r ratio.

d. Paradoxes of spectrophotometry.

Unfortunately, not all phytochrome controlled responses have yielded to spectrophotometric analysis. There may be no spectrophotometrically assayable phytochrome in peas, and yet it is possible to show red/far-red reversibility of lateral root initiation and stem elongation (Furuya and Hillman, 1964; Hillman, 1965; and see Hillman, 1967a). Briggs and Chon (1966) found a slightly different discrepancy in corn coleoptiles; saturation energies for physiological responses were several orders of magnitude below the energies required for spectrophotometric detection of phytochrome conversion.

Hillman (1967a), in discussing these paradoxes between spectrophotometry and physiological response postulated the existence of bulk and active pools of phytochrome. In some situations the concentration of the active phytochrome pool may be too low for spectrophotometric detection but it is possible that extremely low threshold levels of P_{fr} could be saturating for some responses as found by Oelze-Karrow et al (1970). Saturation at a low threshold energy may explain the control of petiole elongation in pea plants. Elongation was found to be

maximally sensitive to as little as two seconds irradiation with red light of 120 $\mu w/cm^2$ energy (Haupt and Linder, 1968) but the total energy these authors employed was one to two orders of magnitude below that which was required to simultaneously saturate the inhibition of elongation of the third internode. Hence, it is possible that, because of instrumentation limitations, spectrophotometric changes may not always be detectable, particularly when saturation is achieved at a low threshold concentration of P_{fr}.

Three recent discoveries have indicated the possible existence of further complications during phytochrome transformations.

(i) Phytochrome in the P_r form is synthesized in darkness (Butler & Lane, 1965; Clarkson and Hillman, 1967) or in light (Marme, 1969; Wetherell, 1969). As P_{fr} decay is more rapid than P_r synthesis the detectable level of phytochrome (P_{tot}) may approach zero in continuous red irradiation (Clarkson and Hillman, 1967; Kendrick and Frankland, 1968; Marme, 1969).

(ii) In germinating seeds phytochrome appears as P_r during their hydration (Tobin and Briggs, 1968) and may undergo rapid conversion from P_r to P_{fr}. This effect, described as "inverse-reversion" had been postulated by Toole (1961) but was first observed spectrophotometrically by Boisard et al (1968) in lettuce seeds. They found that after photoconversion of spectrophotometrically assayable phytochrome P_{fr} to P_r, the level of P_{fr} rose again rapidly in darkness. Confirmation of these findings has been reported subsequently for cucumber seeds (Spruit and Mancinelli, 1969) and Amaranthus seeds (Kendrick et al, 1969). "Inverse-reversion" occurs even in dry seeds and may proceed to completion within thirty minutes in darkness. Use of a physiological assay for

the proportion of phytochrome (P_{fr}:P_{tot}) present in green tissue also suggested the possibility of inverse reversion of P_r to P_{fr} (Evans and King, 1969) and there were indications of a comparable phenomenon in Chenopodium rubrum (Cumming et al, 1965).

(iii) Working with extracted phytochrome, Anderson et al (1969) reported that low pH (< 6.2) or low temperature favoured the formation of an acid form of phytochrome - $P_{fr}H$. Light absorption was maximal at 650 nm for $P_{fr}H$ (cf. 720 nm for P_{fr}) and $P_{fr}H$ formation in plants would depend on the in vivo pH and temperature conditions and it has been postulated by Borthwick et al (1969) that $P_{fr}H$ reversion to P_{fr} in darkness is the basis of the apparent inverse reversion of P_r to P_{fr}.

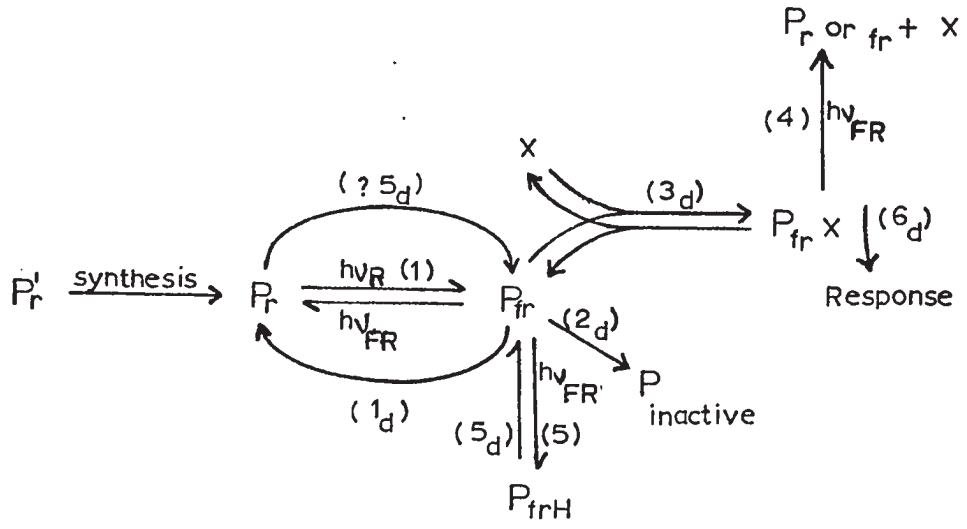
e. Phytochrome transformations summarized.

Equation 2, redrawn from Borthwick et al (1969) and Hartmann (1966), summarizes the transformations of phytochrome as currently observed and postulated. The red/far-red photoreversible step (1) inducing the formation of P_{fr} and P_r, respectively, is probably the best understood of all these responses and appears to involve an isomerization of the bilitriene chromophore followed by a change in its covalent bonding to protein, causing changes in protein configuration and activity. The active form of phytochrome (P_{fr}) may revert to P_r in darkness or decay (2). In continuous light, and sometimes in darkness, P_{fr} decay will be more important than reversion to P_r in darkness. Total levels of phytochrome may be lower in green tissues because of P_{fr} decay although the evidence for this assumes that the active pool reflects the bulk levels of detectable phytochrome. P_r synthesis occurs in light or darkness and thus an equilibrium level of P_{tot} will be attained in continuous light dependent on the balance of synthesis, decay and

Equation 2.

A kinetic model of phytochrome light and dark interconversions. After Hartmann (1966) and Borthwick et al (1969).

P = phytochrome; X = substrate or reaction partner ; hν = light of red (R) or far-red (FR) wavelengths.



Reactions:

(1) P_r and P_{fr} photointerconversion.

(2) P_{fr} decay or destruction.

(3) P_{fr} substrate interaction

(4) Absorption of FR radiation by $P_{fr}X$ causing its dissociation.

(5) Absorption of FR radiation by P_{fr} causing $P_{fr}H$ formation.

5d may be $P_{fr}H \rightarrow P_{fr}$ or $P_r \rightarrow P_{fr}$.

(6) $P_{fr}X$ complex controlling photomorphogenesis.

reversion. In darkness P_r synthesis will proceed but will not alter the level of P_{fr} that is available unless P_r undergoes inverse reversion to P_{fr}. At present it is debatable whether "inverse reversion" of P_{fr} occurs (5d) or whether P_{fr} is formed in darkness from $P_{fr}H$ (5d). There is as yet no *in vivo* evidence about $P_{fr}H$ formation but changes in the cellular pH in darkness might allow such a conversion. As will be discussed in the next section substrate availability for P_{fr} action (3d) may prove important and the light sensitivity of the substrate - P_{fr} complex (4) may differ from that of P_{fr}.

VI THE HIGH ENERGY REACTION OF PHOTOMORPHOGENESIS.

Many of the red/far-red phytochrome controlled photomorphogenic responses of plants can also be induced by the use of prolonged irradiation with higher intensities but wavelengths in the blue (450 nm) and far-red (720 nm) regions are maximally effective in these high-energy reactions (HER). Table 2 summarizes some of the HER phenomena observed to date. Effectiveness in the blue and far-red regions does not favour the involvement of photosynthesis and, except in the case of anthocyanin synthesis in apple skins (Downs *et al*, 1965), the action of photosynthetic pigments has been excluded either by the use of aetiolated seedlings containing no chlorophyll or by the application of DCMU to inhibit photosynthetic electron transport (see Evans *et al*, 1965a). These results do not suggest that chlorophyll acts in photoreception in green tissue, but the use of DCMU does not exclude the possibility of chlorophyll photoactivation of cyclic or pseudocyclic photophosphorylation. In *Scenedesmus*, photoactivation of cyclic photophosphorylation saturates at 100 ft-c (Urbach and Gimmler, 1970).

Additive phytochrome mediated responses have been reported for

several responses to high energy radiation (see Mohr and Noble, 1960; Evans et al, 1965a;Lane and Kasperbauer, 1965). Sequential or parallel relationships between two photoreceptors have been postulated to explain these observations of interaction of an unknown HER and phytochrome. However, Hartmann (1966) presented a simplified interpretation of these results which suggests that, at least for wavelengths greater than 550 nm HER phenomena could be explained on the basis of photoreception via phytochrome. He found that for suppression of lettuce seed germination by prolonged irradiation, maximal effectiveness was at a wavelength of 720 nm but equal suppression of germination could also be achieved by mixing very low energies of red (658 nm) with relatively high energies of longer wavelengths of far-red irradiation (766 nm). With either pure or mixed irradiations maximum effectiveness for an HER occurred at levels of P_{fr} estimated to be about 3% of total phytochrome. Hartmann suggested prolonged far-red radiation,or the red/far-red mixture,maintained a minimal loss of P_{fr} by decay because the photochemical steady-state setting of P_{fr}:P_r was low but sufficient P_{fr} would still be present for its action over a prolonged period of time. To convert more P_r to P_{fr}, thus enhancing the loss of P_{fr} by decay, would only reduce the absolute levels of P_{fr} below those maintained by prolonged far-red irradiation.

The rapidity of morphogenetic or biochemical response to the presence of P_{fr} becomes quite important in this model (see equation 2). Also the interactions discussed earlier between HER responses and phytochrome could be understood, because both P_{fr} decay and the rate of P_{fr}-substrate interaction in darkness would be determined by light quality and the duration of a prior HER as well as by the quality of irradiation terminating the HER.

For hypocotyl elongation (Hartmann, 1966), and seed germination

TABLE 2. Representative high-energy reaction (HER) responses of plants.

a. Photomorphogenesis.

ENTRY	SPECIES	RESPONSE	MOST EFFECTIVE WAVELENGTHS (nm)		REFERENCE
1	Sinapis alba	Anthocyanin synthesis		710	Mohr (1957)
2	Sinapis alba	Anthocyanin synthesis	450	730*	Bertsch and Mohr (1965)
3	Sorghum	Anthocyanin synthesis	470	no response	Downs and Siegelman (1963)
4	Lactuca sativa	Hypocotyl elongation	450	720	Mohr and Wehrung (1960)
5	Lactuca sativa	Hypocotyl elongation	450	720	Hartmann (1967)
6	Lactuca sativa	Hypocotyl elongation	-	746*	Hartmann (1966)
7	Petunia	Hypocotyl elongation	430-450	660	Evans et al (1965a)
8	Chenopodium polyspermum	Stem elongation	-	710	Jacques (1969)
9	Chenopodium polyspermum	Stem elongation	-	730*	Jacques (1969)
10	Mimosa pudica	Leaflet opening	430	710	Fondeville et al (1967)
11	Lactuca sativa	Seed germination	-	717	Hartmann (1966)
12	Lactuca sativa	Seed germination	-	766*	Hartmann (1966)
13	Amaranthus arenicolor	Seed germination	-	720	Hendricks et al (1968)
14	Amaranthus arenicolor	Seed germination	-	720*	Hendricks et al (1968)
15	Cuscuta	Twining	450	730	Lane and Kasperbauer (1965)

* Auxilliary red irradiation provided.

Table 2 continued.

b. Flowering.

ENTRY	SPECIES	FLOWERING RESPONSE TYPE	MOST EFFECTIVE WAVELENGTHS (nm)		REFERENCE
16	Hyoscyamus niger	long-day plant	blue	far-red	Stolwijk and Zeevaart (1955)
17	Hyoscyamus niger	long-day plant	450	710-720	Schneider et al (1967)
18	Sinapis alba	long-day plant	blue	far-red	Stolwijk (1954)
19	Sinapis alba	long-day plant	blue	red*	Hanke et al (1969)
20	Wheat	long-day plant	-	700-720	Friend (1968)
21	Brassica campestris	long-day plant	-	710	Friend (1968)
22	Blitum capitatum	long-day plant	-	710-730	Jacques and Jacques (1969)
23	Lolium temulentum	long-day plant	-	700-725	Blondon and Jacques (1970)
24	Lolium temulentum	long-day plant	-	725*	Blondon and Jacques (1970)
25	Xanthium pennsylvanicum	short-day plant	-	600-700	Salisbury (1965)
26	Pharbitis nil	short-day plant	450	650	Salisbury (1965)

* Auxilliary red irradiation provided.

(Hendricks et al, 1968), the effectiveness of an HER has been observed to change with the intensity at any one wavelength. The photoequilibrium of P_{fr}:P_r should remain unaltered under these conditions and Hartmann (1966) suggested that excessive stimulation of photoreaction 2 (equation 2) led to "competitive self inhibition" of the P_{fr}-X reaction. On the other hand, Borthwick et al (1969) attributed the intensity dependence of HER phenomena to a possible photoreaction 5 (equation 2) that gave rise to the formation of $P_{fr}H$ and, as well, to changes in the absorption properties of P_{fr} when complexed with its reaction partner X (photoreaction 4, equation 2). Action maxima in the red in which P_{fr}:P_r ratios would exceed 0.5 might be explained in Hartmann's model on the basis of species differences in their rates of P_{fr} decay; P_{fr}-X complexing or P_r synthesis.

Hartmann's evidence that the HER action maxima shifts to longer wavelengths with the superimposition of a red background irradiation has been verified subsequently (entries 2, 6, 9, 24 in Table 2). However, at the time that Hartmann developed his model, the only responses that had been examined were those in which the optimal ratios of P_{fr}:P_{tot} were low; 0.03 or 3.0% P_{fr} for hypocotyl elongation and 0.10 for lettuce seed germination. In a recent study of HER displays, the ratio of P_{fr}:P_{tot} was held above 0.4, yet the action maxima remained unchanged at 720 nm (see entry 14, Table 2). These latter results suggest the presence of additional photoreactions to those proposed by Hartmann, and Borthwick et al (1969) postulated the occurrence of photoreactions 4 and 5 as outlined in equation 2. The extension of Hartmann's model in this way complicates the interpretation of an HER in any one organism but as Borthwick and coworkers have emphasised, "the nature of the HER is best analysed for detail in this multiplicity of

displays where individual component reactions might be dominant in any one of the displays".

b. HER and flowering.

Action spectra for HER phenomena controlling flowering in long-day plants support the concept of a phytochrome mediated HER (Table 2). Schneider et al (1967) have presented a detailed action spectrum for the HER controlling flowering in the long-day plant Hyoscyamus niger. Their finding of maximum effectiveness at 710 to 720 nm confirmed earlier studies (e.g. Stolwijk and Zeevaart, 1955) in which broad band blue and far-red radiation was most effective. However, the most convincing evidence of a phytochrome mediated HER in long-day plants was published by Hanke et al (1969) for Sinapis alba and Blondon and Jacques (1970) for Lolium temulentum. In each case irradiations were with a standard red background mixed with increasing energies of far-red. In Lolium, the shift in the action maxima in the far-red to longer wavelengths on the addition of a red background irradiation is reminiscent of the experiments of Hartmann (1966) on lettuce seed germination. Also a photosynthetic HER was excluded in earlier experiments of Evans et al (1965b). Thus taken together, the experiments of Blondon and Jacques (1970) and Evans et al (1965b) leave little doubt that phytochrome is the photoreceptor for the HER controlling flowering in Lolium and probably other long-day plants.

For short-day plants, knowledge of the photoreceptor is not as certain. Photosynthetic mediation is unlikely, although photosynthate supply may control floral development (Parker and Borthwick, 1940; Liverman and Bonner, 1953; Carr, 1957; Deltour, 1970). Several investigators have examined the influence on flowering of light intensity, duration and quality when given as a light interruption beginning at the

seventh or eighth hour of darkness. A normal flowering response in a subsequent dark period was achieved by 6 to 8 hours of white light of either 150 or 2000 ft-c given to Xanthium strumarium (Salisbury, 1965) and in Pharbitis nil a similar treatment showed saturation at an intensity of 10 to 50 ft-c (Takimoto, 1967; Zeevaart and Marushige, 1967). As shown by the action spectra determined by Salisbury (1965), red light was most effective in both Xanthium and Pharbitis.

A different approach to these questions has been adopted in experiments with Chenopodium amaranticolor (Konitz, 1958; Bunning and Moser, 1969), Chenopodium rubrum (Cumming, 1963, 1969c), Xanthium strumarium (Salisbury, 1963) and Perilla ocymoides (Bunning and Moser, 1969). In the hope of maintaining steady state conditions of irradiation, light was used to replace the dark period. Only Konitz (1958) and Cumming (1963, 1969c) used energies adequate for rapid phytochrome photoconversion and both workers found that continuous far-red irradiation or light with relatively low red/far-red ratios was more effective than red or white light. These results suggest the importance of a phytochrome mediated HER in short-day plants. Since the experiments of Cumming (1969c) indicated that darkness could not be replaced as effectively by the same period in light, it is difficult, at present, to determine the significance of these results in relation to plants given daily light : dark cycles.

CHAPTER II

RHYTHMIC CONTROL OF FLOWERING.

I. INTRODUCTION.

The change from vegetative to reproductive growth in plants is a most dramatic and important aspect of plant growth and it is well established that the length of the daily light period is particularly significant in controlling this response. By using artificial means to lengthen or shorten photoperiods it was found that some plants flowered in response to increased length of the photoperiod (long-day plants), others to decreased photoperiod length (short-day plants). Initially it was suggested by Hamner and Bonner (1938) that the length of the dark period was crucial and this led to a number of experiments in which plants were given repeated light:dark cycles; the light period was generally held constant and the length of the dark period was varied to give a total period longer or shorter than 24 hours. When the length of the cycle was varied there were rhythmic changes in the capacity to flower as the length of the dark period increased for both Hyoscyamus niger (Claes and Lang, 1947; Hsu and Hamner, 1967) and soybean (Hamner, 1960). However, for Kalanchoe blossfeldiana (Harder and Gummer, 1949; Schmitz, 1951), Perilla nankinensis (Rupcheva, 1948: quoted by Zeevaart, 1969), Silene armeria (Takimoto, 1955) and Xanthium pensylvanicum (Hamner and Bonner, 1938; Schwabe, 1955) the same type of experiment suggested a mutual interdependence between the lengths of the light and dark periods. There was no evidence of rhythmic fluctuations of effectiveness of light:dark cycles in these latter species. To say the

least, these results with cycles of light and darkness are confusing, and, in view of their complexities, it could be suggested that "it does not seem likely that the rhythmic fluctuations which can be observed under certain circumstances are an inherent part of the photoperiodic response" (Lang, 1965). However, as discussed later, this conclusion may be too extreme.

To emphasise and clarify the concepts important to a discussion of photoperiodic time measurement, some of the results presented in this chapter were derived from published experiments on the flowering response of soybean, Kalanchoe blossfeldiana, Hyoscyamus niger and Silene armeria when given repeated light:dark cycles. Experiments are also reported that illustrate the flowering response of C. rubrum to such light:dark cycles; to daily photoperiodic cycles and to a single dark period of varied duration. Although experimental conditions differed slightly from those generally used with C. rubrum (see Cumming, 1967b) responses are comparable to those published previously. The conditions of experimentation outlined here have been adhered to throughout this thesis.

II MATERIALS AND METHODS.

a) Growing conditions.

Chenopodium rubrum L selection 374 (Origin 60°47'N 137°32'W) has been used exclusively in these investigations. Many aspects of the photoperiodic response of this selection have been reported previously (cf. Cumming, 1969a) and the seed used in these experiments had been multiplied from the original selection over 5 to 7 generations. In the early experiments seed was obtained from plants grown in the summer of 1966 under long days in a glasshouse. Most of the experiments involved seed grown again under long days in the summer of 1969. There were no

differences in the photoperiodic response of the seed samples.

Before sowing, the seeds were cleaned and washed for 30 minutes in a 5% solution of a wetting agent - "Aerosol" (product of American Cyanamid Co.) followed by 30 minutes in a 10% Javex solution. About 100 to 150 seeds were planted on 7 layers of 4.25cm 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, together, autoclaved.

Successive temperature alternations: 32.5°C for 12 hours, 10°C for 12 hours; for $4\frac{1}{2}$ days, in the light, gave 95% germination. Light was from fluorescent lamps (Westinghouse F20 T12 CW) adjusted to give 600 ft-c at 20°C at plant height. Because the lamps were subjected to the same temperature fluctuations as the plants, the light intensity actually changed from about 700 ft-c at 32.5°C to 500 ft-c at 10°C. Light intensities were measured with a selenium photovoltaic cell (Weston illumination meter model 756). Four and a half days after sowing the temperature was changed to 20°C, light intensity was now 600 ft-c, and Hoagland's No. 1 nutrient solution was applied (Fe^{3+} ions chelated with 6.0 mg/l sequestrene). Dark period treatments commenced $5\frac{1}{2}$ days after sowing. The temperature during the dark period was 20°C and following darkness the plants were returned to 20°C and 600 ft-c fluorescent light. At least two further applications of Hoagland's solution were given, one at the end of the dark period and another two days later. When dark periods longer than 15 hours were used in a single experiment some of the dishes were sown 12 hours earlier than the other dishes and, hence, in a different cabinet. Cabinet temperatures were monitored automatically. In some experiments involving long dark periods a solution of glucose - 0.25M, 0.3M or 0.4M - dissolved in Hoagland's solution was applied once

to the plants in place of the normal first application of Hoagland's solution $4\frac{1}{2}$ days after germination. The glucose - Hoagland's solution was replaced later by Hoagland's solution but only after the last dishes of a particular treatment were returned to light following the long dark period.

The percentage flower initiation was determined by dissection (25X magnification under a dissecting microscope) of 15 plants per dish no earlier than one week after the end of the dark period. Irrespective of the absolute level of flowering in a population of plants, a maximal response was achieved 5 to 6 days after the beginning of an inductive dark period (Figure 1). Plants kept in continuous light or given a shorter than critical dark period showed no sign of flower initiation after 3 weeks.

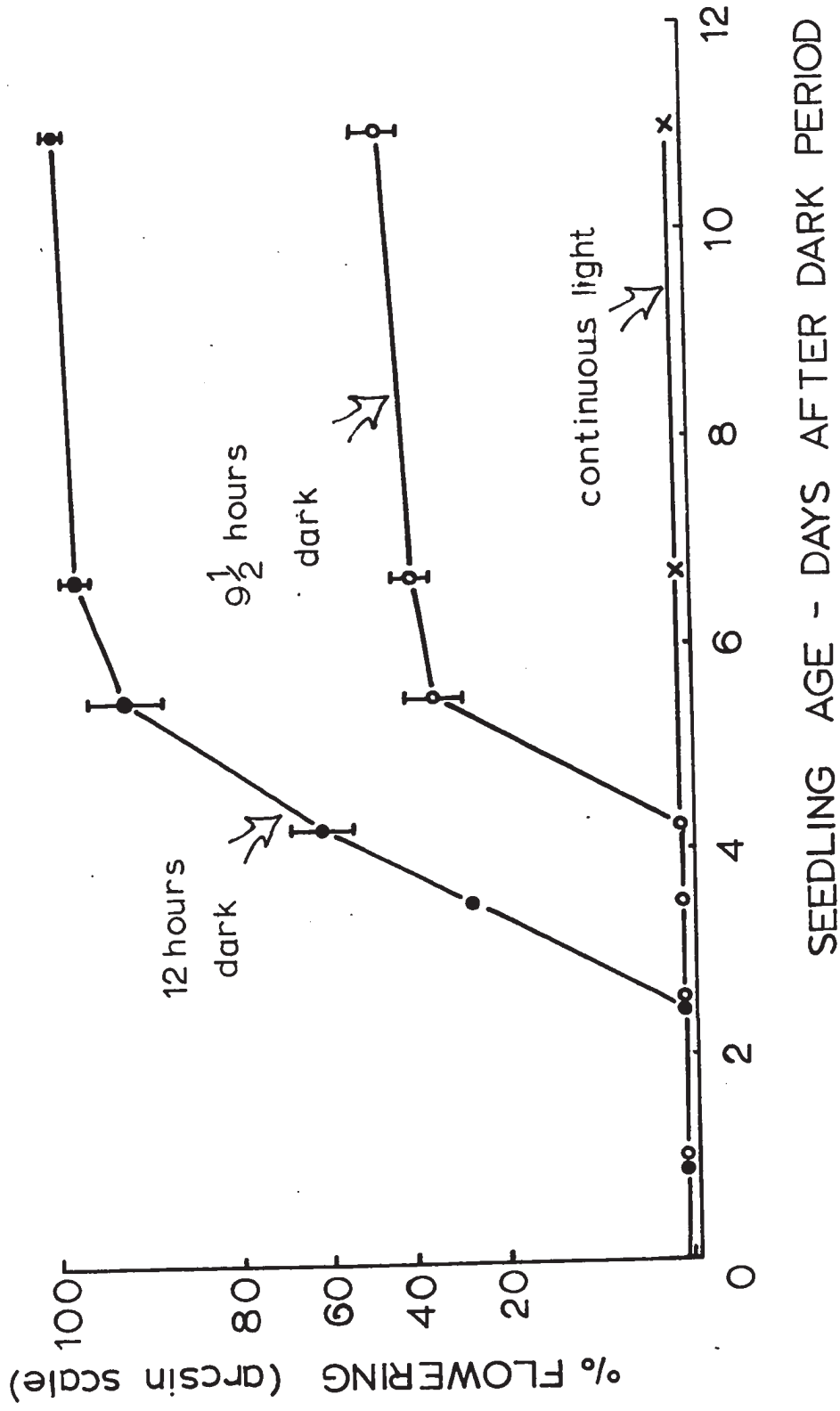
There was a minimum of two dishes per treatment (30 plants dissected) and sometimes as many as eleven dishes. All experiments have been repeated at least twice and, where possible, analysis of variance was applied to angular transformed values of the percentage flowering data. All curves have been fitted by eye to the raw data.

b) Special light treatments.

Single or multiple light interruptions of a dark period were given with irradiation obtained from red fluorescent lamps filtered through a No. 14 ruby Cinemoid filter giving a total energy of 115 $\mu w/cm^2$. Far-red light of a total energy of 420 $\mu w/cm^2$ was obtained from incandescent tubes filtered through heat absorbing glass and a layer of far-red plastic. Fluorescent light used for dark period interruptions was from Westinghouse F20 T12 CW fluorescent lamps adjusted to 600 ft-c at plant height. The spectral intensity distribution of red and far-red sources was measured with an ISCO SR spectroradiometer. More detail of

FIGURE 1.

Time of appearance of flowers (% flowering on an arcsin scale) on seedlings of C. rubrum exposed to dark periods of: (●) 12 hours; (O) 9½ hours or (×) continuous light commencing 5½ days after sowing. Day 0 on the time scale is the beginning of the dark period. After the single dark period plants were held in continuous fluorescent light of 600 ft-c intensity; temperature 20°C. Steel and Torrie (1960) discuss the need to apply an arcsin (angular) transformation in the analysis of parameters that follow a binomial distribution.


% FLOWERING (arcsin scale)
100
80
60
40
20
0
2
4
6
8
10
12
SEEDLING AGE - DAYS AFTER DARK PERIOD
12 hours dark
9½ hours dark
continuous light

the spectral energy distribution of the sources of radiation is given in Chapter V (Table 9 Figure 24). Five minutes of red or far-red irradiation achieved photostationary equilibrium in the photoconversion of P_r to P_{fr} and vice versa - detail of the experiments that establish this point is also given in Chapter V.

III RESULTS.

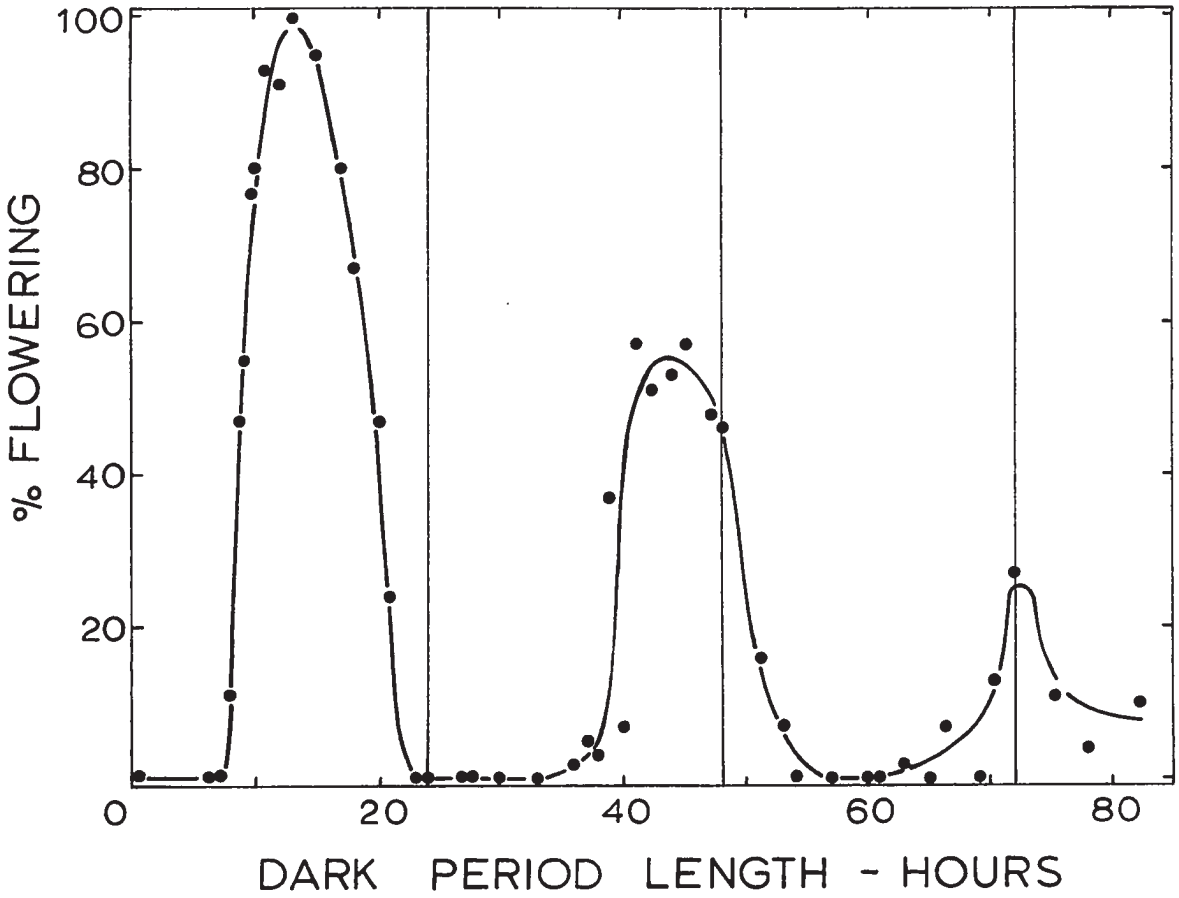
a) Rhythmicity in the flowering response of Chenopodium rubrum.

The results presented in Figure 2 illustrate a typical rhythm of flowering observed with C. rubrum when it is given a single dark period of various durations interrupting continuous light $5\frac{1}{2}$ days after sowing. Fluorescent light of 600 ft-c was given before and after darkness and flowering was assayed no earlier than one week after the end of the dark period. The individual points in Figure 2 are the averages of 15 plants sampled per dish for 6 to 7 dishes pooled from three different experiments. Homogeneity of experimental variances was not tested. Frequent sampling established the shape of the oscillation unambiguously and the curve was fitted by eye. Although in earlier work higher intensities of light were used (Cumming et al, 1965; Cumming, 1967a), the oscillation reported above is identical in its timing to the many published curves.

Flowering only resulted after dark periods of 8 hours or longer and the critical dark period was between 7 and 8 hours under these conditions. Since all plants flowered when given a 13 hour dark period, it can be suggested that there was a high degree of synchrony in the population. For longer dark periods the capacity to flower decreased to zero but increased again after 36 to 39 hours of darkness. The

FIGURE 2.

The flowering response of C. rubrum following a single dark period of different lengths that commenced $5\frac{1}{2}$ days after sowing. Temperature alternations: 32.5°C for 12 hours, 10°C for 12 hours; were applied during the $4\frac{1}{2}$ day germination period. Thereafter the temperature was 20°C and the light intensity was 600 ft-c. Vertical lines drawn at 24, 48 and 72 hours of darkness.


100
80
60
40
20
% FLOWERING
0
20
40
60
80
DARK PERIOD LENGTH - HOURS

oscillations evident in this response were consistent between experiments and from the raw data of Figure 2, and other experiments, it was possible to calculate the average peak times (the mode). Peaks fell on the average at 13±0.7 hours for the first peak and at 44.1±1.2 and 71.1±0.9 hours for the second and third peaks, respectively. This gives a period length of about 30 hours and in the subsequent discussion peaks have been assumed to fall at 13, 43 and 73 hours of darkness.

The light-off signal at the beginning of darkness apparently sets the phase of this rhythm as discussed by Cumming et al (1965). Since the rhythm persisted in continuous darkness in the absence of external stimuli and the period remained fairly constant, the oscillation conforms to the general conceptions of a free running endogenous rhythm. (see Aschoff, 1960). The phase of the rhythm when darkness is terminated determines the degree of flowering. The oscillation tended to fade out with prolonged darkness and plants were often dead after more than 4 to 5 days of continuous darkness. It is possible that depletion of the metabolic reserves causes this fade out of the response and Cumming (1967a) found that the oscillation could be sustained by supplying sugar (sucrose or glucose in Hoagland's solution) during the dark period.

In connection with the experiments reported in Figure 2, it was important to know whether light intensity had any specific effect on dark period time measurement. In earlier published work (Cumming, 1967a) it was reported that time measurement (i.e. rhythm peak times) was not influenced by growing plants in low (600 ft-c) or higher (3000 ft-c) intensity fluorescent light and the data presented in Figure 2 for plants grown in 600 ft-c fluorescent light provide additional support for this concept. Lower intensities of light do, however, reduce the degree (i.e. the amplitude) of flowering (Cumming, 1967a). It follows that if it

could be shown that the degree of flowering depends on the light intensity given before darkness there might be a specific interaction with dark period time measurement. When C. rubrum was given a 1½ day period at either 600 or 3000 ft-c prior to a dark period commencing 4½ days after germination, flowering was greater for the higher light intensity (Cumming, 1967a). Furthermore, in experiments reported in Chapter V, after 4½ days of germination and a period of only 12 hours at 600 ft-c the degree of flowering was reduced by offering light of very low intensities (less than 10 ft-c) for the 12 hours immediately prior to darkness. However, contradictory results were obtained in other experiments in which plants were grown in 3000 ft-c after a dark period but which had received 3000 or 600 ft-c fluorescent light prior to darkness. Results of one such experiment are presented in Figure 3 and a similar response was observed in a duplicate experiment. There was no increase in the flowering response with a five-fold increase in the intensity of light given before darkness and, if anything, with dark periods 8 or 9 hours long, the lower intensity might have been superior to the higher intensity. Clearly these experiments showed that there was no specific requirement for higher intensities of light prior to darkness. The key to the conflict between these latter results and those of Cumming (1967a) and those presented in Chapter V is the observation that lower intensity light can reduce flowering if also given after darkness (Figure 2). In other words the degree of flowering depended quite sensitively on the light intensity but there was no necessity for the higher intensity light to precede darkness. The effect of light intensity on the amplitude of the rhythm of flowering is apparently specific not for time measurement but for the ability of the apex to express dark period induction. These responses to light intensity probably reflect

the degree of photosynthetic input. In subsequent experiments, to avoid any complications from possible limitations to expression of floral induction, timing has been assessed only on the basis of the timing of rhythm peaks, irrespective of amplitude. Nevertheless, since the net effect of photoperiodic cycles can only be estimated in terms of the amount of flowering i.e. percentage or earliness of flowering, in this particular instance the importance of amplitude cannot be ignored unless equal total energies of light are given in all photoperiods.

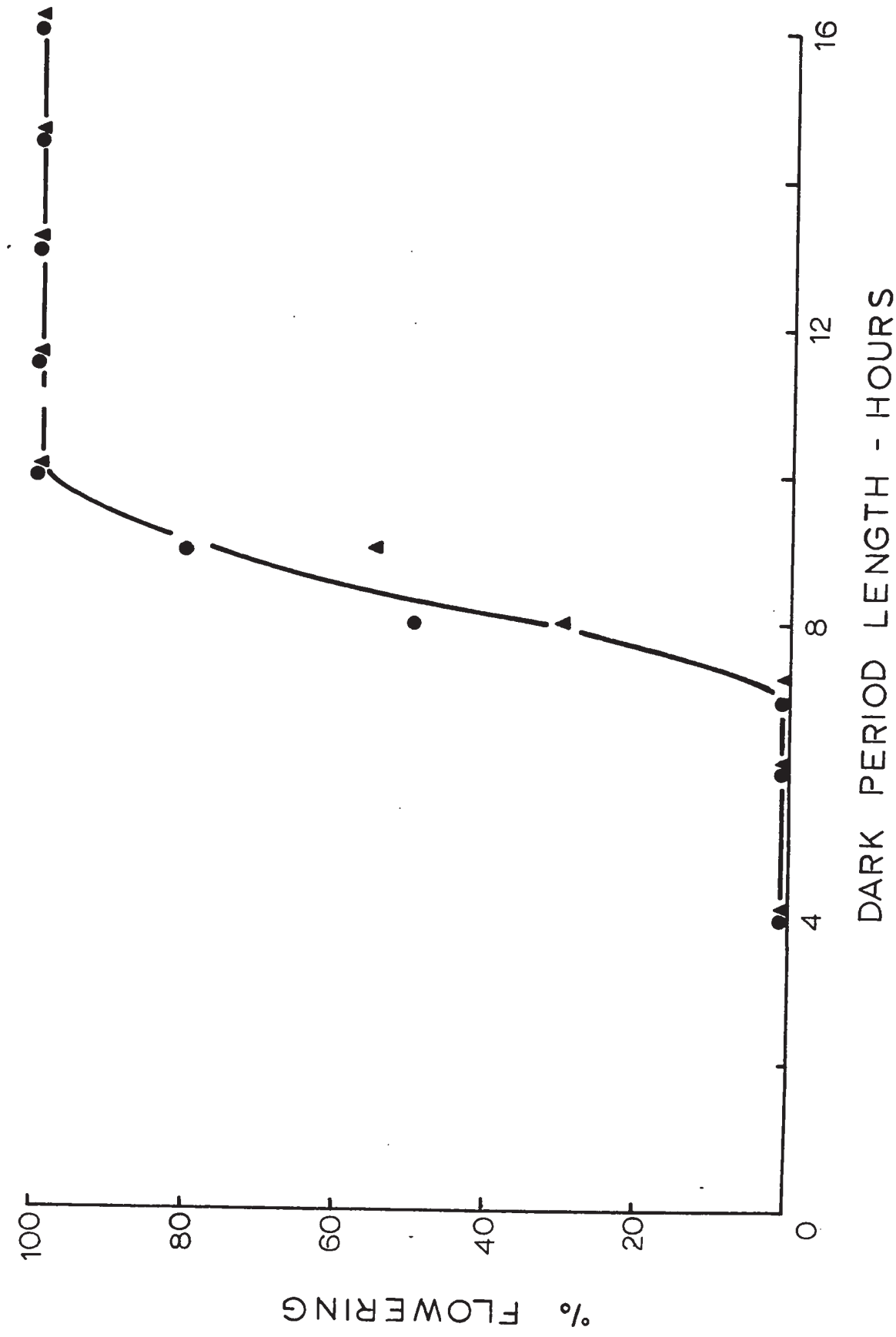
b. The photoperiodic response of Chenopodium rubrum.

The photoperiodic response of C. rubrum was determined in a series of experiments by subjecting $5\frac{1}{2}$ day old seedlings to a single dark period or two to eight cycles of various daily photoperiods. The first dark period of all cycles commenced $5\frac{1}{2}$ days from sowing and the percentage of plants flowering was determined one week after the last photoperiodic cycle. To restrict the level of flowering, Hoagland's nutrient solution was applied at 1/5 its normal strength in these experiments.

The flowering response to a single period of darkness or to 3 and 6 cycles of various daily photoperiods is shown in Figure 4a. Complete data for the same experiments are given in Figure 5 in which the relationship between the flowering response and the number of cycles of a photoperiod is presented for photoperiods varying from continuous light ($24:\bar{0}$) to continuous darkness ($0:\bar{24}$). When these experiments were repeated an additional treatment was included; a single 12 hour dark: 12 hour light period was given to all plants starting $4\frac{1}{2}$ days after sowing, then, 3 or 6 photoperiodic cycles were begun $5\frac{1}{2}$ days after sowing (Figure 4b). There were differences in the degree of flowering between the two experiments as is evident for the response to a single 12 hour

FIGURE 3.

Flowering response of C. rubrum following a single dark period that commenced 5½ days after sowing: 4½ days germination then 24 hours of 600 ft-c (●) or 3000 ft-c (▲) fluorescent light prior to the dark period; 3000 ft-c fluorescent light after the dark period for both treatments.


% FLOWERING
100
80
60
40
20
0
4
8
12
16
DARK PERIOD LENGTH - HOURS

dark period (Figure 4a 91% flowering; Figure 4b 12 hour dark control, 69% flowering). However, the pattern of the photoperiodic response was comparable in the two experiments.

With photoperiods less than 6 hours or longer than 12 hours in duration little or no flowering was obtained (Figures 4 and 5) and an 18 hour photoperiod was not inductive even after 8 cycles (Figure 5). In Figures 2 and 3 the length of the critical dark period was about $7\frac{1}{2}$ to 8 hours but under repeated photoperiodic cycles it may be even shorter since in the results presented in Figure 4b it is evident that 6 cycles of an 18 hour photoperiod were partially inductive when given in summation with a 12 hour dark period (control single 12 hour dark period 69% flowering; 12 hour dark period plus 6 cycles of an 18 hour dark period 83% flowering). There is also some evidence in Figure 5 of the summation of the response to repeated photoperiodic cycles of 6 hours light: 18 hours darkness. Reduced flowering under photoperiods shorter than 6 hours (Figures 4 and 5) must be attributed in part to a shortage of photosynthate as plants given more than 6 cycles of continuous darkness died.

Cumming (1967b) obtained a similar parabolic relationship to photoperiod length when seedlings of this ecotype ($60^{o}47'$N) were given continuous photoperiodic cycles from sowing; the light intensity was 250 ft-c. He estimated the days from sowing until 4 out of 25 plants showed visible flowering and, under maximally inductive conditions (8 to 16 hour photoperiods), flowering was obtained in 10 to 15 days. Flowering was much later or plants were still vegetative after 62 days under photoperiods shorter or longer than 8 or 16 hours. No flowering was obtained in continuous light in the experiments reported here and it could be that the flowering observed after 58 days of continuous light (Cumming, 1967b) reflects a less direct response to photoperiod.

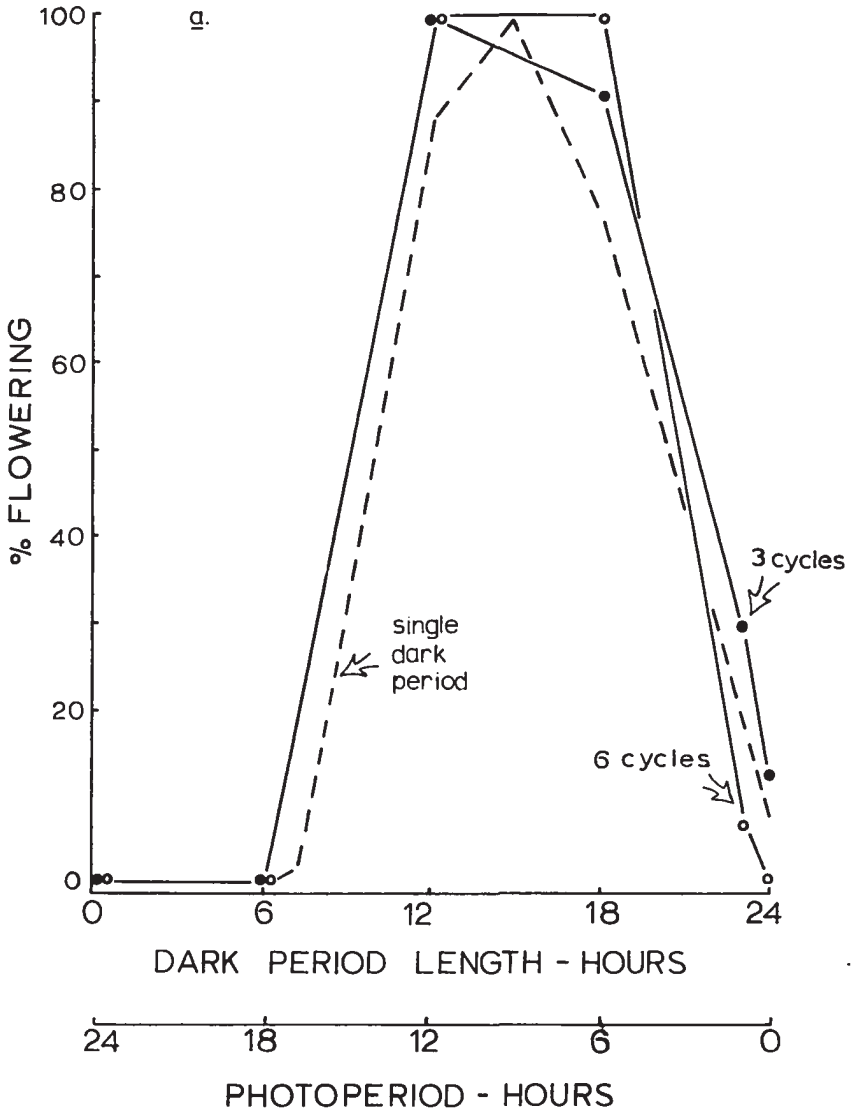
FIGURE 4.

Flowering response of C. rubrum given a single dark period of varied durations (- - -) or given 3 (●) or 6 (o) cycles of daily photoperiodic treatments:

(a) dark period of the first cycle begun $5\frac{1}{2}$ days after sowing,

(b) all plants given a 12 hour dark period commencing $4\frac{1}{2}$ days after sowing, 3 or 6 photoperiodic cycles were begun $5\frac{1}{2}$ days after sowing.

Temperature 20°C, fluorescent light of 600 ft-c intensity.


a.
100
80
60
40
20
0
% FLOWERING
3 cycles
single
dark
period
6 cycles
0
6
12
18
24
DARK PERIOD LENGTH - HOURS
24
18
12
6
0
PHOTOPERIOD - HOURS

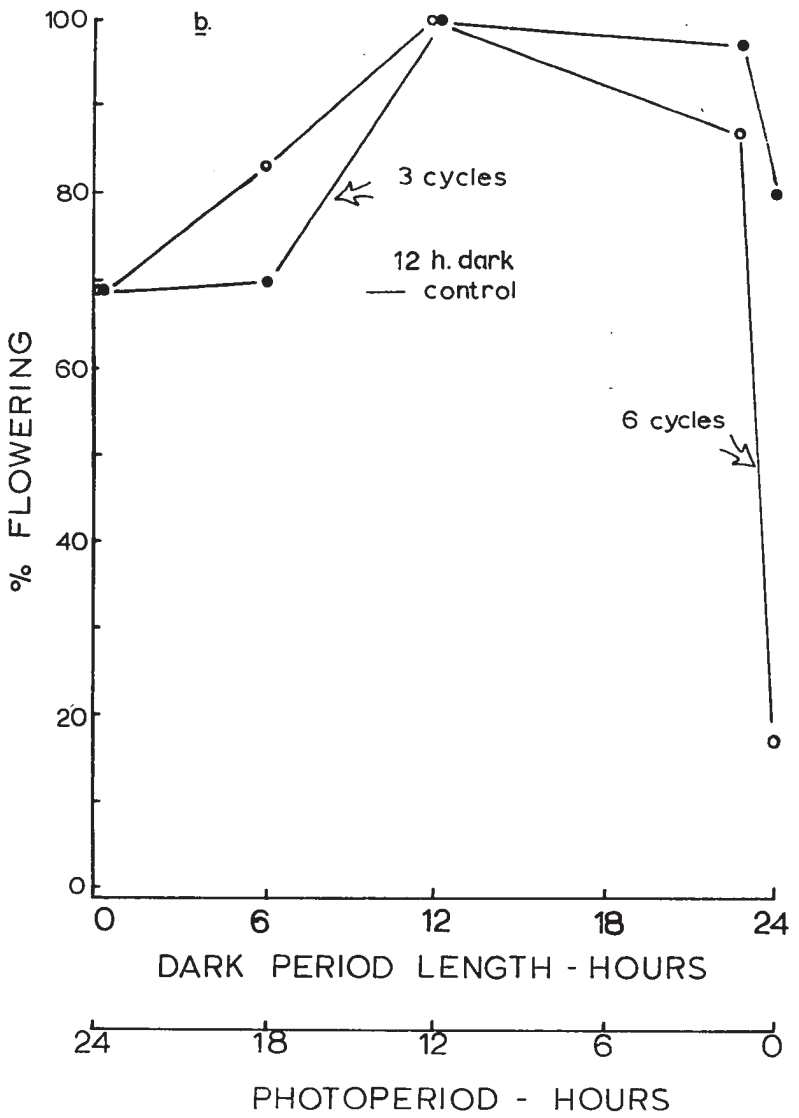
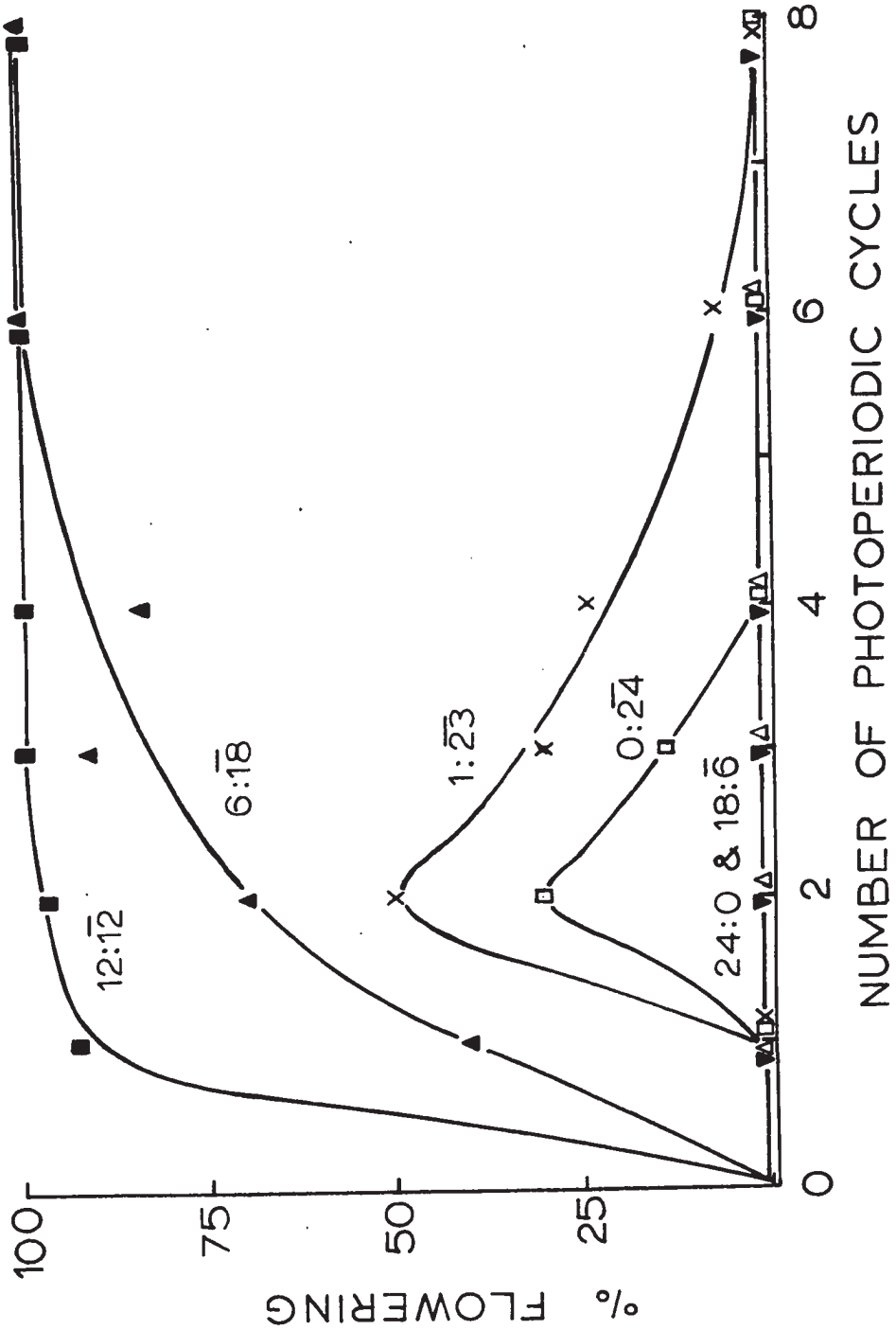

b.
100
80
60
40
20
0
% FLOWERING
3 cycles
12 h. dark
control
6 cycles
0
6
12
18
24
DARK PERIOD LENGTH - HOURS
24
18
12
6
0
PHOTOPERIOD - HOURS

FIGURE 5.

Flowering response of C. rubrum to increasing numbers of daily photoperiodic cycles (data from Figure 4a). Photoperiod lengths in hours of light : hours of darkness per day: (□) 0:$\overline{24}$; (X) 1:$\overline{23}$; (▲) 6:$\overline{18}$; (■) 12:$\overline{12}$; (▼) 18:$\overline{6}$; (Δ) 24:$\overline{0}$.


% FLOWERING
100
75
50
25
0
2
4
6
8
NUMBER OF PHOTOPERIODIC CYCLES
12:12̄
6:18̄
1:23̄
0:24̄
24:0 & 18:6̄

Depending on the method of presentation a parabolic relationship between flowering and the length of the photoperiod may appear as either a "U-shaped" (Cumming, 1963) or a "bell-shaped" curve (Figure 4).

c. Flowering in relation to cycle length.

In a large number of experiments with different species, studies of the flowering response to daily photoperiodic cycles have been extended to light:dark cycles of total durations divergent from a 24 hour length. In most instances these data were used only to show the importance of the length of darkness in a cycle and, hence, the significance of interactions between the lengths of light and darkness were often lost. However, by modifying the method of graphical presentation developed by Schmitz (1951), a three dimensional representation can be derived that combines all information in the one figure. This approach is illustrated for the short-day plants soybean and Kalanchoe blossfeldiana in Figure 6 and for the long-day plants Hyoscyamus niger and Silene armeria in Figure 7. Increasing flowering response - represented by increased shading of the squares - for each combination of a light:dark cycle has been expressed in relation to the length of both the dark period (abscissa) and the light period (ordinate).

There is very clearly a rhythmic pattern of flowering in the response of soybean and Hyoscyamus to combinations of light:dark cycles. The results with Silene, although suggestive, do not appear sufficient to establish this point and for Kalanchoe it might be concluded, as others have (cf. Lang, 1965) that there is no evidence of rhythmicity in this plant.

In soybean and Hyoscyamus rhythmicity was manifested with increasing lengths of darkness and rhythm period lengths were similar for both plants - about 24 hours. Light, however, had opposite effects on flowering when terminating darkness given to short-day and long-day

FIGURE 6.

Flowering response of soybean and Kalanchoe blossfeldiana to different combinations of light and dark periods tested in photoperiodic cycles of varying total lengths.

Soybean; number of flowering nodes per 10 plants: 0 (▣); 1 - 8 (▨); 9 - 17 (▨); 18 - 26 (▧); 27 - 35 (▩); >35 (■). Drawn from data of Wareing (1953); Blaney and Hamner (1957); Nanda and Hamner (1958, 1959).

Kalanchoe; number of days to first open flower: vegetative > 140 (▣); 110 - 140 (▨); 80 - 109(▧); 50 - 79 (▩); < 50 (■). Data redrawn from Schmitz (1951) and Harder and Gummer (1949).

The dotted line indicates the approximate border between inductive and non-inductive light:dark combinations. The line at 135° for soybean indicates the flowering for cycles totalling 24 hours in length.

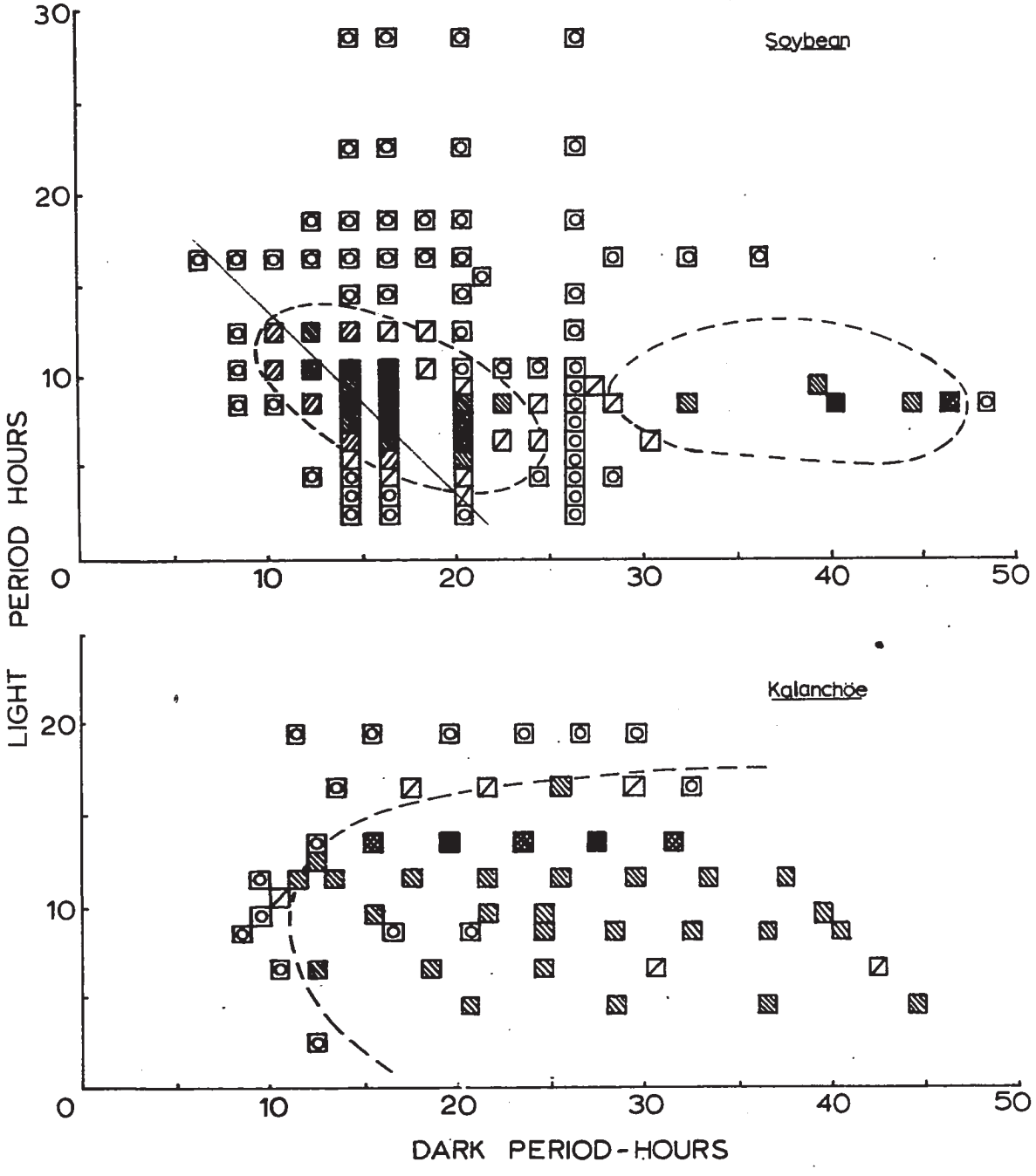

Soybean
Kalanchöe
LIGHT PERIOD HOURS
DARK PERIOD - HOURS
0
10
20
30
40
50

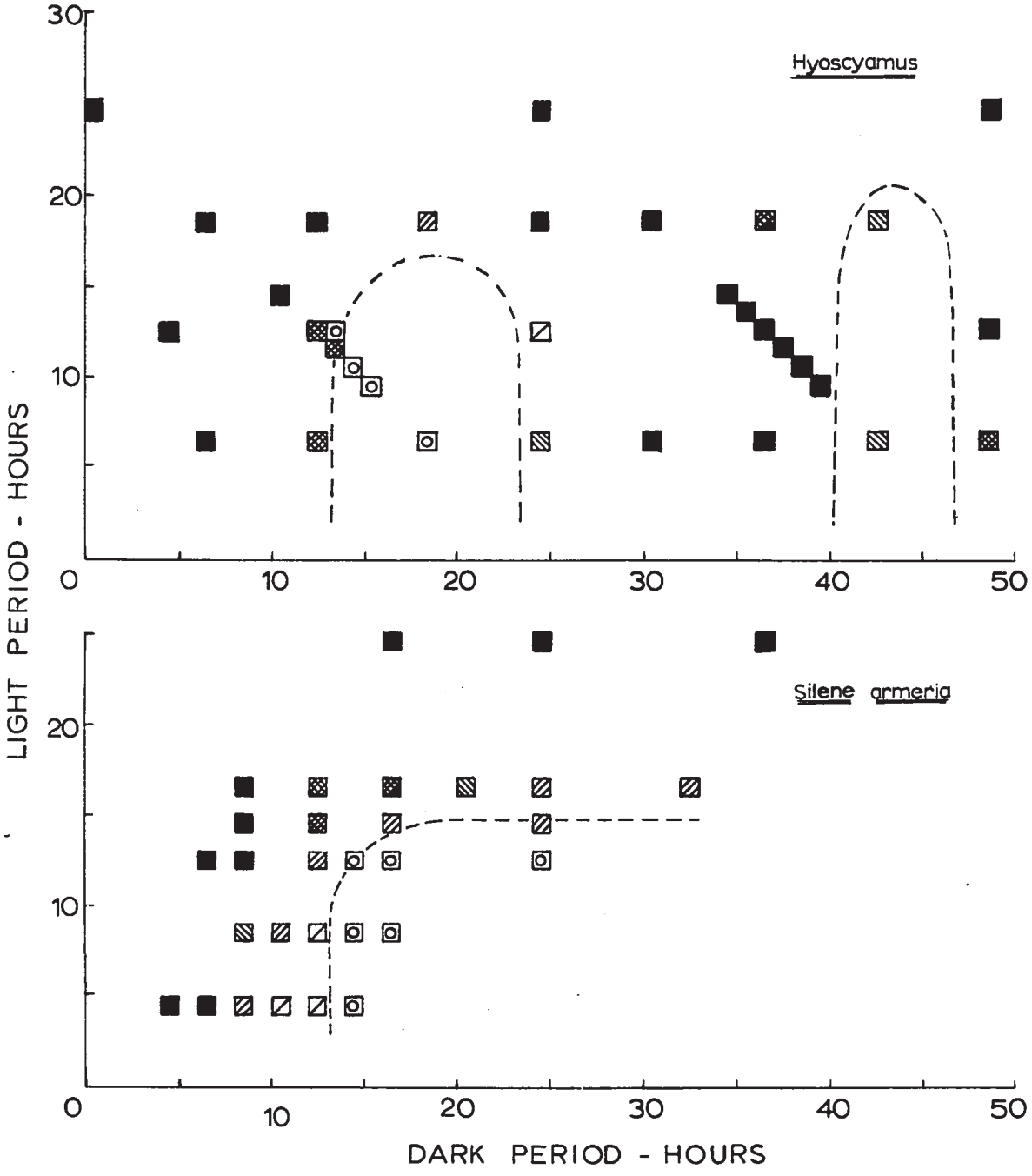
FIGURE 7.

Flowering response of Hyoscyamus niger and Silene armeria to different combinations of light and dark periods tested in photoperiodic cycles of varying total lengths.

Hyoscyamus; % plants bolted or flowering: 0 - 16 (▫); 17 - 32 (▨); 33 - 49 (▨); 50 - 66 (▧); 67 - 82 (▩); 83 - 100 (■). Drawn from data of Claes and Lang (1947) and Hsu and Hamner (1967).

Silene; % plants flowering drawn from Takimoto (1955).

The dotted lines indicate the approximate border between inductive and non-inductive light:dark combinations.


Hyoscyamus
Silene armeria
LIGHT PERIOD - HOURS
DARK PERIOD - HOURS
0
10
20
30
40
50

plants. These results also indicate that in long dark periods plants endogenously reached a phase of light or dark "liking" every 12 hours. If endogenous rhythmic changes also occur in light periods a response similar to that to darkness could be expected as the length of the light period is extended. The data in Figures 6 and 7 are insufficient to establish this point but the sudden changes from light to dark "liking" after light periods of 13 hours (soybean) or 16 to 18 hours (Kalanchoe) suggest effects on a rhythm. When the light period exceeded a critical upper value, irrespective of the length of the subsequent dark period, flowering was no longer possible.

In order to study the flowering response of C. rubrum to light: dark cycles, after the normal germination period seedlings were given 4 cycles of dark and light commencing $5\frac{1}{2}$ days after sowing. The durations of light and darkness were varied independently between 0 to 36 hours. To reduce the level of flowering, Hoagland's solution was supplied at 1/5 its normal strength. The results from such an experiment, presented in Table 3, show that flowering occurred only when the dark period exceeded 6 hours in length. Maximum flowering resulted with dark periods of 12 hours but declined with longer periods of darkness. It is probable that the rhythmic response to the length of darkness observed with a single cycle of light and darkness (Figure 2) also occurs in long dark periods of repeated photoperiodic cycles (Table 3). If a rhythm continued in long light periods as was suggested for soybean and Kalanchoe, in a light:dark cycle of $36:\overline{12}$ given to C. rubrum, light should impinge on a phase of the rhythm in which flowering would be prevented. However, increasing the length of the light period up to 36 hours did not reduce the response to a subsequent 12 hour dark period (Table 3).

TABLE 3.

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 at 5½ days after sowing. Hoagland's solution supplied at 1/5 normal strength. Temperature 20°C, 600 ft-c fluorescent light.

LIGHT PERIOD LENGTH (HOURS)	% FLOWERING						
	DARK PERIOD LENGTH - HOURS						
	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				

IV DISCUSSION.

It is evident from the results presented in this chapter that C. rubrum is a short-day plant showing sensitivity to one short day as was reported previously by Cumming (1959, 1963, 1967b). A "bell-shaped" photoperiodic response may be characteristic not only of this species but of other short-day plants such as Chenopodium amaranticolor (Konitz, 1958); Kalanchoe blossfeldiana (Bunsow, 1960); Perilla ocymoides (Moshkov, 1939); and soybean (Blaney and Hamner, 1957).

During darkness the reaction(s) controlling flowering undergoes rhythmic changes in sensitivity to light in the short-day plants C. rubrum (Cumming et al, 1965 and Figure 2) and soybean (Figure 6) and in the long-day plant Hyoscyamus niger (Figure 7). Judging from the experiments of Finn (quoted by Hamner, 1960), Silene armeria, too, may undergo rhythmic changes in sensitivity to light although this was not evident in Takimoto's results (Figure 7). Comparing the rhythmic responses of long-day and short-day plants (Figures 6 and 7) it might be suggested that an "identical rhythm or clock is read by the short-day plants in exactly the opposite fashion as by the long-day plant" (Hamner, 1960).

With Kalanchoe, despite the apparent lack of rhythmicity in the response to combinations of light:dark cycles, failure to flower in long photoperiods implies the action of a rhythm and this was also suggested for soybean (Figure 6). Furthermore, in Kalanchoe rhythmic fluctuations can be evoked by light interruptions in a long dark period (Claes and Lang, 1947; Carr, 1952; Melchers, 1956; Engelmann, 1960) or by giving a single extended dark period following five marginally inductive dark periods (Engelmann, 1960). Lack of rhythmicity in response to

light:dark cycles in Kalanchoe cannot therefore be taken as disproof of the action of a rhythm measuring photoperiod duration but, how can the response to light:dark cycles be understood? That the flowering response of Kalanchoe was insensitive to cycle length (Figure 6) but a 24 hour cycle was optimal in soybean (Figure 6 points on any horizontal line) suggests as a tentative explanation that, in Kalanchoe, the period length of an endogenous rhythm may have been entrained to different values by the various photoperiodic cycles. Certainly cycles of 4 to 24 hours in duration can entrain the period of the rhythm of petal movement in Kalanchoe (Bunsow, 1953). Offering brief light interruptions during the dark period of cycles of various durations might provide information on entrainment of a rhythm of flowering in Kalanchoe and such an approach when applied to Lemna gibba indicated that the period of the rhythm controlling flowering could be entrained by photoperiodic cycles 15, 24 or 36 hours in duration (Nakashima, 1968). On the other hand in soybean and possibly Hyoscyamus it appears that entrainment might be unimportant.

To observe a rhythm of flowering does not prove that it is acting as a clock measuring the duration of a daily photoperiodic cycle. In approaching this question Cumming and Wagner (1968) and Cumming (1969a) pointed out that a parabolic response to photoperiod might result if phasing of the rhythm of flowering in C. rubrum was controlled by the timing of the daily dusk signal. Evidence for the importance of a light-off signal in rhythm phasing had been previously reported by Cumming et al (1965) and the data in Table 3 further support this conclusion. Other workers also suggested that the light-off signal controls timing in Xanthium pensylvanicum (Hamner and Bonner, 1938; Schwabe, 1955); Pharbitis nil (Takimoto and Hamner, 1964) and Lemna perpusilla (Hillman, 1969). On the other hand, for soybean, Hamner (1960) suggested that the

light-on or dawn signal was dominant in rhythm phasing and this relationship was also favoured by Bunning in 1960. In this instance a sigmoidal and not a parabolic relationship would then result under various daily photoperiodic cycles. However it is also possible that with extremely short daily photoperiods, flowering might decline as a result of a limitation of photosynthate supply (see Parker and Borthwick, 1940, for soybean and Figures 3 and 4 for C. rubrum). Another interpretation is that sole control of rhythm phasing might not reside with the daily dawn or dusk signals alone and, as found for Pharbitis nil, both signals may control the phase of a rhythm of flowering (Takimoto and Hamner, 1964).

The inconsistencies in the experimental findings summarized in the preceding discussion emphasize three major shortcomings in our knowledge of how rhythms act as timers. Greater definition is required of both rhythm phasing and entrainment under photoperiodic cycles. Also, it may be quite illusory to stress rhythm phase control predominantly by a dawn or dusk signal of the photoperiod and individual species might well respond in different ways. Finally, too simple a view of the photoperiodic response must be avoided and response to a particular photoperiod may also be influenced quite independently by other factors such as photosynthetic supply. Experiments presented in the next chapter outline a possible approach to the question of rhythm phasing and entrainment in the photoperiodic control of flowering of C. rubrum. The question of photosynthetic effects has been avoided in this approach by estimating rhythm phasing on the basis of rhythm peak timing alone.

CHAPTER III

PHOTOPERIODIC CONTROL OF PHASING OF THE RHYTHM OF FLOWERING.

I. INTRODUCTION.

Experiments discussed in the previous chapter gave general support to the concept of a rhythm measuring the duration of the daily light:dark cycle in the control of flowering. However, demonstration of a rhythm of flowering is neither proof nor disproof of its actual involvement in time measurement in daily photoperiodic cycles, and, before applying Bunning's hypothesis to a photoperiodic situation it must be established to what degree, and how, photoperiod controls the phase of the endogenous rhythm.

In Chenopodium rubrum the dusk or light-off signal can be of major significance (see Cumming et al, 1965 and Chapter II); yet it has not been established that the phase of the rhythm is controlled by the light-off signal under photoperiodic conditions.

To approach the problem of rhythm phasing in the photoperiodic control of flowering, attempts have been made to relate flowering response to the photoperiodic control of an indicator rhythm such as leaf or petal movement (Bunning, 1954; Bunsow, 1960; Holdsworth, 1964; Ioffe, 1968; Halaban, 1968b;Bunning, 1969; Denney and Salisbury, 1970), dark respiration (Hillman, 1970) or betacyanin content (Wagner and Cumming, 1970). Halaban's (1968b)experiments are particularly significant as they suggest that the photoperiodic control of the phase of the rhythm of leaf movement is correlated with the time in the daily dark period of greatest sensitivity of flowering to a brief light interruption of darkness.

Unfortunately, the significance of the results using an indicator rhythm such as leaf movement rests on the assumption that the phasing of the overt indicator rhythm truly reflects the phasing of the putative rhythm controlling flowering. In fact, a relationship between leaf movement and flowering may not hold up under all conditions and recently Denney and Salisbury (1970) have reported that the status of a rhythm of leaf movement was not a valid indicator of the photoperiodic clock controlling flowering in Xanthium strumarium. Furthermore, Ioffe (1968) found that the rhythm of leaf movement of Perilla oleifera was not only rephased by the photoperiod but was suppressed by the action of those light interruptions of darkness which were unfavourable to flowering. Halaban (1968c), however, did not examine thoroughly the effect of giving light interruptions on the phasing under different photoperiods of the rhythm of leaf movement in Coleus. Finally, in Chenopodium rubrum it was evident in the experiments of Wagner and Cumming (1970) that the phase of an indicator rhythm (betacyanin content) could be changed without any effect on the phase of the rhythm of flowering. Hence it is doubtful that indicator rhythms can be used to estimate the phasing of the rhythms controlling flowering.

To avoid the difficulties and ambiguities associated with the use of indicator rhythms, direct measurements are reported here of the phasing of the actual rhythmicity of flowering of C. rubrum under various photoperiodic conditions. C. rubrum has been used because it offers both ease of handling and rhythmic sensitivity of its flowering response to a single dark period of varied duration (see Chapter II). To assess rhythm phase control and entrainment, a single light pulse of 6, 12 or 18 hours duration has been given at different times and hence at different phases of the free running rhythm of flowering. Following the light pulse

an advance or delay in the phase of the rhythm was assayed by allowing the rhythm to run free in a subsequent dark period of varied duration, then the plants were returned to continuous light until dissected for flowering. A detailed analysis of photoperiodic entrainment by a daily 6 hour photoperiod (6:$\overline{18}$) was also undertaken by measuring the phasing of the rhythm following 1 to 5 photoperiodic cycles. Shifting of the phase of the rhythm by a single light pulse and by repeated photoperiodic cycles supports the concept of a rhythm that is sensitive to photoperiod and thus capable of measuring photoperiod duration. For 6, 12 or 18 hour daily photoperiods the steady state phasing of the rhythm reflected the effect of photoperiodic cycles on flowering.

II MATERIALS AND METHODS.

Chenopodium rubrum (origin 60°47'N 137°32'W) was used in all these experiments and the materials and methods were as described in Chapter II.

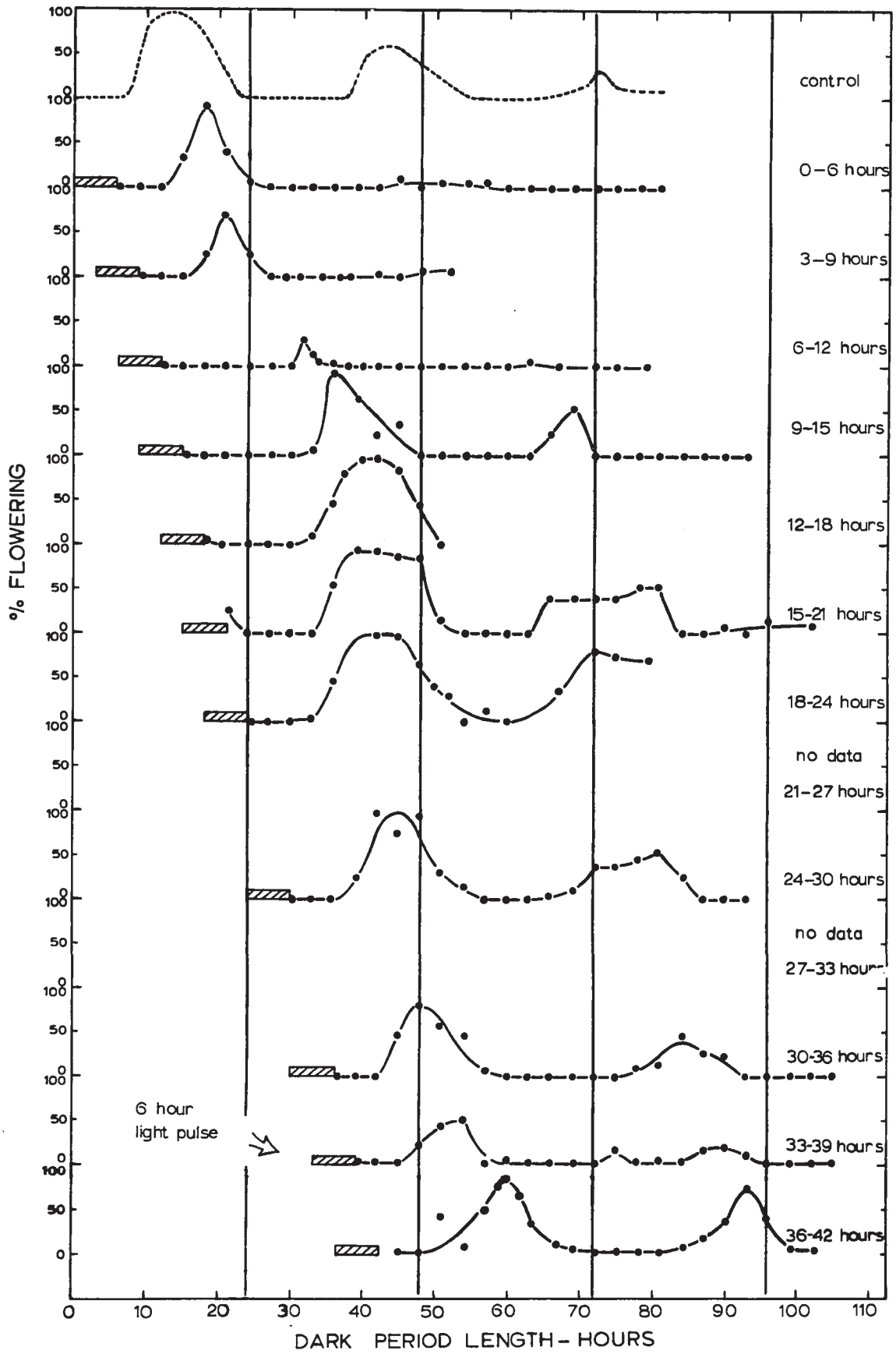
III RESULTS

a. Phasing response of the rhythm to a six hour light interruption.

As was shown in the previous chapter (Figure 2), in dark periods of varied duration the capacity to flower undergoes regular oscillations with a free running period of about 30 hours and a phase fixed by the time of the light-off signal. To analyse phasing of this rhythm by photoperiodic treatments, plants germinated and grown in continuous light were placed in darkness $5\frac{1}{2}$ days after sowing of the seed; in all figures the time scale commences at a point representing $5\frac{1}{2}$ days after sowing.

FIGURE 8.

Flowering response of C. rubrum to a single dark period of varied duration interrupted with a 6 hour pulse of red light (115 $\mu w/cm^2$) that was given at different times in darkness. Dark period temperature 20°C; temperature in red light 26°C. Upper curve (broken line) control redrawn from Figure 2.


% FLOWERING
DARK PERIOD LENGTH - HOURS
control
0-6 hours
3-9 hours
6-12 hours
9-15 hours
12-18 hours
15-21 hours
18-24 hours
no data
21-27 hours
24-30 hours
no data
27-33 hours
30-36 hours
33-39 hours
36-42 hours
6 hour
light pulse

Single 6 hour light periods were used to interrupt darkness at various times between 0 and 36 hours and the phase of the rhythm of flowering was assayed by varying the length of the subsequent dark period by three hour increments. In the bulk of the experiments low intensity (115 $\mu w/cm^2$) red irradiation was used although, as shown later, it was possible to interchange red light with a higher intensity of fluorescent light (600 ft-c) In Figure 8 the bulk of the results are from the one experiment (C 79) there being two dishes and hence 30 plants sampled for each value. Identical responses were obtained in other experiments. As shown later rhythm phase shifting was identical at temperatures of 26°C or 20°C (Table 8).

By comparison to the rhythm of flowering in non-irradiated control plants (Figure 8, upper curve), a 6 hour light period, when interrupting darkness at different times, could shift the position of the peaks of the rhythm. Rhythm phase shifting was most noticeable when light impinged on the positive slope of either the first or the second peak of the free running rhythm (Figure 8). If the light period was superimposed on or fell later than the peak, e.g. interruptions commencing between 12 and 24 hours in darkness, there was little influence on rhythm phase although the peaks of the rhythm were now rather broad. This was particularly true for dark periods 70 to 80 hours in length. In part, broadening of the rhythm peak may have reflected a summation of the inductive effect of the first dark period (e.g. 12 hours dark, 6 hours light) with that of the second dark period.

Before dealing in detail with the question of rhythm rephasing by the light interruption, it should be noted that the phase of the whole rhythm could be altered and not just the shape and slope of the rhythm. To emphasise this point, the results in Figure 9 show that relative to the uninterrupted controls the timing of both the peaks and troughs of the rhythm were shifted by a 6 hour period of fluorescent light starting

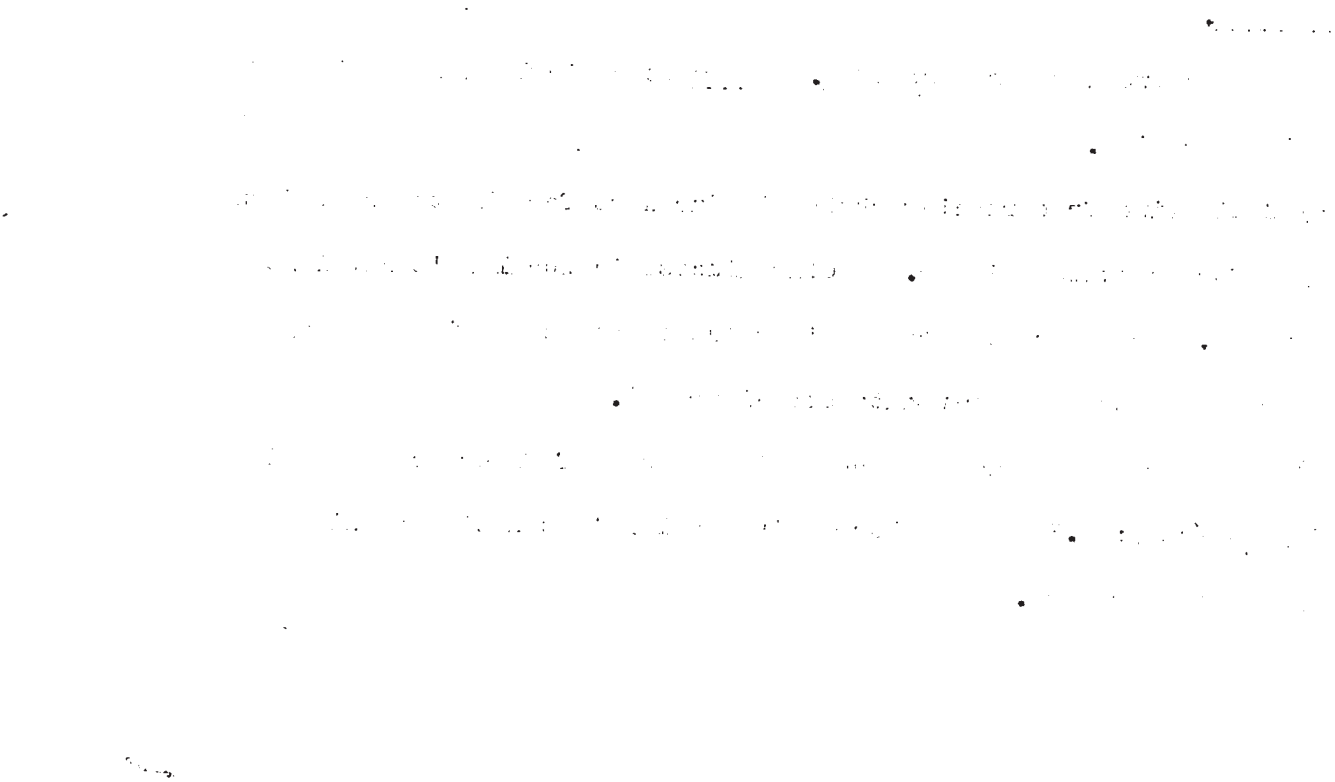
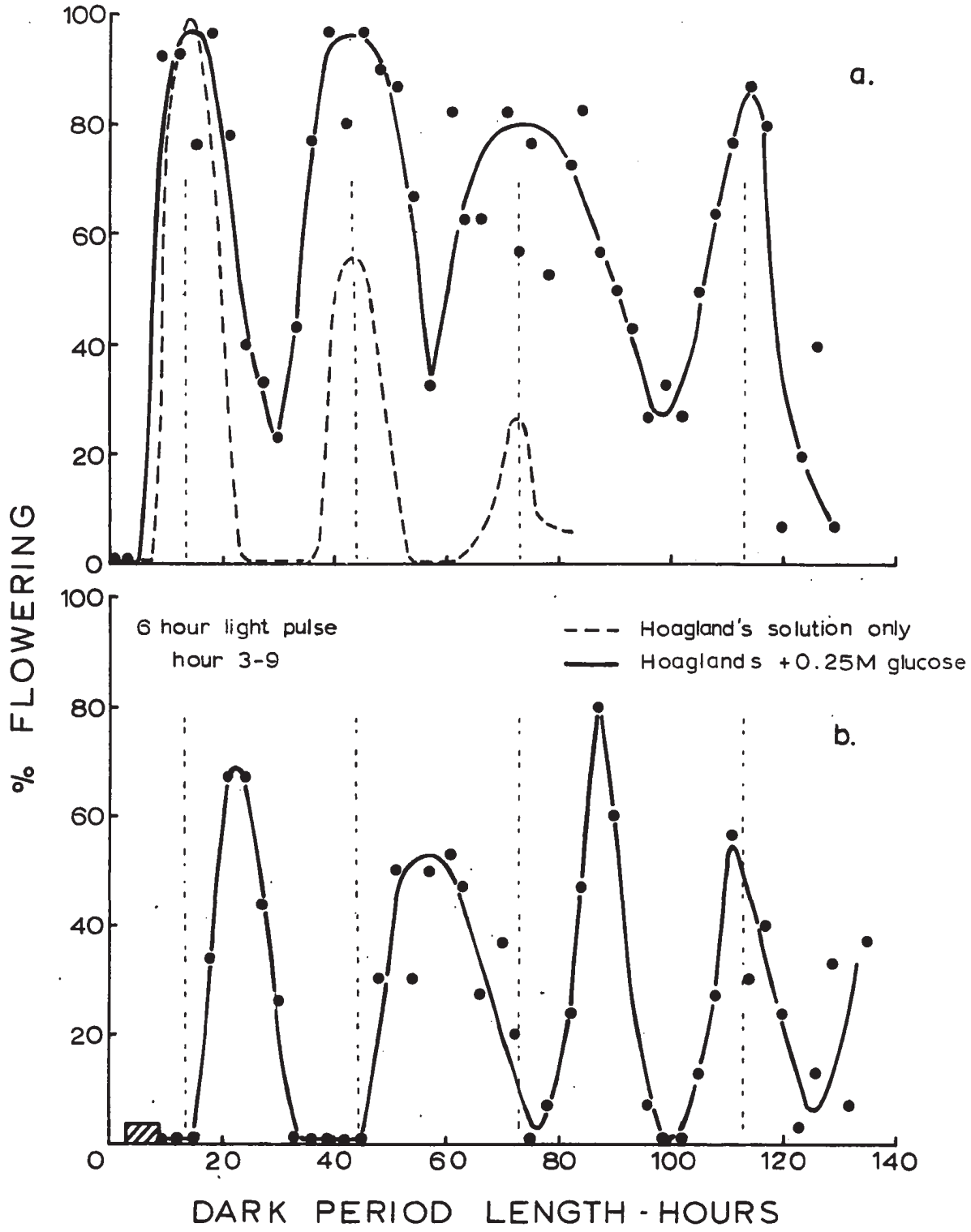


FIGURE 9.

Flowering response of C. rubrum to a single dark period of varied duration.

(a) Solid line: free running rhythm in darkness for plants treated one day before darkness with 0.25 molar glucose in Hoagland's nutrient solution. Broken curve: Hoagland's solution alone applied one day before darkness (for raw data see Figure 2).

(b) Dark period interrupted once with 6 hours of fluorescent light (1037 $\mu w/cm^2$); 0.25 molar glucose in Hoagland's solution applied one day before darkness.


100
80
60
40
20
0
a.
100
80
60
40
20
0
b.
6 hour light pulse
hour 3-9
--- Hoagland's solution only
—— Hoagland s +0.25M glucose
% FLOWERING
0
20
40
60
80
100
120
140
DARK PERIOD LENGTH - HOURS

at hour 3 in darkness. Therefore, as applied in the subsequent discussion, the timing of rhythm peaks will accurately reflect the phasing of the rhythm.

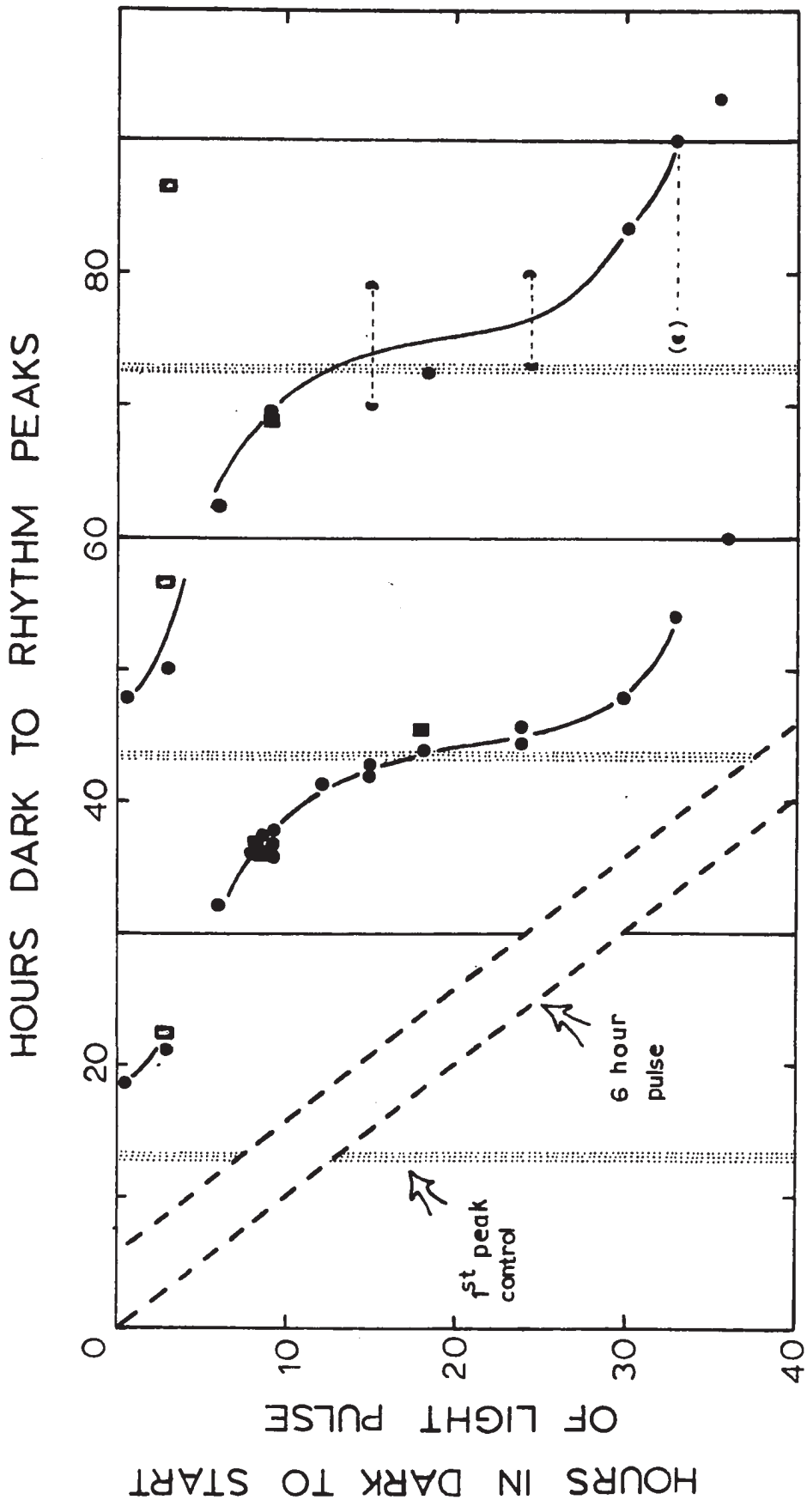
Two further comparisons are evident in the results in Figure 9. The application of glucose sustained the rhythm of flowering (Figure 9a) although the timing of peaks for glucose treated and non-glucose treated plants was unaltered. A similar response to glucose treatment was reported by Cumming (1967a) and in his results and those presented in Figure 9, rhythm period lengths became rather variable after 90 hours of darkness. This effect was reflected in a severe aetiolation and often death of the plants after 125 hours of darkness even with the supply of glucose. Thus measurement of rhythm phasing has been restricted subsequently to measurements in dark periods no longer than 100 hours. Secondly, the similarity of the response to 6 hours of red light (Figure 8) compared to 6 hours of fluorescent light (Figure 9b) suggest that the effective wavelengths for rhythm phasing are those in the red region of the spectrum. This latter point is confirmed in Chapter IV.

Figure 10 has been derived from the data in Figure 8 and illustrates the relationship between rhythm phasing, i.e. peak times, and the timing of exposure to a 6 hour light period. As an example of the derivation, from Figure 8, a 6 hour light period starting at hour 9 in darkness induced rhythm peaks at hour 37 and hour 69 in darkness. Peaks for the uninterrupted (i.e. control) rhythm, however, occurred at 13, 43 and 73 hours in darkness (dotted lines in Figure 10). On combining all results from Figure 8 with those from duplicate experiments it can be seen that there was good agreement between experiments and between rephasing by red or fluorescent light.

As an indication of the accuracy of these derived values, when a 6 hour red light period starting at hour 9 in darkness was given a

FIGURE 10.

The relationship between the time in the dark period when a 6 hour light exposure was given and the time of maximum flowering at the first, second or third peaks of the rhythm of flowering of *C. rubrum* given dark periods of varied duration. (●) red light pulse; (■) fluorescent light pulse; (□) fluorescent light pulse plus glucose (0.25 M) one day before darkness (Representative raw data in Figures 8, 9 and Chapter IV).


HOURS DARK TO RHYTHM PEAKS
0
20
40
60
80
HOURS IN DARK TO START OF LIGHT PULSE
0
10
20
30
40
1st peak control
6 hour pulse

maximum of 70% flowering (average of five experiments) resulted after a dark period of 37 ± 0.1 hours of darkness. The position of the peak was significantly different ($p = 0.001$) from the timing of control peaks. The response curves in Figure 10 for the first and second peaks of the new free running rhythms were symmetrical and therefore rhythm period length remained close to 30 hours even when phase was changed.

The type of graphical presentation illustrated in Figure 10 also shows that it is the time in the cycle of the rhythm at which the 6 hour light pulse was administered that determined whether or not the phase of the rhythm was reset. Generally, it is desirable to follow more than two free running cycles to assay for a new phase but as discussed earlier it was not possible to assess accurately the phase of the rhythm in dark periods longer than 100 hours. In other organisms similar phase shifting of rhythms has been reported (see Bruce, 1960; Winfree, 1970).

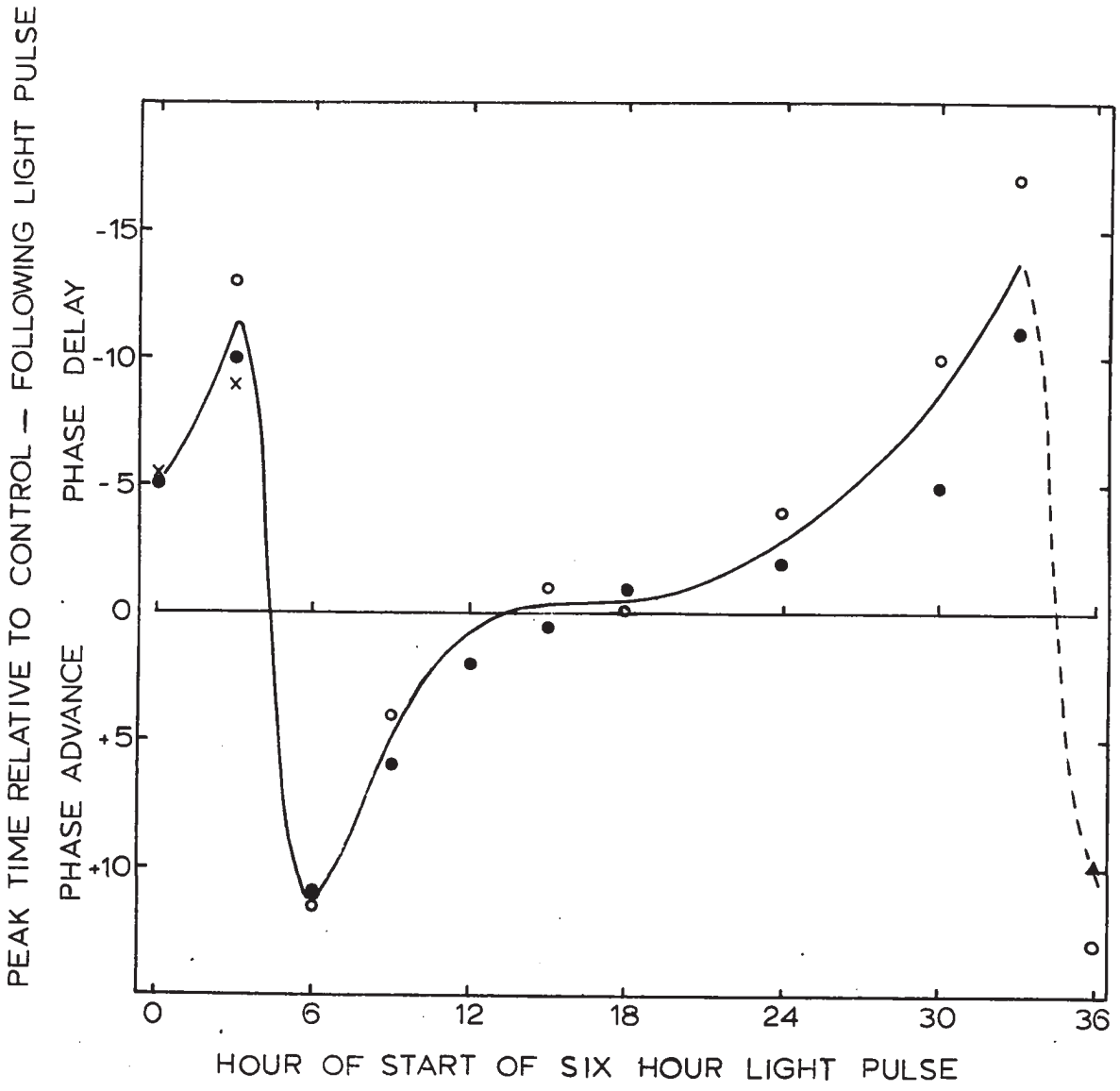
It was difficult sometimes to estimate rhythm peak times for the second peak of the rephased flowering rhythm because the peaks became rather broad and reduced in amplitude (see Figure 8). Transient changes in the phase of a rhythm could account for peculiarities of rephasing as these are known to occur under conditions where light pulses induce a change in the phase of the rhythm (Bunning, 1967). On the other hand, in the experiments reported in Figures 8 and 9 the plant population as a whole was resynchronized immediately upon rephasing to a new rhythm phase since all plants, or 8 out of 10 plants, flowered at the time of the next peak. However, by the second peak it appeared that the plant population had split into two physiologically distinct groups of plants, those with a stable rephasing and those reverting to the original phase of the control rhythm that gave a peak at 73 hours of darkness.

For the present discussion the timing of the light-on signal has been chosen arbitrarily as a reference point although, as will be discussed later (Figure 19), these experiments may indicate that changing sensitivity to light was related to the timing of both the light-on and the light-off signals. It may be relevant to emphasise here that, irrespective of how light pulses controlled rhythm phase, there always appeared to be a preparatory length of darkness between the light period and the next rhythm peak. A light exposure could be no closer than 12 hours to the next peak of the rhythm. The time between the light-off signal and the next peak of the rhythm in darkness approached this lower limit for light periods that commenced at 0, 3 or 30 hours in darkness.

The results in Figure 10 can also be expressed in terms of phase advances or delays of the rhythm peaks relative to the timing of the uninterrupted control rhythm peaks. A phase advance occurs when the peak occurs earlier than that of the control and by convention it is given a positive sign ($+\Delta\phi$). In reality designation of a phase delay relative to a specific peak of the control rhythm might be confused experimentally with an advance of the subsequent peak of the control rhythm. However, since a complete period of the rhythm - 30 hours - would be covered by light pulses commencing between 0 and 30 hours in darkness rhythm rephasing can be inferred by arbitrarily dividing the abscissa time scale into 30 hour segments (Figure 10). This also appears to give a true picture of which peaks are rephased and in which direction. For instance, a light pulse from 0 to 6 hours in darkness can accurately be designated as inducing a phase delay of the first and subsequent rhythm peaks. Also experiments to be described in Chapter IV (Table 5) established that a 6 hour light pulse starting at hour 9 in darkness effectively advanced the timing of the second peak from the

FIGURE 11.

Rhythm peak timing - hours 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 hour light period. Rephasing of the first (×), second (●), third (○) or fourth (▲) peak of the free running rhythm. Values derived from Figure 10.


PEAK TIME RELATIVE TO CONTROL — FOLLOWING LIGHT PULSE
PHASE DELAY
PHASE ADVANCE
-15
-10
-5
0
+5
+10
0
6
12
18
24
30
36
HOUR OF START OF SIX HOUR LIGHT PULSE

forty-third to the thirty-seventh hour of the dark period ($+\Delta\phi$ = 6 hours). Thus, at some time between hour 0 and hour 9 in darkness resetting changed from a delay of the first and subsequent peaks to an advance of the second and subsequent peaks. Inspection of Figure 10 suggests that phase delay of the first peak changed to an advance of the second peak with light pulses commencing at the sixth hour of darkness. No or little phase shift of the second and third peaks resulted from light pulses commencing between hours 12 and 24 in darkness and it appears reasonable to suggest that light interruptions at hours 30 and 33 acted to delay the timing of the second and third control peaks.

In Figure 11 phase advance or delay for each peak has been plotted on the ordinate scale against the time of commencement of the 6 hour light pulse (abscissa). Light pulses commencing between 0 and 30 hours in darkness would cover one complete cycle of a rhythm with a 30 hour period. Hence, pulses beginning at hour 30, hour 33 or hour 36 are comparable in time to interruptions commencing at hours 0, 3 and 6 and, as shown in Figure 11, there are only small differences in the overlapping portions of the curve. As a light period given from 0 to 6 hours in darkness provided only a light-off signal, similar rephasing that resulted from a light period given from 30 to 36 hours in darkness may have depended on timing of the light-off signal alone. Nevertheless, as discussed later (Figure 19) at other times in darkness changing sensitivity to the timing of the 6 hour light exposure probably reflected action of both light-on and light-off signals.

It has been customary in the literature to present resetting data in terms of phase shifts and on a "Subjective Circadian Time" scale. In Figure 11 for a rhythm with a 30 hour free running period this means that light pulses commencing at hours 0, 15 and 30 in darkness would

have commenced at circadian times 0, 12 and 24 respectively; i.e. $\frac{24}{30}$ or $\frac{4}{5}$ of a 30 hour period. A circadian time scale adds some interpretive potential to the data in Figure 11. Rhythm phasing by a daily 6 hour photoperiod can then be assessed by assuming that the circadian period (e.g. 30 hours) is entrained to a period of 24 hours; this will be discussed later.

By definition, no phase advance or delay occurs when the timing of rhythm peaks is identical for control and irradiated plants. This point of no rephasing falls at the time in darkness of the peak of the control rhythm of flowering. Rhythm phase delays or advances greater than 10 hours resulted during the early hours of darkness and there was a dramatic change from a delay to an advance at hours 3 to 6 whether the first, second or third rhythm peaks were measured. A similar change from a delay to an advance is apparent for the light period commencing at hour 36 in darkness.

A significant feature of a phase response curve such as the one in Figure 11 is that it can be used to predict the relationship between phase advances and delays that will result under photoperiodic conditions. As an example of such usage, Figure 12 presents the calculated phasing resulting from the imposition of a 6 hour photoperiodic regime commencing with 18 hours darkness and then 6 hours light per day. With a single photoperiodic cycle a light-on signal would impinge on the control free running rhythm at the eighteenth hour of darkness. From the upper curve, Figure 12 or Figure 11, it can be seen that this would generate, at the most, a phase delay of the next peak of the rhythm of half an hour ($-\frac{1}{2}$). Accepting that the first peak occurs at hour 13 in darkness and that the period of the rhythm is 30 hours (see Figure 2) a single photoperiodic cycle delays the next peak from hour 43 (i.e. 13+30

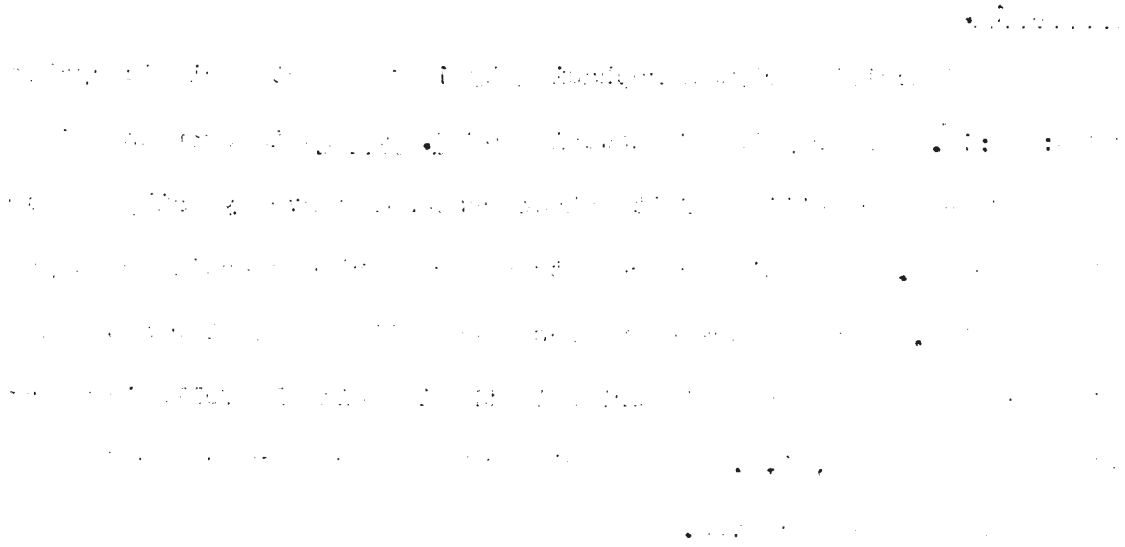
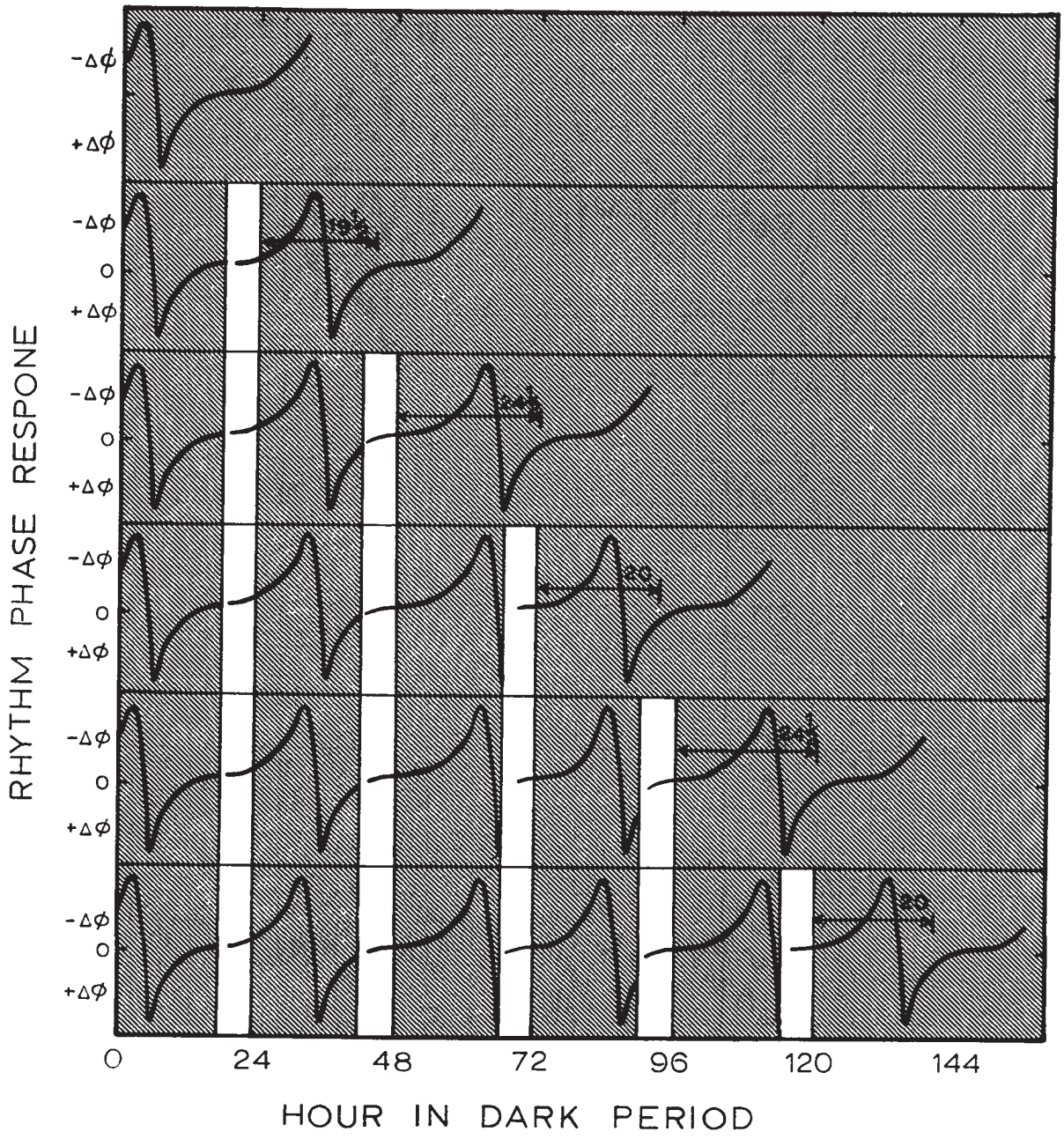
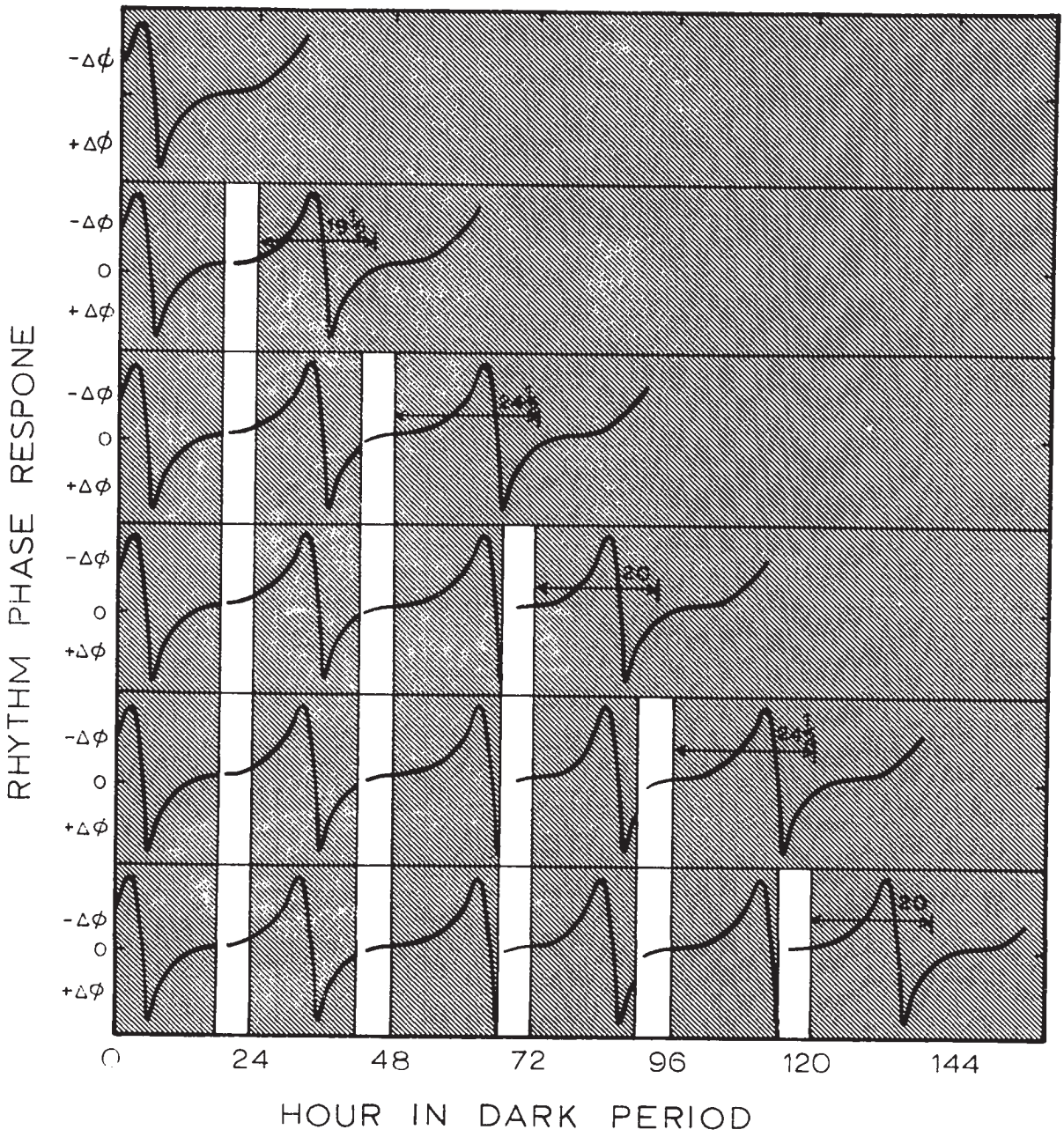


FIGURE 12.

Calculated rhythm rephasing by 1 to 5 photoperiodic cycles of L:D 6:$\overline{18}$. The rhythm of flowering of *C. rubrum* is expressed in terms of phase resetting of its phase response curve by daily 6 hour light periods. Zero time represents a dark period starting $5\frac{1}{2}$ days after sowing. Phase resetting has been estimated in terms of the time from the end of a photoperiod to the immediately following peak time of the rhythm, i.e. at the point of no phase advance or delay of the phase response curves.


RHYTHM PHASE RESPONE
-Δφ
+Δφ
-Δφ
0
+Δφ
19½
24
20
24½
20
0
24
48
72
96
120
144
HOUR IN DARK PERIOD


RHYTHM PHASE RESPONE
-Δφ
+Δφ
-Δφ
0
+Δφ
19½ hr
24½ hr
20 hr
0
24
48
72
96
120
144
HOUR IN DARK PERIOD

hours) to hour $43\frac{1}{2}$ ($13+30-(-\frac{1}{2})$) hours and the time from the light-off signal of the 6 hour photoperiod to the peak will be $19\frac{1}{2}$ hours ($43\frac{1}{2}-24$): timing from the light-on signal of the photoperiod $25\frac{1}{2}$ hours. As illustrated in Figure 12, similar calculations can be made for a second photoperiodic cycle but now the photoperiodic light pulse commences at hour 42 or $1\frac{1}{2}$ hours before the calculated rhythm peak time at $43\frac{1}{2}$ hours. This generates a phase advance of +1 hour (Figure 11) so that the next peak falls at $72\frac{1}{2}$ hours ($43\frac{1}{2}+30-(+1)$) and the time from the light-off signal at the forty-eighth hour to the next peak is $24\frac{1}{2}$ hours. With more cycles the phasing of the rhythm approached a fixed value, each peak of the rhythm falling 20 to $24\frac{1}{2}$ hours after the last light-off signal of the photoperiod. An actual steady state of rhythm resetting was not achieved in these calculations (Figure 12) but would be expected when there was a daily phase advance of 6 hours. In the latter instance the rhythm would be reset repetitively by each photoperiod so that successive peaks of flowering would be 24 hours apart (i.e. a rhythm period of 30 hours with a phase advance of 6 hours). From the phase response curve (Figure 11) a 6 hour phase advance would result if the photoperiod commenced at about 8 hours and the peak of the rhythm would therefore fall at about 23 hours after the last light-off signal of the photoperiod.

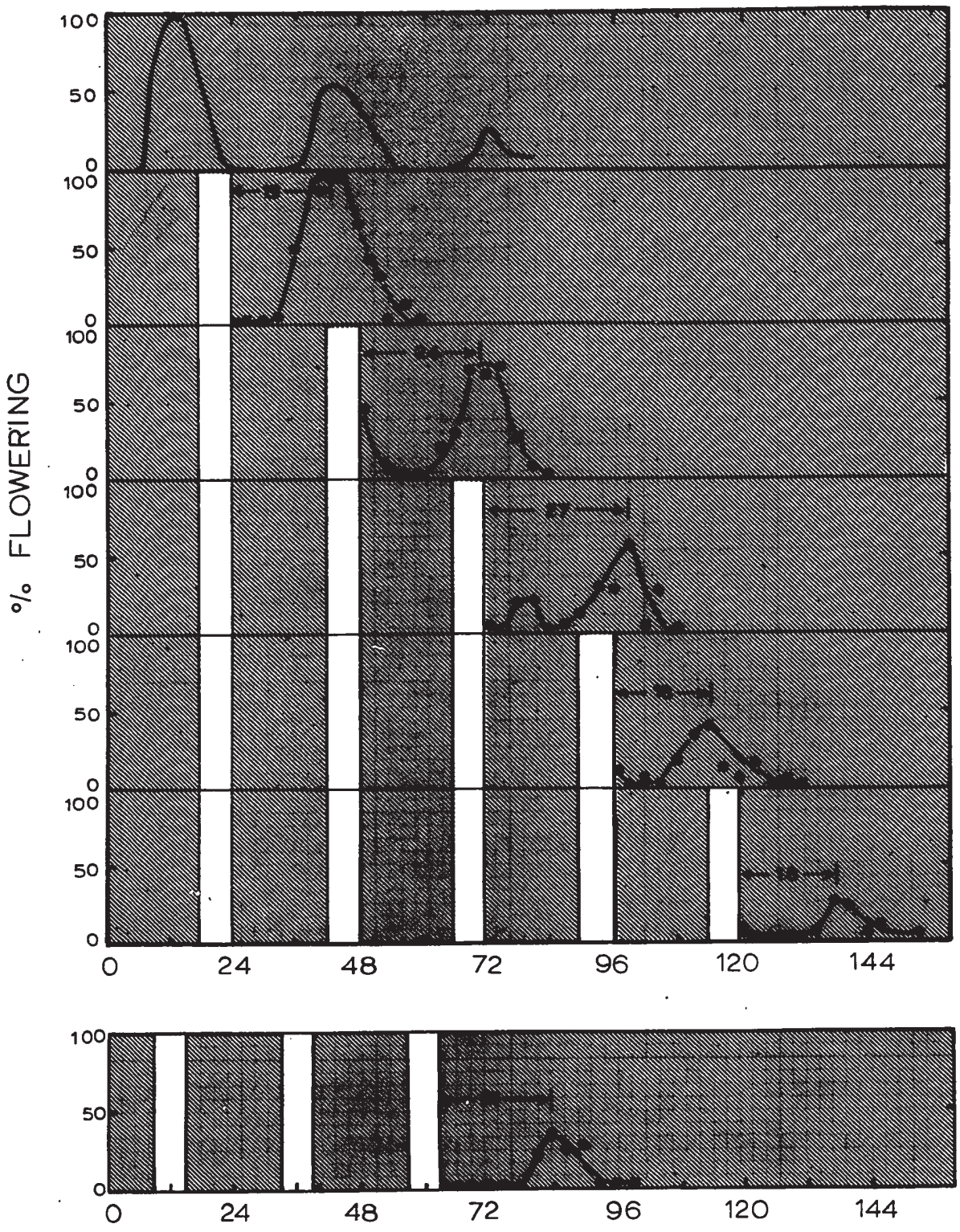
On the other hand in addition to rhythm phase being repetitively reset by daily photoperiodic cycles it might be assumed that the period of the rhythm is also entrained to a 24 hour value. Imposition of a 24 hour or Subjective Circadian Time scale on the abscissa time scale of Figure 11 allows calculation of phasing of a rhythm that is entrained to a 24 hour period. Then the peak of the rhythm would occur at the start of a daily 6 hour photoperiod (no daily phase advance or delay) or, in other words, 18 hours after the last light-off signal of the photoperiod.

Results of actual measurements of steady state phasing of the rhythm by 6 hour daily photoperiods are presented in Figure 13. The first dark period commenced $5\frac{1}{2}$ days after sowing and following 1 to 5 photoperiodic cycles plants were given a single dark period of varied duration so that the phasing of the rhythmic flowering response could be assessed. A steady state value of the phasing of the rhythm was apparent after four photoperiodic cycles and the peak occurred about 18 hours after the last light-off signal of the photoperiod. The gradual decline in flowering with age was to be expected as plants were exposed to photoperiods of low intensity red light; i.e. virtually non-photosynthetic conditions. Replicate experiments confirmed that once the phasing of the rhythm approached a steady state value, i.e.after four cycles, the rhythm peak occurred between 18 to 20 hours after the light-off signal of the photoperiod. No explanation is apparent for the small and early peak of flowering obtained after three photoperiodic cycles. Possibly this peak reflects plants whose free running rhythm was not rephased and hence gave a peak at 73 hours,but the occurrence of this peak has not been verified.

Agreement between observed and calculated values of rhythm phasing (Figures 12 and 13) was evident only for the first two photoperiodic cycles. Small changes in the positioning of the phase response curve (Figure 11) and in the value assumed for rhythm period length could have large cumulative effects on calculations of rhythm phasing. Hence, the values for the time from the light-off signal to rhythm peak times shown in Figure 12 must be regarded as an estimate only. On the other hand divergence between observation and calculation may be indicative of rhythm period entrainment as well as repetitive phase resetting. With entrainment the peak of the rhythm was calculated to occur at the start of the daily 6 hour photoperiod. This value agrees with that observed in

FIGURE 13.

Rephasing of the period length of the rhythmic flowering response of C. rubrum subjected to 1 to 5 photoperiodic cycles of 18 hours darkness, 6 hours red light per day. Zero time is $5\frac{1}{2}$ days after sowing. Uppermost curve the normal free running rhythm of flowering in darkness (see Figure 2). Lowermost curve 2 cycles of 18 hours dark, 6 hours light after an initial cycle of 9 hours dark, 6 hours light. Phasing is estimated as the time from the end of the last light pulse to the time of the rhythm peak.


% FLOWERING
100
50
0
0
24
48
72
96
120
144
HOUR IN DARK PERIOD

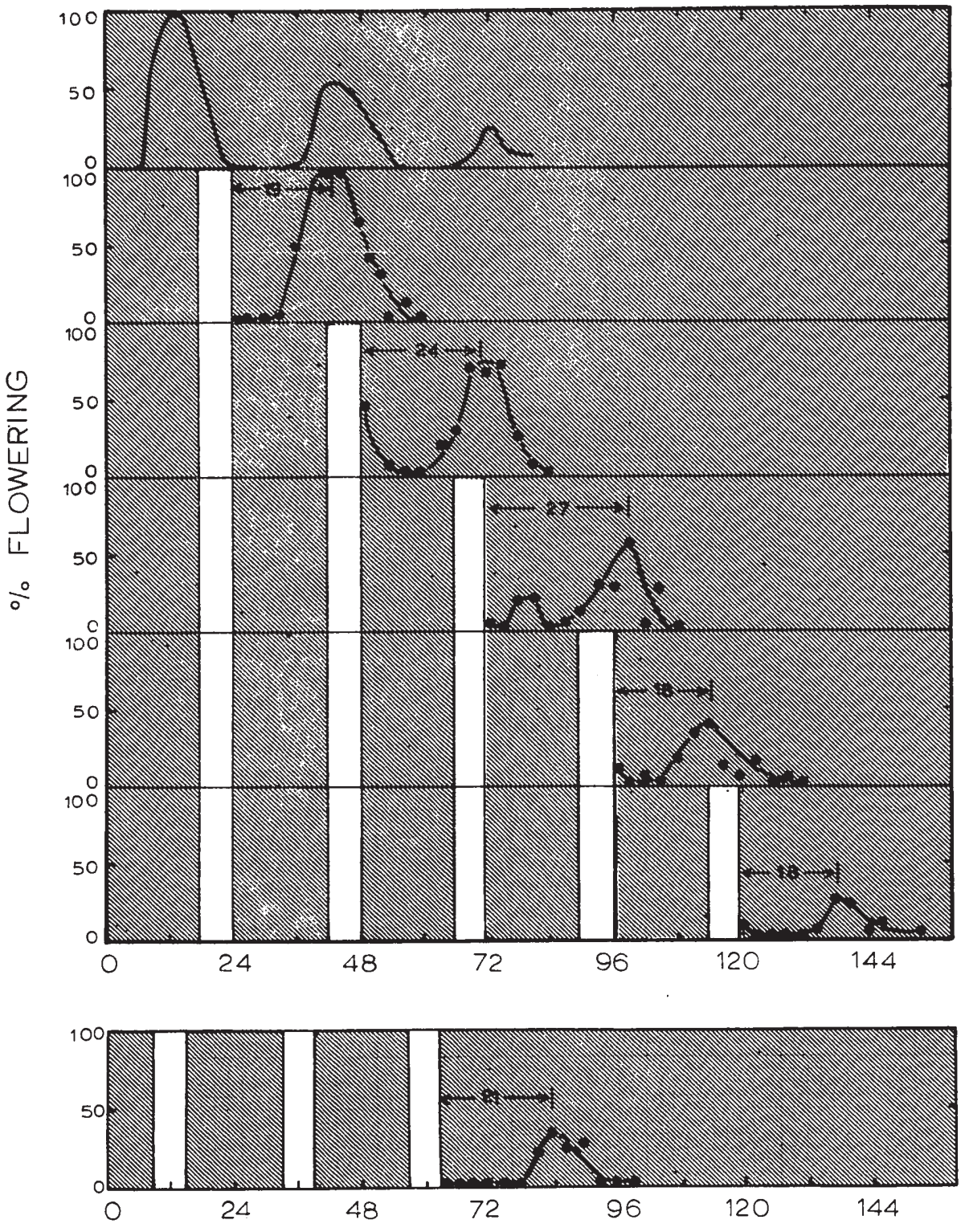

% FLOWERING
100
50
0
24
27
18
21
0
24
48
72
96
120
144
HOUR IN DARK PERIOD

Figure 13, i.e. 18 hours after the light-off signal of the last photoperiod.

In an attempt to obtain more rapid rephasing of the rhythm without significant entrainment, a further experiment was performed in which a light:dark cycle of 9 hours darkness: 6 hours light preceded two cycles of 18 hours dark: 6 hours light (lower curve, Figure 13). Almost immediate attainment of steady state rephasing might be expected under these conditions. Indeed, while the first cycle gave a time from the light-off signal to rhythm peak of 22 hours (Figure 8), two cycles gave a value of 20 hours and three cycles (Figure 13) a value of 21 hours. These values are close to that of 23 hours calculated on the basis of repetitive phase resetting and little or no entrainment might be expected when only one or two 24 hour cycles were given.

b. Phasing response to 12-hour light interruptions.

To examine the control of rhythm phase in 12 hour photoperiods, single 12-hour red irradiations were inserted at different times in darkness in the same manner as previously elaborated in Figures 8 and 10 for 6-hour light pulses. The raw data presented in Figure 14 and the derived relationship between rhythm peak times and the time of initiation of the light pulse (Figure 15) both indicate that the phase of the first rhythm peak maintained a fixed relationship to the timing of the light pulse and hence to the timing of the light-on or the light-off signal. Combining all the results for the first and second rhythm peak times in Figure 15, the period of the rhythm was close to 30 hours, rhythm peaks occuring on the average at $13\frac{1}{2}$ and 46 hours from the light-off signal. Unlike the results obtained with 6-hour light interruptions (Figures 8 and 10) there was no indication of changing sensitivity to the timing of the light period. Therefore a 12-hour light interruption was capable not only of rephasing the rhythm but of suspending it at a fixed phase

FIGURE 14.

Flowering response of C. rubrum to a single dark period of varied duration interrupted with a 12-hour period of red light (115 $\mu w/cm^2$) that was given at different times in darkness. Dark period temperature 20°C; temperature in red light 26°C. Upper curve (broken line) control redrawn from Figure 2.

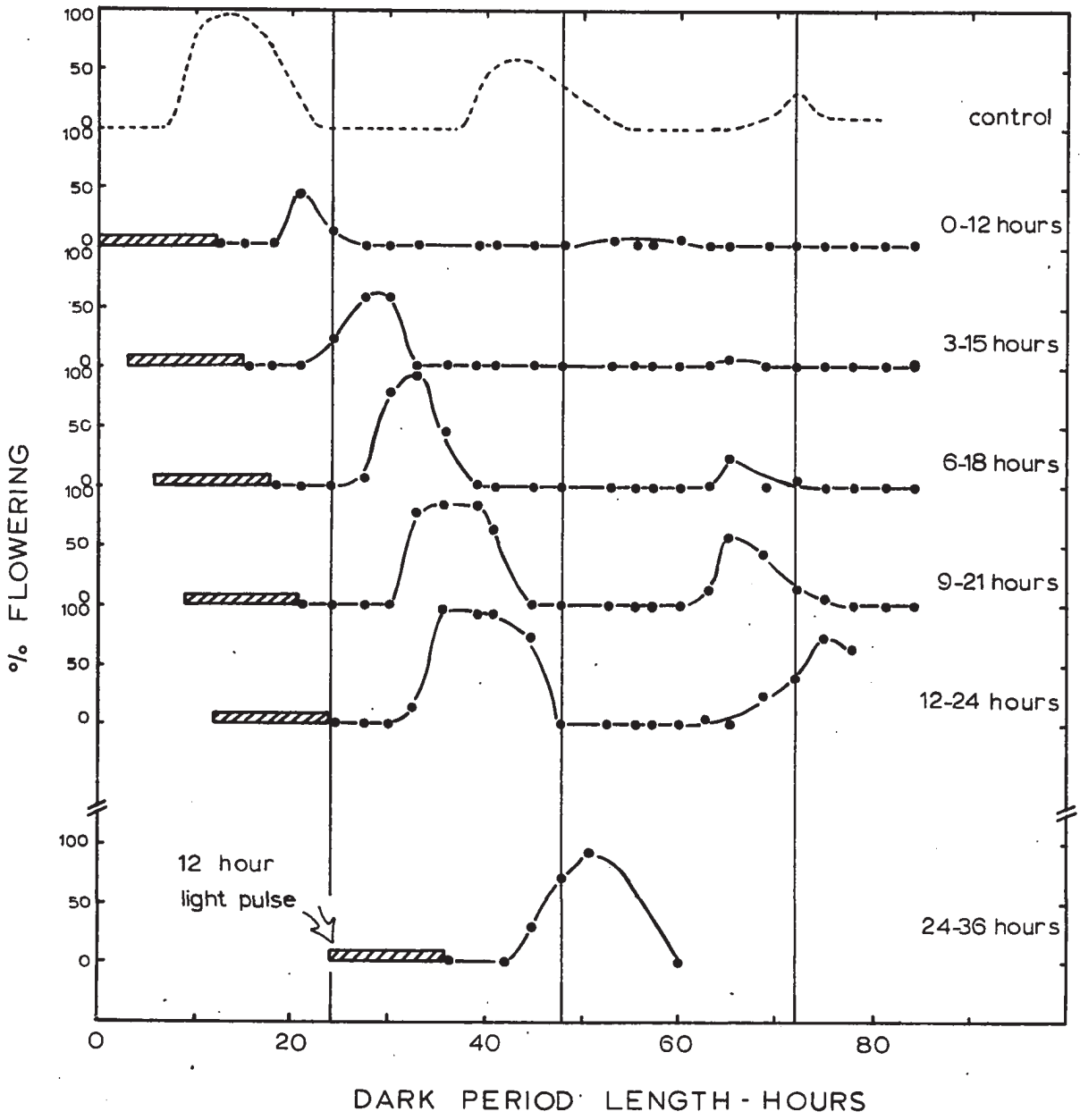
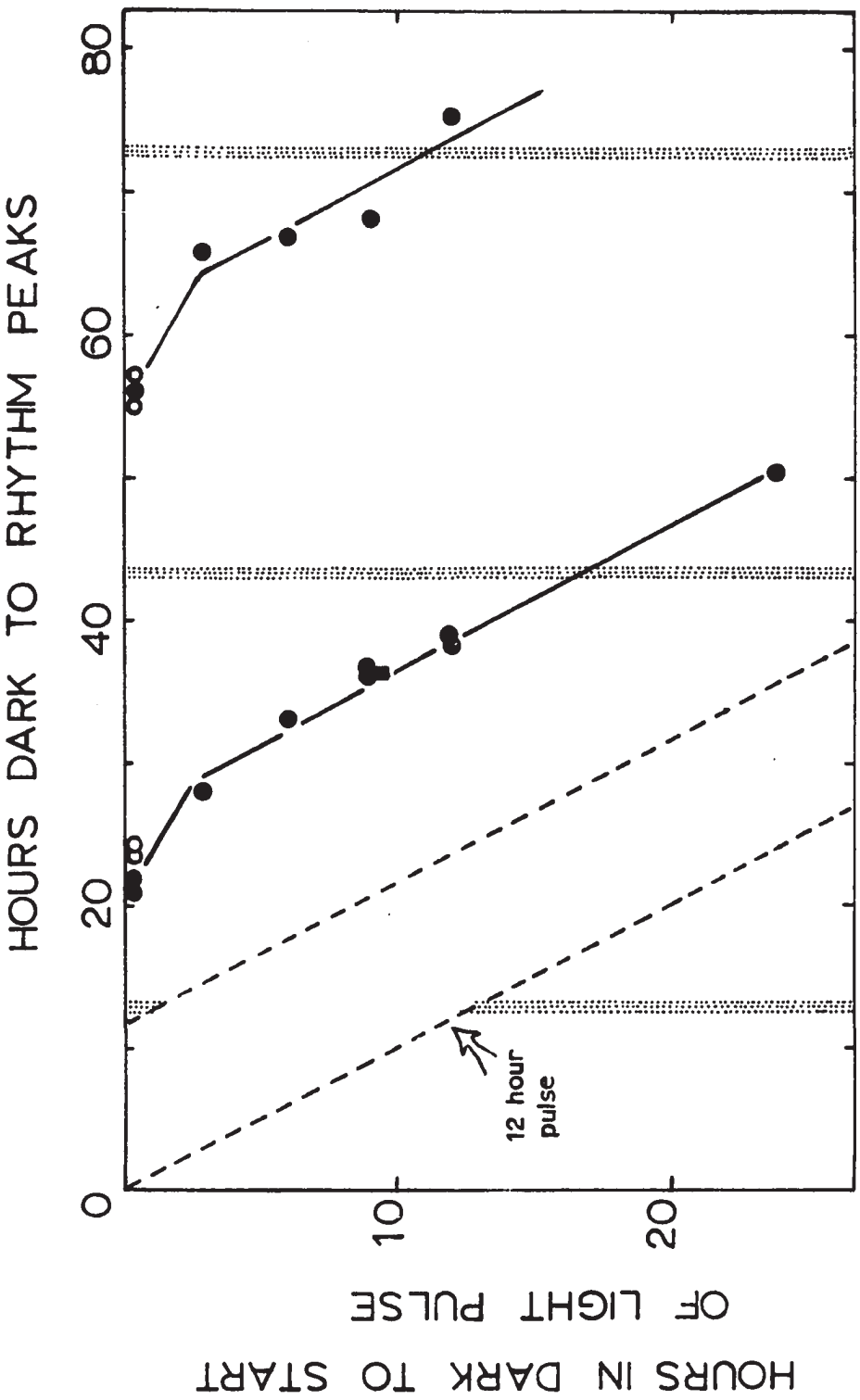

% FLOWERING
100
50
0
control
0-12 hours
3-15 hours
6-18 hours
9-21 hours
12-24 hours
12 hour
light pulse
24-36 hours
0
20
40
60
80
DARK PERIOD LENGTH - HOURS

FIGURE 15.

The relationship between the time in the dark period when a 12-hour light exposure was given and the time of maximum flowering at the first or second peaks of the rhythm of flowering of *C. rubrum* given dark periods of varied duration.

(●) red light pulse (representative raw data in Figure 10),

(■) fluorescent light pulse, (○) red light pulse given to seedlings 5 days old (see Figure 16).


HOURS DARK TO RHYTHM PEAKS
0
20
40
60
80
10
20
HOURS IN DARK TO START OF LIGHT PULSE
12 hour pulse

irrespective of the phase of the control rhythm at the time of giving the light pulse. It is not clear whether there is a real indication of incomplete resetting for the second peak of the rhythm at 60 to 80 hours. These results might have been the consequence of experimental error. Nevertheless, with repeated 12 hour photoperiods the phase of the rhythm would be completely reset each day and there would be no interaction with the rhythm of the previous day. With repeated photoperiodic cycles, if the length of the period was entrained to 24 hours the rhythm peak time would be at 10.4 hours in darkness (i.e. 13.0 x $^{24}/_{30}$ hours).

Somewhat puzzling was the observation that light interruptions during the early hours of darkness gave a reduced amplitude of the flowering response compared to pulses given later in darkness (Figure 14). A similar effect was observed when 6 hour light interruptions were given during the early hours of darkness (Figure 8). One explanation of these responses was that the reduced amplitude of flowering was determined by seedling age. This led to an experiment in which darkness was begun 12 hours earlier than normal; to avoid the added complication of giving a light-off; light-on; light-off sequence of signals a 12 hour light pulse was given from hour 0 to 12 in darkness. The curves in Figure 16a are for replicate experiments in which 12 hours of low intensity red light was given from 5 to $5\frac{1}{2}$ days and the dark period commenced at $5\frac{1}{2}$ days. The curves in Figure 16b are for experiments in which a red light exposure was given from $5\frac{1}{2}$ to 6 days and the dark period commenced on day 6. Or, put another way, a dark period commencing after $5\frac{1}{2}$ days was interrupted with 12 hours of red light from $5\frac{1}{2}$ to 6 days.

The amplitude of the responses was clearly dependent on seedling age at the start of darkness and, thus, for light periods commencing during the early hours of darkness, seedling age was an important factor

contributing to the reduced flowering response observed in Figure 14 and probably in Figure 8. The timing of the flowering rhythm was identical whether low intensity red light (Figure 16a) or fluorescent light of 600 ft-c (Figure 2) was given from day 5 to $5\frac{1}{2}$, although, with red light, amplitude was slightly reduced. By contrast, red light from day $5\frac{1}{2}$ to 6 consistently gave a rhythm in a subsequent dark period with a peak at 9 hours rather than at the normal 12 to 13 hours of the dark period. A change in the peak timing with seedling age could bias the measurements of rephasing in Figure 11 but by no more than 3 to 4 hours. Other data confirm the reality of the effect of seedling age on rhythm amplitude (Chapter VI) and possibly rhythm timing (Chapter V).

c. Phasing response to 18 hour light interruptions.

As with 12 hour light periods, after 18 hours of light the next peak of the rhythm of flowering occurred about 13 hours after plants were returned to darkness. The results presented in Figure 17 are for one such experiment in which an 18 hour light period interrupted darkness from hour 6 to hour 24 in darkness. The response was identical when fluorescent or red light was used as the light interruption, although, as noted before, the amount of flowering was greater when fluorescent light was applied. In another experiment an 18 hour light period commenced after 9 hours of darkness. Again the next peak of the rhythm was 12 to 13 hours after the end of the light period.

d. Phase control by the light-off signal of a photoperiod.

It was shown in parts (b) and (c) that after a 12 or 18 hour light period the time of the rhythm peak fell at a fixed time after the light-off signal. When the photoperiod was 12 hours or longer, the importance of the light-off signal was indicated in Chapter II. In further experiments light interruptions up to 62 hours in length were

FIGURE 16.

Flowering response of C. rubrum to a single dark period of varied duration for replicate experiments (▲, △), (●, ○).

(a) $4\frac{1}{2}$ days germination; 12 hours fluorescent light 600 ft-c; 12 hours red light (115 $\mu w/cm^2$) then darkness commencing $5\frac{1}{2}$ days after sowing.

(b) $4\frac{1}{2}$ days germination: 24 hours fluorescent light 600 ft-c; 12 hours red light (115 $\mu w/cm^2$) then darkness commencing 6 days after sowing.

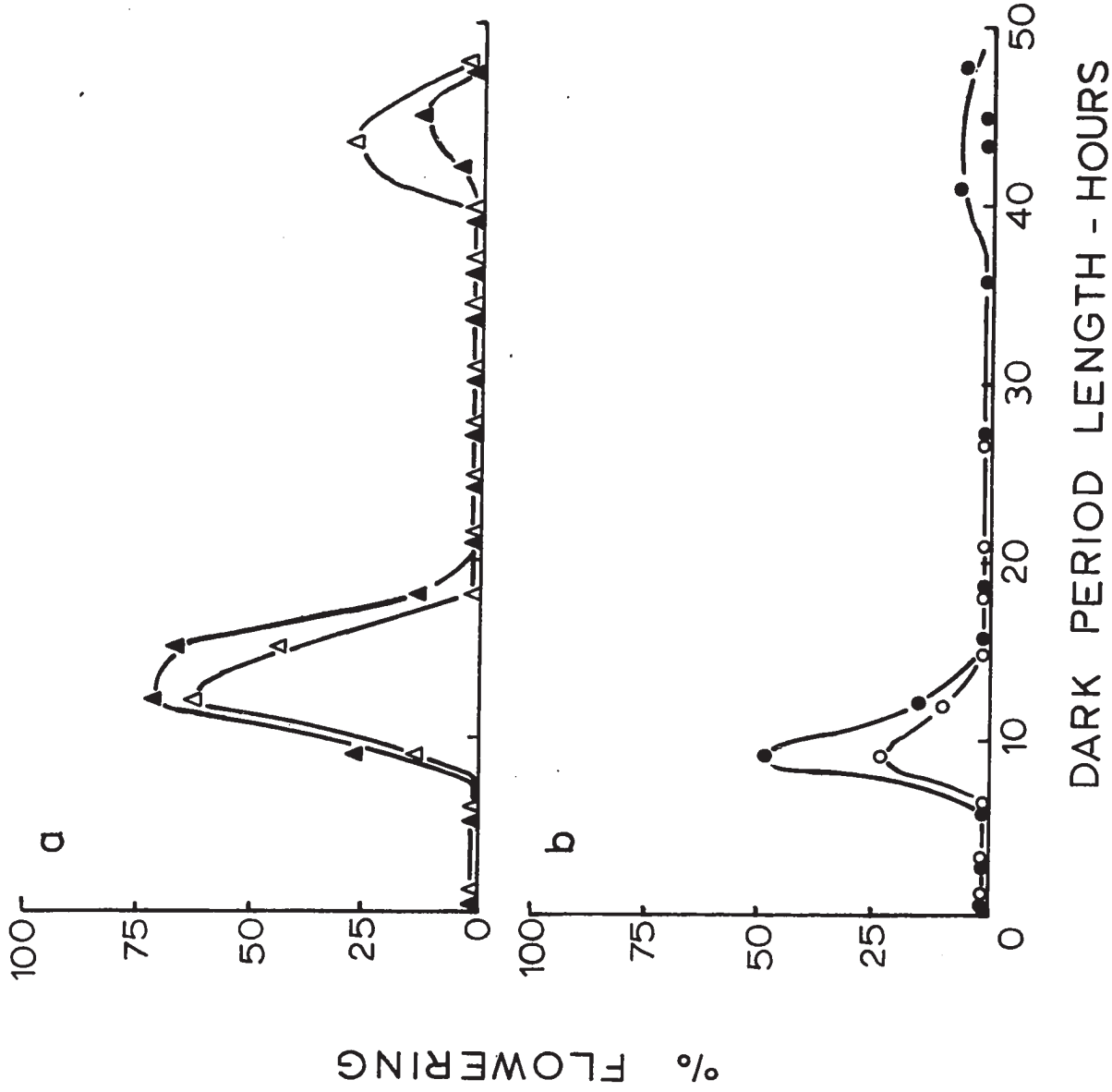
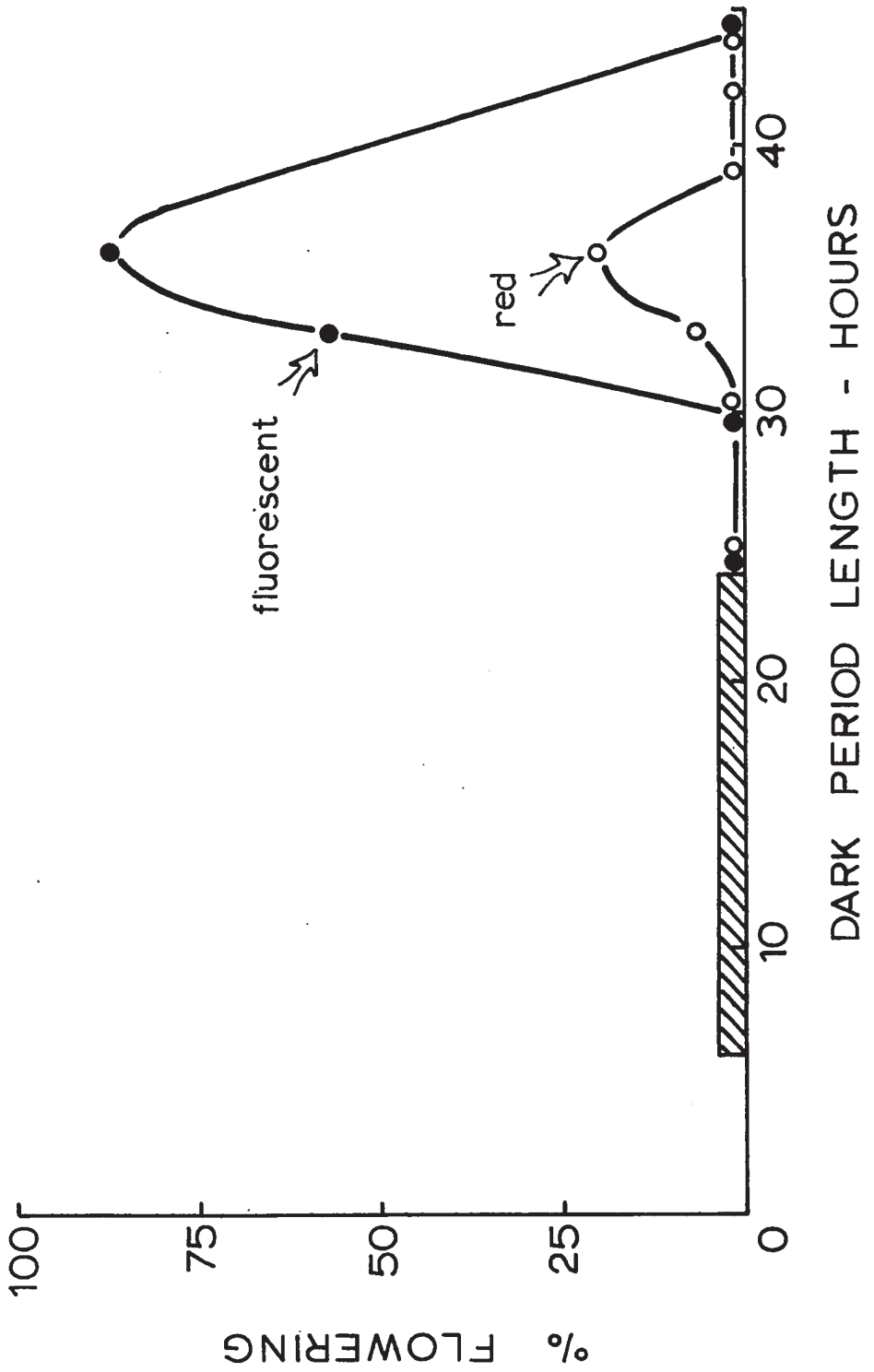

a
b
100
75
50
25
0
10
20
30
40
50
% FLOWERING
DARK PERIOD LENGTH - HOURS

FIGURE 17.

Flowering response of C. rubrum to a single dark period of varied duration interrupted at the sixth hour in darkness by 18 hours of fluorescent light of 600 ft-c (●) or 18 hours of red light (115 $\mu w/cm^2$) (○).


% FLOWERING
100
75
50
25
0
10
20
30
40
DARK PERIOD LENGTH - HOURS
fluorescent
red

begun at a fixed time in darkness - the ninth hour. Thus the time of the light-on signal was held constant but timing of the light-off signal was varied. Representative results are presented in Figure 18 for a single experiment in which fluorescent light was used for the light interruption. The mid point of the rhythm peak fell from 12 to 15 hours after the light-off signal for light interruptions 15 to 39 hours in duration.

A similar graphical presentation to that in Figure 18 has been applied in Figure 19 but only the time in darkness to rhythm peaks has been plotted against the length of the light interruption. Figure 19 illustrates that, for all light interruptions longer than 12 hours, the peaks of the rhythm occurred approximately $13\frac{1}{2}$ hours after the light-off signal. Thus 12 hours or longer duration light interruptions reset and suspended the phase of the rhythm so that it was always reinitiated at a fixed phase relative to the time of the light-off signal preceding darkness. Red or fluorescent light were equally effective in shifting rhythm peak time but after red interruptions longer than 18 hours the amount of flowering was insufficient for accurate measurement of rhythm phase.

It can be concluded that when a single 12 hour light period was used to reset the free running rhythm at its different phases (Figure 15) the timing of the light-off signal alone was important. When a 6 hour light period was applied it was suggested earlier that the light-off signal also played a role in rhythm phasing. However, compared to a 12 hour light period (Figure 15) with a 6 hour light period (Figure 10) there was no fixed relationship between the timing of the light-off signal and phasing of the rhythm. The only possible explanation for this observation is that phasing by a 6 hour light period is controlled by the timing of both the light-off and light-on signals.

FIGURE 18.

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 fluorescent light (600 ft-c) for various durations and commencing at the ninth hour of darkness. Temperatures 20°C.

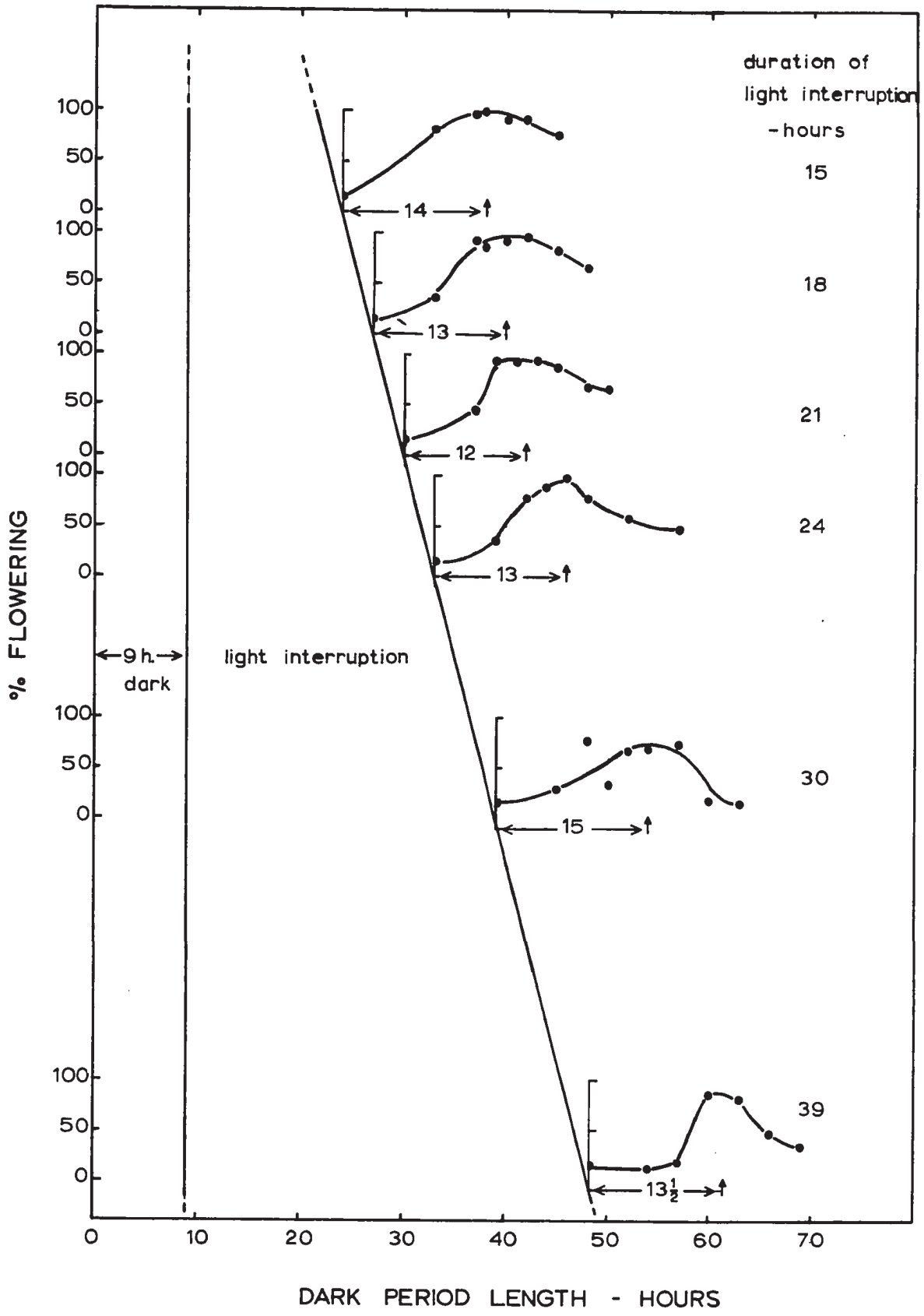

duration of
light interruption
-hours
15
18
21
24
30
39
14
13
12
13
15
13½
9 h.
dark
light interruption
% FLOWERING
100
50
0
0
10
20
30
40
50
60
70
DARK PERIOD LENGTH - HOURS

FIGURE 19.

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 fluorescent (○) or red (●) irradiation for various durations commencing at the ninth hour of darkness. Dark period temperature 20°C, fluorescent light 600 ft-c (1037 $\mu w/cm^2$) at 20°C, red light (115 $\mu w/cm^2$) at 26°C.

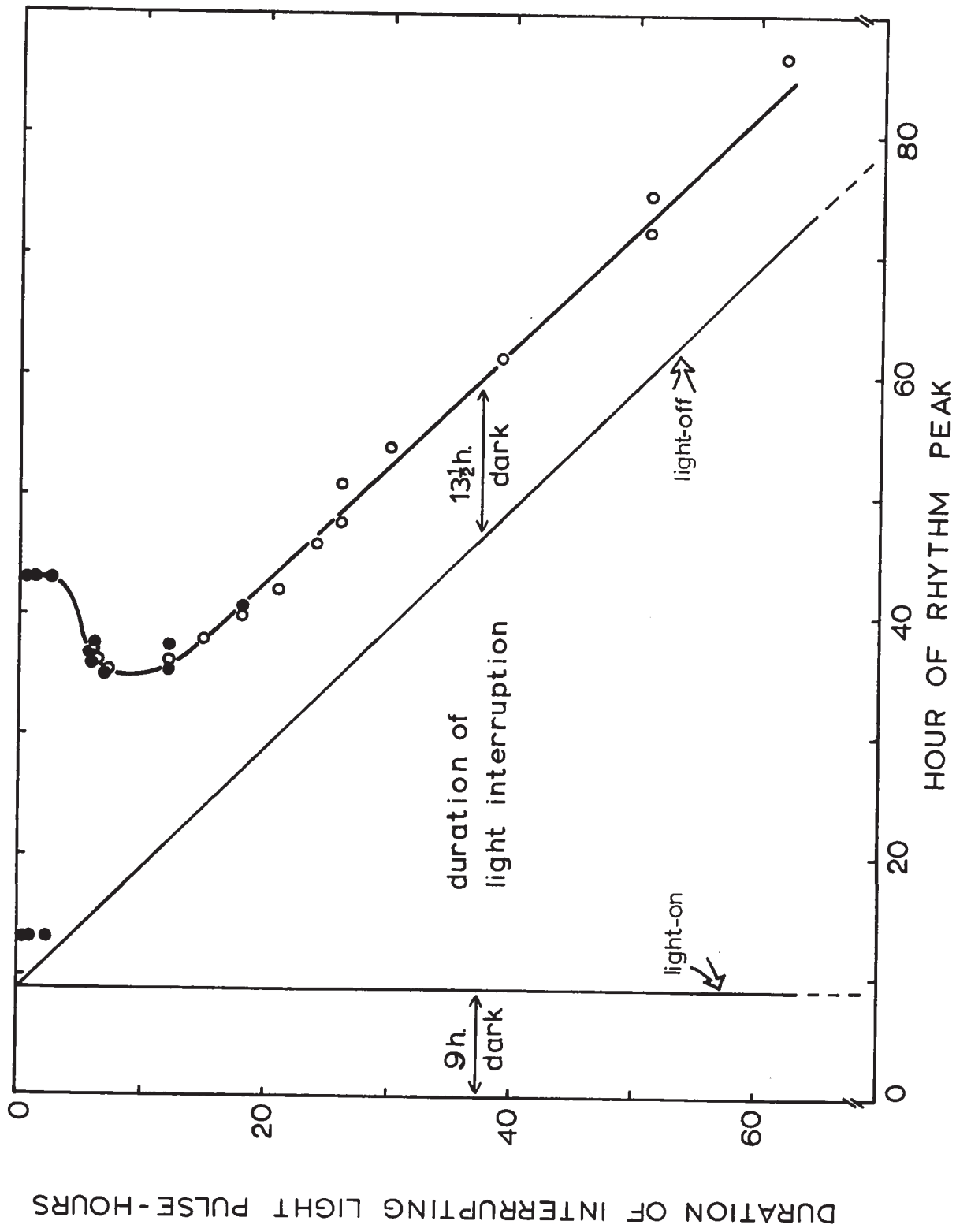
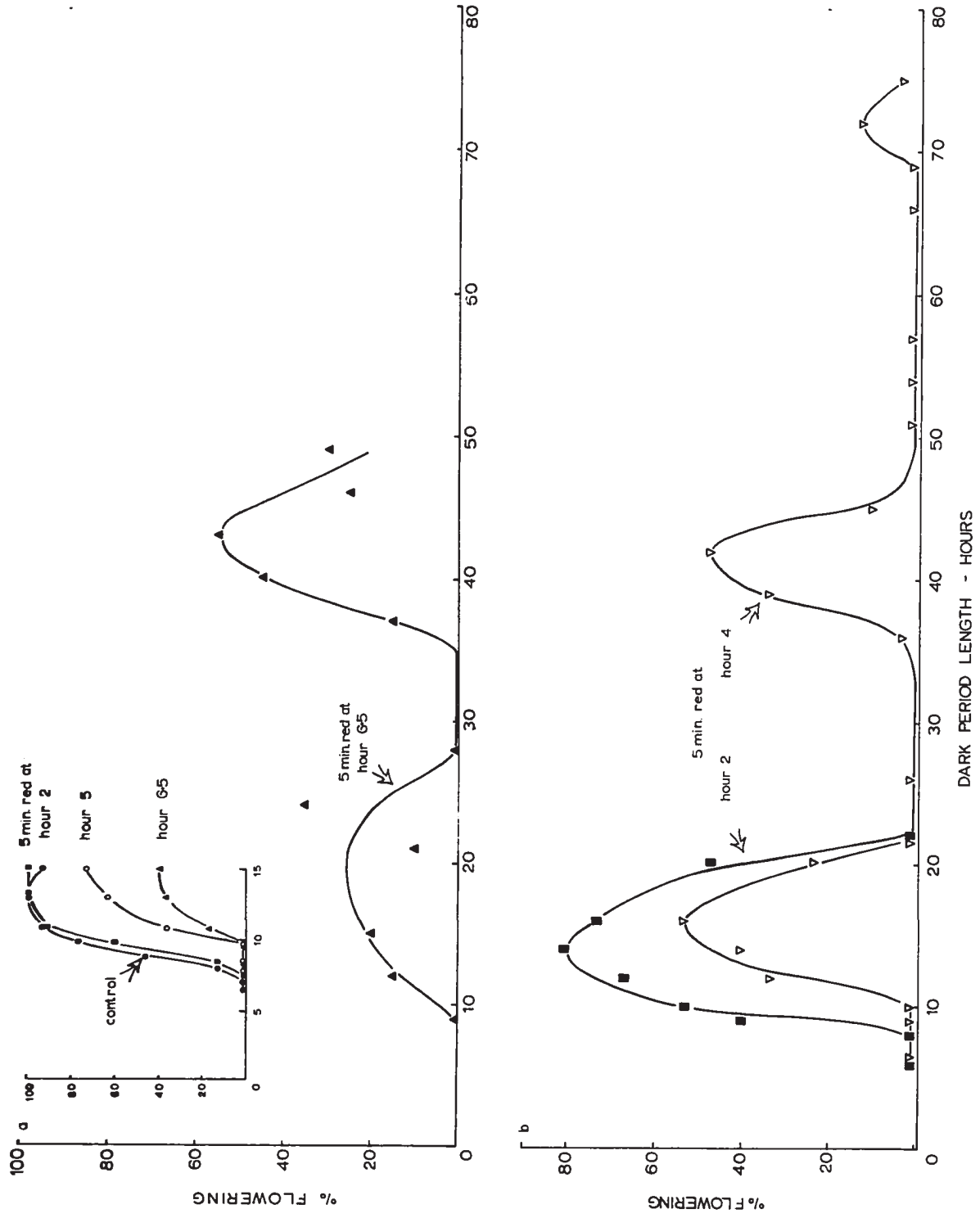

DURATION OF INTERRUPTING LIGHT PULSE-HOURS
0
20
40
60
HOUR OF RHYTHM PEAK
0
20
40
60
80
9h.
dark
13½h.
dark
light-on
light-off
duration of
light interruption

FIGURE 20.

a. Flowering response to dark periods of varied duration that commenced $5\frac{1}{2}$ days after sowing. A solution of 0.4 molar glucose in Hoagland's solution was applied at $4\frac{1}{2}$ days. For all plants the dark period was interrupted once with a 5 minute period of red irradiation (115 $\mu w/cm^2$) after 6.5 hours of darkness (▲).

Insert: flowering response to dark periods of varied duration. Five minutes red irradiation was given at 0 (● - control); 2 (■); 5 (○); or 6.5 (▲) hours of darkness to different groups of plants. Only Hoagland's nutrient solution was applied to these plants.

b. Flowering response to dark periods of varied duration that commenced $5\frac{1}{2}$ days after sowing. A 5 minute red irradiation was given to different groups of plants at 2 (■) or 4 (▽) hours of darkness. Only Hoagland's nutrient solution was applied to the seedlings.


a
b
% FLOWERING
DARK PERIOD LENGTH - HOURS
5 min. red at hour 2
hour 5
hour 6·5
control
5 min. red at hour 6·5
5 min. red at hour 2
hour 4

The length of the light period had to be in excess of $2\frac{1}{2}$ hours but less than 6 hours if an interruption was to be effective for rephasing the rhythm (Figure 19). Previously, Cumming et al (1965) reported that a brief light pulse - 5 minutes of red irradiation - interrupting darkness at the sixth or twelfth hour of darkness did not change the timing of the second and third peaks of the flowering response. The results presented in Figure 20 confirm their findings. Five minutes of red light given at hour 6.5 in darkness, while influencing the amplitude and timing of the first peak of the rhythm had little affect on phasing of the second or third peaks of the rhythm. In two other experiments this shift was confirmed for the first peak of the rhythm of flowering. Interruptions given at hour 0, 2, 4, 5 or 6.5 in darkness partially shifted the first peak of the rhythm of flowering (Figure 20a, inset and b).

IV. DISCUSSION.

a. Photoperiodic time measurement.

The results presented in this chapter provide substantial evidence for the involvement of an endogenous rhythm as a timer measuring the length of the daily photoperiod. The essence of these experiments lies in their direct measurement of phasing of the rhythm of flowering of Chenopodium rubrum in response to different photoperiods. Although flowering encompasses many partial processes including the production and action of a floral stimulus (see Chapter VI), as a physiological parameter, flowering expresses the net response to all the biochemical sequences that are involved in and controlled by photoperiodic time measurement. Therefore, the difficulties that might be associated with the use of overt indicator rhythms are bypassed and, mechanistically, no aasumptions need be made

about the nature of a rhythm.

It might be inferred that measurements of the percentage of plants flowering indicate only the response of a population of plants and do not reflect the oscillations occurring in an individual plant. However, earlier experiments of Cumming (1965, 1967a) and those reported in Figures 8 and 9 indicated that there was a high degree of synchrony in the population. All individuals or at least 80% of all plants could progress alternately through maxima and minima in their capacity to flower, and this was consistent for 3 cycles (90 hours) of the free running oscillation whether measurements were of the normal ("control") rhythm (Figure 9a) or of the rhythm after rephasing (Figures 8 and 9b). Repeat experiments were also very consistent in response. Therefore, the flowering percentage of the population as a whole is representative of the capacity of an individual plant to flower and hence of the endogenous, free running rhythm in each plant.

In plants (Zimmer, 1962; Moser, 1962; Hastings, 1964; Feldman, 1967; Halaban, 1968a) and animals (Pittendrigh, 1960; Pittendrigh and Minis, 1964; Winfree, 1970) the sensitivity of an endogenous oscillation to light has been found to vary with the phase of the oscillation. Similar sensitivity was also found for the rhythm of flowering of C. rubrum when a single 6 hour light period interrupted darkness at various phases of the oscillation (Figures 8, 10 and 11). In a comparable way to the responses just referred to greatest phase advance or delay of the rhythm occurred for red light pulses commencing over the 10 hours immediately prior to the rhythm peak.

This capacity for rhythm rephasing raises the possibility that the rhythm could act as a "clock" so that, in photoperiodic cycles, such as 6 hours light: 18 hours dark (6:$\overline{18}$), repeated phase advances and delays

may reach an eqilibrium at which point the rhythm peak would occur at a fixed time each day, i.e. 23 hours after the light-off signal. However, when this prediction was examined it became apparent that repetitive phase resetting each day did not always account for the observed response. After 3 cycles of a 6 hour photoperiod calculated and actual values differed by 8 hours and at a steady state the peak occurred at 18 hours (Figure 13) and not at the estimated 20 to 24 hours (Figure 12). Possibly the accuracy of the values for rhythm period length of the phase response (Figure 11) limited the accuracy of calculation. On the other hand, repetitive phase resetting by daily photoperiods may occur in association with entrainment of the period of the rhythm to a value of 24 hours as observed by Pittendrigh (1960). On incorporating period entrainment into the calculations from the phase response curve of Figure 11, actual steady state rhythm phasing was now simulated by calculation. Rhythm peaks would occur 18 hours after the light-off signal of a 6 hour daily photoperiod.

Turning to the question of rhythm phasing under longer daily photoperiods, in some organisms little difference has been observed in the shape of phase response curves obtained with both short (15 minutes) or long (12 hours) light pulses (e.g. Pittendrigh, 1960 and see Hastings, 1964; Halaban, 1968a). However, in C. rubrum a completely different pattern resulted for 12 hour compared to 6 hour light periods. With a 12 hour light period there was a fixed relationship between the time of the light-off signal and the time of the next peak and this was quite independent of the phase of the control rhythm at that time (Figures 15 and 19). A similar response was evident for an 18 hour photoperiod, although this length of light period was not used as extensively. It may, nevertheless, be concluded that rhythm phase would be fixed by the

TABLE 4.

Action of daily photoperiodic cycles on the phasing of the rhythm of flowering of C. rubrum and hence on the degree of flowering.

DAILY HOURS OF LIGHT AND DARKNESS	CALCULATED PEAK TIMES(H)		PHOTOPERIODIC FLOWERING RESPONSE	
	REPHASING ALONE	REPHASING + PERIOD ENTRAINMENT	EXPECTED	PERCENTAGE FLOWERING OBSERVED
6:$\overline{18}$	23(29)*	18(24)	+	91
12:$\overline{12}$	13.0(25.0)	10.4(22.4)	++	100
18:$\overline{6}$	13.0(31.0)	10.4(28.4)	0	0

* times from the last dusk or dawn signal, e.g. 23(29), respectively.

timing of the light-off signal in 12 or 18 hour photoperiods. If period entrainment was of no significance rhythm peaks would occur about 13 hours after the daily light-off signal but with entrainment peaks would occur 10.4 hours after the dusk signal (13.0 X 4/5).

The importance of rhythm phase control in photoperiodic time measurement for flowering is summarized in Table 4 for the response to 6, 12 or 18 hour photoperiods. In accordance with published reports of the photoperiodic control of flowering in C. rubrum (Cumming, 1963, 1967b and see Chapter II) maximal flowering would be expected under a daily 12 hour photoperiod. Rhythm peak times under 12 hour photoperiods fall 13 hours after the light-off signal and the critical dark period would be reached 6 hours earlier. Slightly less flowering is obtained in 6 hour photoperiods (Figure 4) and rhythm peak times also occur slightly later than under 12 hour photoperiods. No flowering would occur in an 18 hour photoperiod as the length of darkness does not exceed the critical dark period. Photoperiods less than 3 hours in duration gave no rhythm rephasing (Figure 19) so that each cycle light would fall on different phases of the 30 hour oscillation; and coincidence of light on all phases of the rhythm coupled with reduced photosynthesis would limit flowering under these photoperiods. Clearly an oscillatory clock that changes its rhythm phasing according to photoperiod duration can account for the photoperiodic control of flowering in C. rubrum.

b. Interaction of light and the rhythm.

A quantitative type of rephasing of the rhythm of C. rubrum may best fit the results presented above where 6 hours of light were far less effective than 12 hours of light. In this respect, after a 6 hour light pulse despite the synchrony of response of the first peak, the second peak sometimes showed dispersion into two apparently physiologically

distinct populations (Figure 8). With a 12 hour light pulse there was stronger rephasing and little apparent population dispersion at the second peak.

Often, when discussing rhythm rephasing an arbitrary reference point such as the timing of "dawn" or "dusk" has been adopted. In the instance of a 6 hour light period (Figures 10 and 11) the reference point was taken as the time of the dawn signal. This infers nothing about the importance of a light-on, a light-off, or a differential response to both signals and it was suggested that phasing with a 6 hour light period was determined by both dawn and dusk signals. On the other hand, there was clear evidence (Figures 15 and 19) that the timing of the light-off signal alone was controlling the phase of the oscillation for light periods of 12 hours or longer. These latter results do not suggest a durational influence of light but favour a purely "impulse-generated" phasing response to the light-off signal. Therefore, a skeleton photoperiod consisting of short flashes of light should simulate dawn and dusk of the complete photoperiod (see Pittendrigh and Minis, 1964; Hillman, 1964a; Oda, 1969). However, neither 6 nor 12 hour light interruptions could be replaced by skeleton photoperiods given as either 5 or 15 minute "light-on" and "light-off" signals (see Chapter IV). To explain all these results it may be necessary to accept some cooperativity of durational (quantitative) and impulse generated (qualitive) effects of light on rhythm rephasing.

The reinitiation of the rhythm of flowering at a fixed phase after long periods in continuous light (12 to 62 hours - Figure 19) suggested that one durational effect of light may be to suspend the oscillation. In similar experiments with Drosophila, Pittendrigh (1960, 1966) also found there was a fixed phase relationship to the time of the light-off signal of light interruptions 12 to 34 hours in duration. He

described the response as one of "damping" of the rhythm but this clearly was not referring to the type of damping previously observed in continuous light in which the rhythm may continue unobserved (Bunning, 1967). Previously, Cumming et al (1965) commented on the dominance of the light-off signal for initiation of the rhythm of flowering of C. rubrum seedlings grown for 4 to 6 days under continuous light. They concluded that the rhythm was either suspended in continuous light or that it continued but was reset to a fixed phase by the light-dark transfer. If this latter interpretation were true it is unlikely that on increasing the duration of the light period from 6 to 12 hours the phase reference point would shift so dramatically to control by the timing of the light-off signal and retain a fixed relationship to this signal for long periods of light (Figure 19). Also the changing sensitivity to rephasing as shown by a 6 hour light period (Figure 8) suggest that if there was a continuing oscillation in the light it would reset to different phases depending on the timing of the light-off signal. Suspension of the photoperiodic timer in continuous light has also been postulated from studies of the flowering of Xanthium (Hamner and Bonner, 1938). In Pharbitis nil (Takimoto and Hamner, 1964, 1965), Lemna perpusilla (Hillman, 1964a) and Lemna gibba (Nakashima, 1966) rhythms of flowering also suspend in continuous illumination but this is not true for soybean and Kalanchoe (see Chapter II).

The concept of rhythm suspension as favoured here appears not to concur with earlier ideas of a completely innate, free running oscillation (see Aschoff, 1960). However, the recent discovery of a "singular state" of non-rhythmicity in Drosophila (Winfree, 1970) provides an explanation for rhythm suspension. Another possibility is that flowering becomes uncoupled from control of the single "master rhythm". Other rhythms need not be uncoupled in continuous light as had been observed for rhythms of

leaf movement in Phaseolus (Hoshizaki and Hamner, 1964), respiration in Lemna gibba (Miyata and Yamamoto, 1969) and, in C. rubrum cell division (Chapter VI) and betacyanin content (Wagner and Cumming, 1970). However, at least for C. rubrum, the period length of the rhythms of cell division and betacyanin content (20 to 22 hours) is distinct from that of the rhythm of flowering (30 hours) and control by separate "master rhythms" is suggested.

c. Bunning's Hypothesis of Time Measurement.

Time measurement in the photoperiodic control of flowering undoubtedly involves the action of an endogenous rhythm and, as suggested by Bunning, rhythm phase is important in determining the response. Nevertheless the results presented here indicate a degree of complexity which has not always been introduced into a model such as that of Bunning (1960) in which the dawn signal was assumed to set the rhythm phase. Nor would it be wise to emphasise control by the dusk signal alone as postulated by Halaban (1968b) or to suggest that there are two rhythms coupled individually to dawn and dusk signals as did Takimoto and Hamner (1964). The only valid generalization may be that the importance of the light-on and light-off signals for phase entrainment of a rhythm will vary with photoperiod. Even then, this statement needs qualification when different species are compared. Flowering of soybean, for instance, shows no evidence of rhythm suspension in long photoperiods (Chapter II).

In the photoperiodic control of flowering light falling too early in a dark period (i.e. before the critical dark period is reached) prevents flowering. This effect was related by Bunning to the timing of the coincidence of light on the scotophile phase of the rhythm. In the experiments presented here it has not been possible to ascertain whether one particular phase of the rhythm was more light sensitive than another. Superficially, the results of Figures 8 and 14 favoured this interpretation but subsequently seedling age was found to be quite important in determining rhythm amplitude. Nevertheless, earlier

experiments with C. rubrum (Cumming et al, 1965) do suggest that there is a rhythmical change in sensitivity to brief light interruptions coincident at different phases of a rhythm that proceeded in darkness. There were no effects of light on rhythm phasing in these experiments and interactions with seedling age probably were not important. As well as a light "coincidence" effect under photoperiodic conditions, the rapidity of rephasing will also determine the relative importance of light for flowering.

d. A "timer" other than a rhythm?

Despite the conclusion that an endogenous rhythm is measuring the length of the daily light and dark cycle in C. rubrum, these results in no way rule out the possible presence and activity of other "timers". In most instances the rhythmic timer may be limiting and dominant in its action. Nevertheless, as suggested by Cumming (1969b) phytochrome conversion could be important for timing at least during the early hours of darkness. Two aspects of the rhythmic control of flowering of C. rubrum favour this concept. Firstly, when 6 hour light pulses impinged on different phases of the rhythm (Figure 10) the closest a light pulse could approach the next peak of the rhythm was 12 hours after the end of the light interruption for pulses beginning at hours 0, 3 or 30 in darkness. There is apparently a preparatory or critical length of darkness setting a lower limit to the action of the rhythm. By contrast, for other rhythms such as those of petal movement in Kalanchoe (Zimmer, 1962); luminescence in Gonyaulax (Hastings, 1964) and leaf movement in Coleus (Halaban, 1968a) at times of greatest phase advance the light pulse may actually be impinging on the next peak without causing any of the displacement evident in Figure 10. Secondly, a sugar solution applied to the plants 24 hours before darkness enhanced flowering of all peaks and

troughs of the rhythm in darkness but failed to give any flowering over the early hours of darkness (Figure 9). Thus the early hours of darkness may differ significantly from the lat er hours.

Takimoto and Hamner (1964) concluded from their experiments with Pharbitis nil that "rhythms" and an"hourglass" timer were operative in that plant. Their hourglass component could relate to phytochrome conversion and the simplest interpretation of Bunning's "coincidence" effect of light certainly points to phytochrome as the photoreceptor for this effect. (Fredericq, 1964; Cumming et al, 1965; Halaban and Hillman, 1970b). These concepts have been examined in experiments reported in Chapter V.

CHAPTER IV

THE PHOTORECEPTOR CONTROLLING RHYTHM PHASING.

I. INTRODUCTION.

Photoperiodic control of flowering, insect diapause or leaf sleep movements requires not only the presence of a "clock" but presupposes the action of a photoreceptor that is coupled in some way to this "clock". Thus definition of the pigment and knowledge of its action may be of considerable importance in understanding time measurement.

In animals and plants a number of action spectra have been measured for phase shifting of circadian oscillations by single light pulses and these experiments have been tabulated in Table 5. To date accurate action spectra have been obtained only for animals and lower plants. In the former, greatest effectiveness of blue light in rhythm phase shifting suggests photoreception by flavins, porphyrins and semiquinones but fine detail has been insufficient to distinguish between the various pigments (see Hendricks, 1969).

In lower plants photoreception for rhythm phase shifting may involve two pigments; one absorbing in the blue (Hastings and Sweeney, 1960; Sargent and Briggs, 1967); the other in the red region (Hastings and Sweeney, 1960). The photoreceptor could be a carotenoid in the blue region as suggested by Sargent and Briggs (1967) for Neurospora crassa and the action of both chlorophyll in the red and carotenoids in the blue could explain the results with the green alga, Gonyaulax polyedra (Hastings and Sweeney, 1960).

TABLE 5.

Effective wavelengths for rhythm phase shifting or rhythm initiation by visible light.

ORGANISM	RESPONSE	EFFECTIVE WAVELENGTHS	ENERGY	REFERENCE
Paramecium bursaria	Phase shift of mating rhythm.	UV and 600-700 nm		Ehret, 1960.
Drosophila pseudoobscura	Phase shift of adult emergence rhythm.	400-500 nm	10^{-11} einstein	Frank and Zimmerman, 1969.
Pectinophora gossypiella	Phase shift of egg hatching rhythm.	400-500 nm	10^{-11} einstein	Bruce and Minis, 1969.
Neurospora crassa	Phase shift of conidication rhythm.	400-500 nm	7×10^{-14} einstein	Sargent and Briggs, 1967.
Gonyaulax polyedra	Phase shift of luminescence rhythm.	475 and 650 nm		Hastings and Sweeney, 1960.

BROAD BAND RADIATION.

Phaseolus multiflorus	Initiation of leaf movement rhythm.	red/ far-red reversible	100 uw/cm^2	Lorcher, 1958.
Phaseolus multiflorus	Phase shift of leaf movement rhythm.	red/far-red reversible		Bunning and Moser, 1966.
Bryophyllum fedtschenkoi	Phasing of carbon dioxide output rhythm.	red not blue	85 uw/cm^2	Wilkins, 1960.
Coleus blumei and *C. frederici*	Phase shift of leaf movement rhythm.	blue & red	2×10^3 uw/cm^2	Halaban, 1969 .

Such a conclusion is supported by the experiments of Senger and Schoser (1966) who observed that wavelengths in the red (674 nm) and blue (485 nm) regions of the spectrum were most effective for photoperiodic entrainment of diurnal oscillations of cell division and nucleic acid synthesis in the green alga Chlorella. Application of DCMU, a specific inhibitor of chlorophyll activated electron transport in photosynthesis, erased the response to red wavelengths whereas the sensitivity to blue light remained unaltered. Furthermore, Senger and Bishop (1966) found that in an achlorophyllous mutant, blue radiation was most effective for synchronizing cell division and the action spectrum and the in vivo and in vitro absorption suggested a carotenoid photoreceptor in the blue. Chlorophyll apparently was the effective pigment in the red region. In Gonyaulax, however, Sweeney (1969b) has been unable to relate her observation of maximal effectiveness in the red to a chlorophyll absorbing in this region.

Another rhythm in Gonyaulax, that of photosynthesis, has been tested for photoreception mediated by photosynthetic pigments. There was no detectable rhythmic change in the content of either carotenoids or chlorophylls (Hastings et al, 1961) and inhibition of either photosynthetic non-cyclic electron transport or of cyclic electron transport did not cause any phase shift (Sweeney, 1969b). It is probable therefore that photosynthetic pigments are neither photoreceptors for this nor other rhythms in Gonyaulax. Red/far-red reversibility in the rhythmic response of another dinoflagellate, Gyrodinium dorsum (Forward and Davenport, 1970) could indicate that phytochrome may also act as the photoreceptor in Gonyaulax in the red and possibly in the blue regions.

In higher plants, evidence for any type of photoreceptor is

not precise because only broad band radiation has been used (see Wilkins, 1960; Bunning, 1967; Halaban, 1969). A low energy requirement for response suggests it is unlikely that photosynthetic pigments play any significant role in higher plants. In Lemna perpusilla for instance, entrainment of flowering (Hillman, 1964a) and of the rhythm of carbon dioxide evolution (Hillman, 1970) can be achieved with a total of 30 minutes red light of 10 $\mu w/cm^2$ (550 - 800 nm) when given as a skeleton photoperiod. These energies are far too low to activate carbon dioxide fixation (Gaastra, 1959) and are well below those energies required to saturate cyclic photophosphorylation (Urbach and Gimmler, 1970).

As found with algae, sensitivity to red radiation suggests the involvement of phytochrome. To date the most convincing evidence for the action of phytochrome is that of Lorcher (1958) who found that the rhythm of leaf movement of Phaseolus multiflorus could be induced by 6 hours of red light (100 $\mu w/cm^2$), prevented by terminating the red radiation with one hour of far-red and, re-induced with a further hour of red light. This implies the action of phytochrome and such a conclusion is supported by the studies of Bunning and Moser (1966) who reported that phase shifting of the leaf movement rhythm of Phaseolus could be controlled by broad band red but not by far-red irradiations.

For flowering substantial evidence was presented in Chapter III showing that low intensity red light is most effective for rhythm phase resetting and, furthermore, a photosynthetic effect appeared unlikely. In this chapter the possibility that phytochrome is the photoreceptor controlling phasing of the rhythm of flowering in Chenopodium rubrum is examined. Rather than attempting the measurement of an action spectrum red/far-red reversibility has been used to characterize phytochrome action.

II. MATERIALS AND METHODS.

Chenopodium rubrum (origin 60°47'N 132°32'W) was used in all these experiments and the materials and methods were as described in Chapter II.

III. RESULTS.

a. The photoreceptor for rhythm phase shifting.

The experiments reported in the previous chapter indicated that a 6 hour interruption of darkness with low intensity red light induced phase shifts of the rhythm of Chenopodium rubrum. Figure 21b summarises this response for a light period interrupting darkness from hour 9 to hour 15; flowering was assayed on plants given various lengths of a subsequent dark period (3 hour increments). Relative to the uninterrupted control (Figure 21a) the peak of the flowering rhythm was shifted significantly (i.e. from hour 43 to hour 37). However, when a 6 hour skeleton light period was given over the same time (i.e. 9 to 15 hours, Figure 21c) with either 5 or 15 minutes of red radiation initiating and terminating the 6 hour period, phasing of the rhythm of flowering (Figure 21c) was not visibly different from that obtained with the unirradiated controls. The designation skeleton light period is applied subsequently to any treatments in which two brief light pulses are used to simulate the beginning and end of a complete light period. As was evident in Figure 19 (Chapter III), rhythm phase shifting only resulted when $2\frac{1}{2}$ hours or more light was given as an interruption, but the total irradiation in the skeleton light pulses was 30 minutes at the most. Skeleton light periods also had little

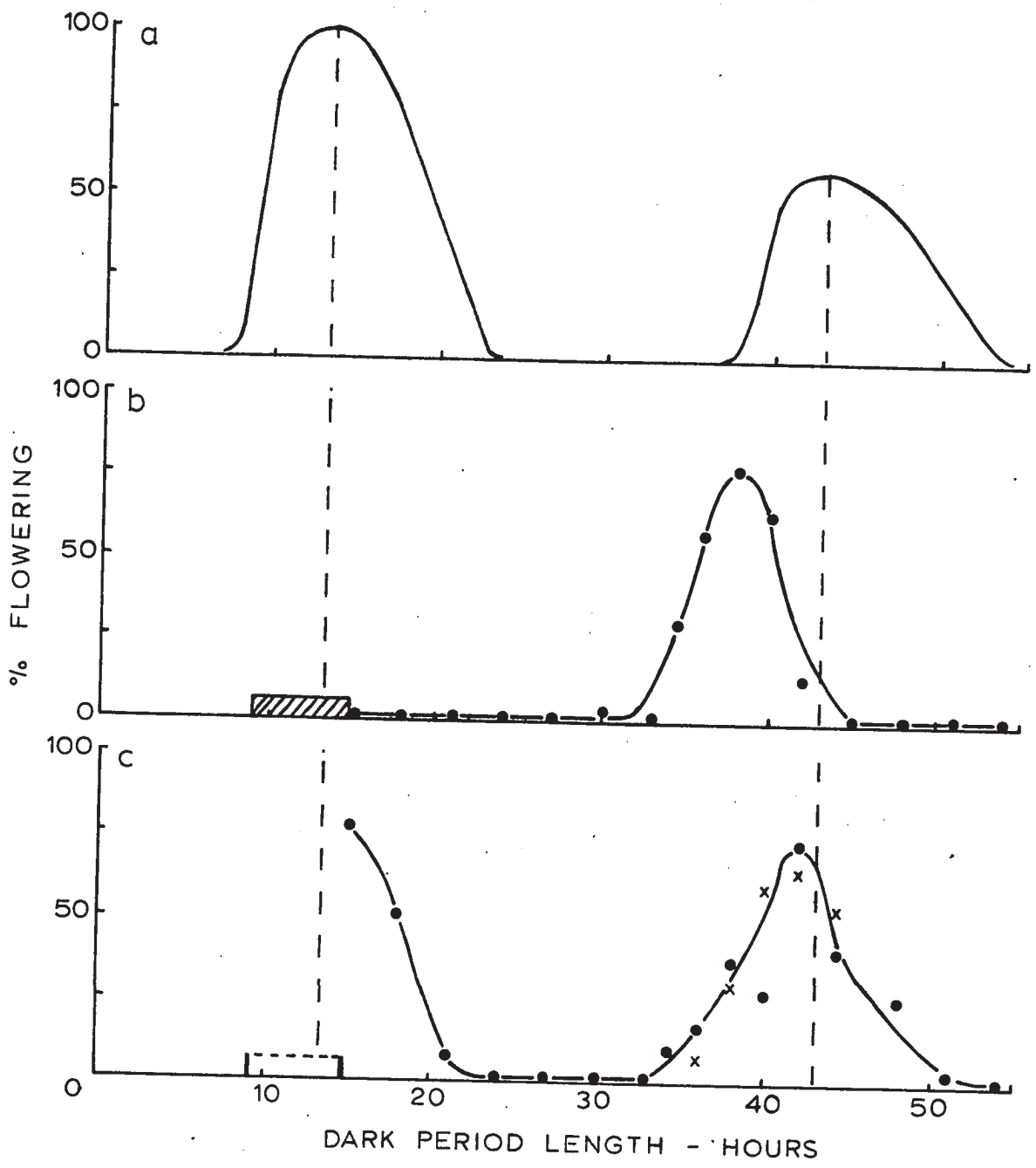
FIGURE 21.

Flowering response of C. rubrum given a dark period of varied duration;

(a) uninterrupted control curve;

(b) 6 hours of red light (115 $\mu w/cm^2$) given from hour 9 to hour 15 in darkness;

(c) 6 hour skeleton red light interruption (115 $\mu w/cm^2$) given as 5 (●) or 15 (x) minute irradiations initiating and terminating the 6 hour period.


100
a
50
0
100
b
50
0
% FLOWERING
100
c
50
0
10
20
30
40
50
DARK PERIOD LENGTH - HOURS

effect on the first peak of the control rhythm while, by comparison, 6 hours of continuous light erased the first peak of the rhythm (Figure 21b,c).

In order to determine how much light was required for a 6 hour skeleton light period to act as a continuous 6 hour irradiation, brief light breaks were introduced into a skeleton light period that was given from hour 9 to hour 15 in darkness (Table 6). Flowering was assayed only for dark periods between 33 and 50 hours in duration (3 hour increments) as this period can be seen to include the times of peaking of both the rephased and the control rhythms of flowering (cf. Figure 21). Six hours of continuous red light rephased the rhythm to give a peak of flowering occuring with a dark period 36 hours in length whereas, with the skeleton light period, peak flowering resulted with a dark period between 39 and 42 hours in duration. In these particular experiments there may have been some slight rhythm phase resetting by a skeleton light period although there was no rephasing in the similar experiments presented in Figure 21. A 5 minute interruption with red light at the mid point of the skeleton light period caused the phase of the rhythm to shift to hour 39 of darkness and, hence, towards the peak time obtained with the continuous light period (i.e. hour 36).

When light interruptions were given every 2 or $1\frac{1}{2}$ hours in the skeleton light period the peak of the rhythm did not appear to shift any further. However the more frequent the light interruptions the more flowering increased for dark periods 36 hours in length and declined for dark periods 45 hours in length (Table 6). The progressive shift from a rhythm similar to that of the dark controls with a peak at hour 43 in darkness (Figure 21) to a rhythm that peaks at the thirty-seventh hour in darkness after 6 hours of continuous light indicates

TABLE 6.

Flowering response of C. rubrum given a dark period of varied duration but with a 6 hour light interruption from hour 9 to 15 in darkness. All interruptions were with red light of 115 $\mu w/cm^2$ intensity and were given either continuously; as a skeleton light period of two 5 minute light pulses (5R) commencing and terminating the 6 hour period (e.g. 5R-6hD-5R); or as an "interrupted" skeleton light period (e.g. 5R-3hD-5R-3hD-5R). Total light period duration was always 6 hours and in designating the hours of darkness as "3hD" for example, this was shorthand for an actual dark period of 2 hours 52.5 minutes.

TREATMENT DURING 6 HOUR RED LIGHT PERIOD.	PERCENTAGE FLOWERING FOR DARK PERIODS OF VARIED DURATION (HOURS)						
	33	36	39	42	45	48	50
6 hours red	10	60	53	30	0		
5R-6hD-5R	0	7	33	33	27	7	0
5R-3hD-5R-3hD-5R	0	17	50	27	10	0	0
5R-2hD-5R-2hD-5R-2hD-5R	0	23	50	23	17	0	
5R-1½hD-5R-1½hD-5R-1½hD-5R-1½hD-5R	0	27	40	33	7	0	0

that rhythm rephasing represented an advance of the second peak of the control rhythm. It can also be concluded that if a skeleton light period was to substitute the effect of a continuous light period, it was essential to have the light interruptions distributed over the 6 hour period. A total of 15 to 25 minutes of red irradiation could rephase the rhythm only when spread over the 6 hours of the skeleton light period (Table 6) but even a 30 minute total irradiation was ineffective when given as 15 minute pulses at the beginning and end of the skeleton light period (Figure 21).

In a number of further experiments using a 6 hour skeleton light period attempts were made with far-red light to reverse the effect of interruptions of the skeleton light period with red light. Red interruptions of the skeleton light period consistently advanced the phase of the rhythm but clear far-red reversal of the red effect could not be achieved for a 6 hour light period.

In another series of experiments a red light period interrupted darkness for 12 hours from hours 9 to 21 in darkness. As for a 6 hour period, the phase of the rhythm controlling flowering could be assessed by giving dark periods 30 to 46 hours in duration (see Figure 14). The results presented in Figure 22 illustrate that continuous red irradiation shifted the peak of the rhythm of flowering from 43 hours to 36 hours in darkness. Rephasing to give a peak at 34 hours was expected on the basis of results presented in Figure 15. A skeleton light period of 5 minutes red - 11 hours 50 minutes darkness - 5 minutes red (Figure 22a) or 5 minutes red - 5 minutes far-red - 11 hours 45 minutes darkness - 5 minutes red (Figure 22b) had only a slight rephasing effect. Rhythm peaks occurred at 40 to 42 hours in darkness. On interrupting the skeleton light period with brief pulses of red light given every 2 or $1\frac{1}{2}$

hours (Figure 22a and b, respectively) the timing of the rhythm peak shifted to 38 hours in darkness and hence towards the peak time that resulted from 12 hours continuous red irradiation. Far-red light pulses did not shift rhythm phase to the same degree as red light but far-red was partially effective when compared to the skeleton light period. A terminal far-red radiation also influenced the red light induced rhythm phase shifting. The results in Figure 22a illustrate red/far-red reversibility of rhythm amplitude and not necessarily peak times, although the level of flowering obtained at 44 hours was higher than in plants which were exposed to red light alone. In Figure 22b far-red appeared to reverse the red induced shift of the rhythm peak times. Red interruptions of the skeleton gave a peak at about 38 hours, far-red at about 40 hours and red + far-red at 39 to 40 hours. The shoulders of the peak of the rhythm showed comparable responses. Assuming that photoreversible control by red and far-red radiation indicates phytochrome action, it is also apparent that even low levels of P_{fr} set by far-red irradiations were partially effective for rhythm phase resetting. Furthermore the action of P_{fr} must have been rapid because complete reversal of the effect of red light was not possible.

b. Light quality and rhythm amplitude.

Rhythm amplitude, i.e. the amount of flowering, was increased by the use of fluorescent light interruptions (see Figure 9). Figure 23 and Table 7 present further evidence to support this conclusion. In both experiments, after 9 hours of a dark period, 6 hours of light were given either from fluorescent (1037 $\mu w/cm^2$) or red (115 $\mu w/cm^2$) lamps. During the subsequent dark period groups of plants were removed from darkness at 3 hourly intervals and their flowering response determined. Compared to red light the amplitude and width of the rhythm peaks were

FIGURE 22.

Flowering response of C. rubrum given a dark period of varied duration interrupted with light from hour 9 to 21 in darkness.

Upper panel: (■) 12 hours continuous red irradiation or, (●) 12 hour skeleton red light period (a) initiated and terminated by 5 minutes of red radiation; (b) initial 5 minutes red only followed by $1\frac{1}{2}$ minutes far-red.

Middle panel: (a) 12 hour skeleton light period interrupted every 2 hours with 5 minutes of red (●) or far-red (○) radiation; (b) 12 hour skeleton with interruptions every $1\frac{1}{2}$ hours with $1\frac{1}{2}$ minutes of red (●) or far-red (○).

Lower panel: 12 hour skeleton light period interrupted every 2 (a) or $1\frac{1}{2}$ (b) hours with red light followed immediately by far-red (○) light.

Irradiation energies; 115 $\mu w/cm^2$ red, 420 $\mu w/cm^2$ far-red. Dashed line (b) lower panel indicates the response to the red interrupted skeleton light period (middle panel,b).

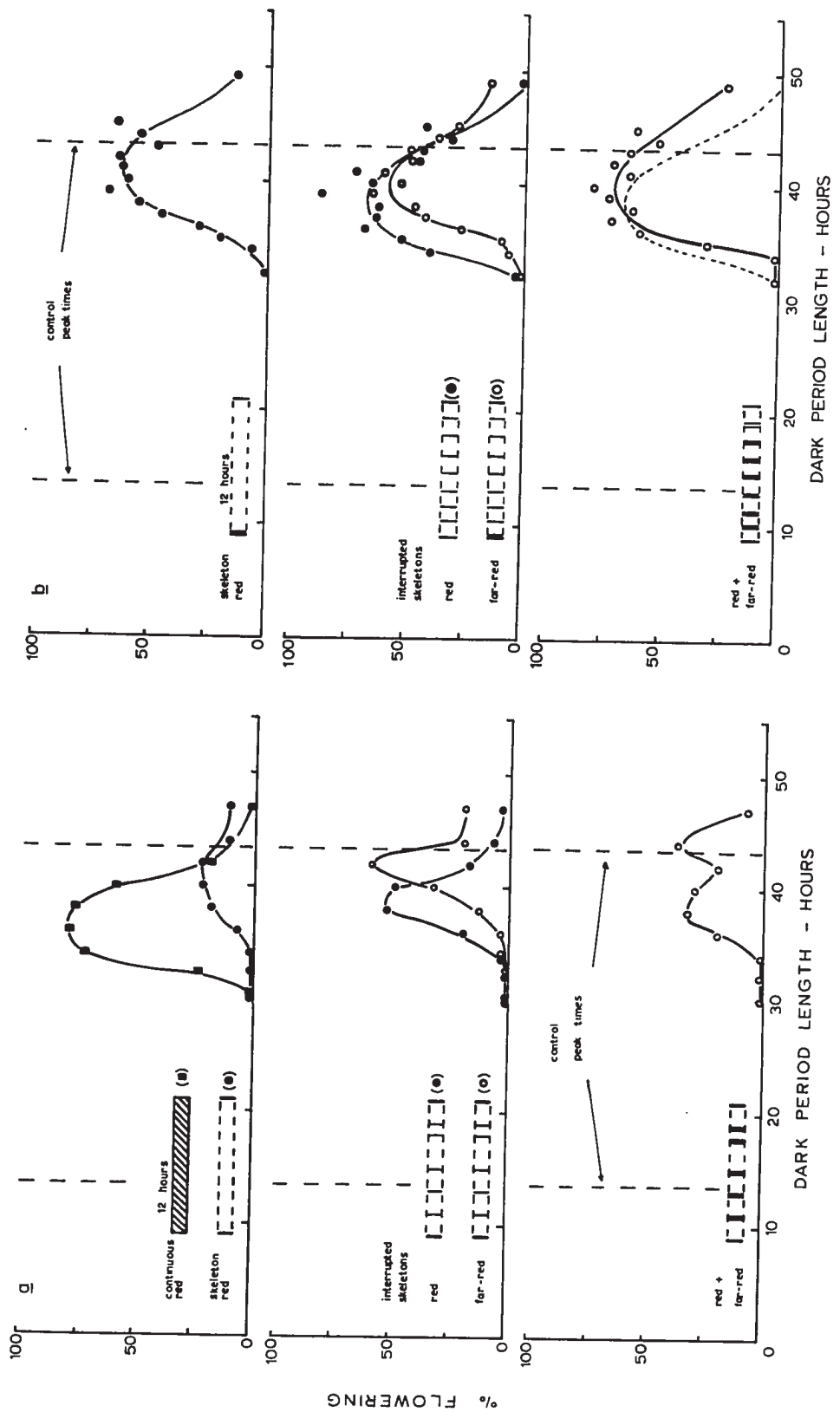

a
b
continuous red
12 hours
skeleton red
interrupted skeletons
red
far-red
red + far-red
control peak times
% FLOWERING
DARK PERIOD LENGTH - HOURS

FIGURE 23.

Flowering response of C. rubrum given a dark period of varied duration.

Upper curve; uninterrupted control curve.

Lower curves; a 6 hour light pulse was given from hour 9 to hour 15 in darkness: (●) 6 hours red light (115 $\mu w/cm^2$),

(○) 6 hours fluorescent light (1037 $\mu w/cm^2$).

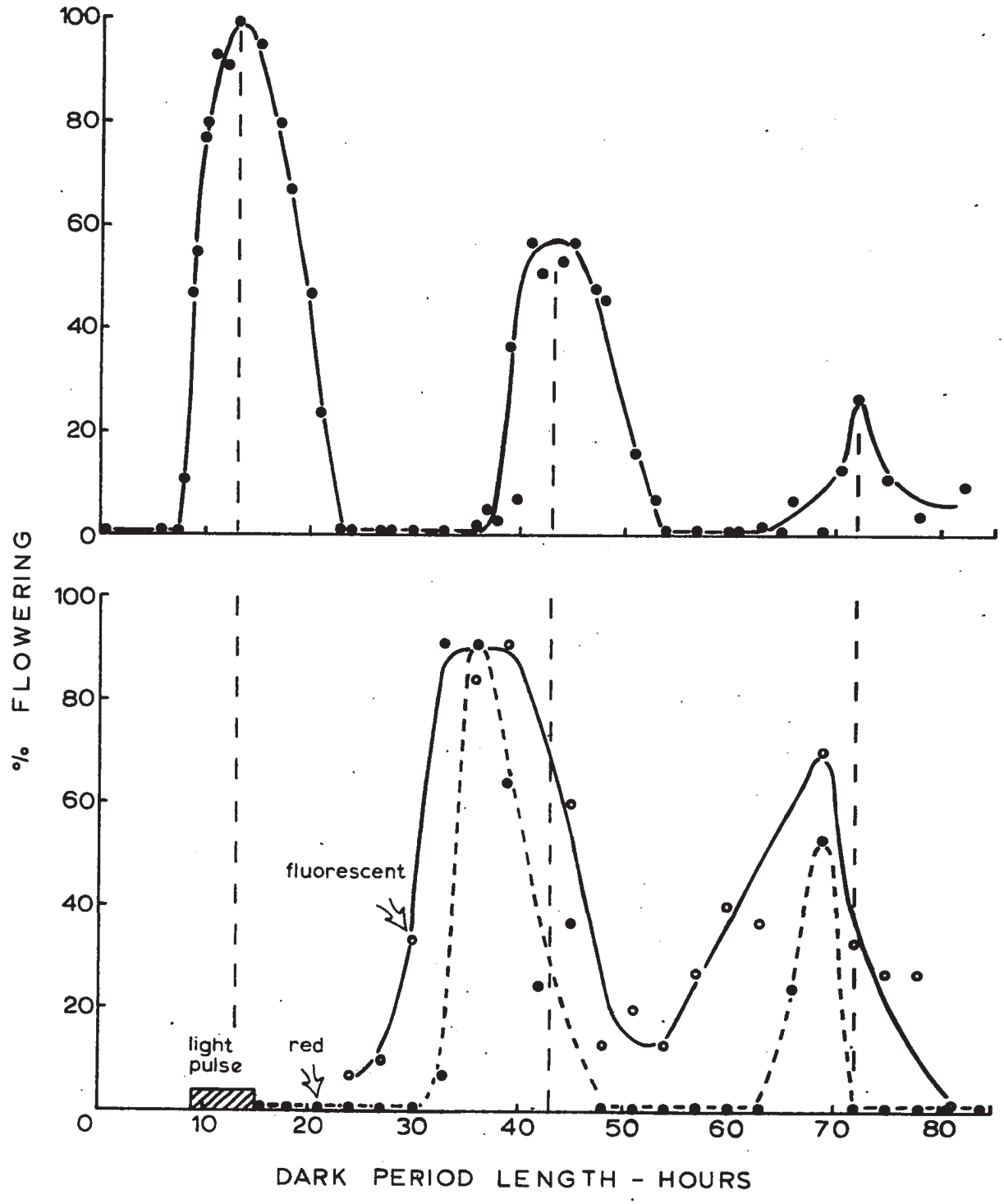

100
80
60
40
20
0
% FLOWERING
fluorescent
light pulse
red
10
20
30
40
50
60
70
80
DARK PERIOD LENGTH - HOURS

TABLE 7.

Flowering response of C. rubrum given a dark period of varied duration but with a 6 hour light interruption from hour 9 to hour 15 in darkness. Irradiations with red (115 $\mu w/cm^2$) or fluorescent (1037 $\mu w/cm^2$) lamps.

DARK PERIOD LENGTH (HOURS)	PERCENTAGE FLOWERING	
	SIX HOURS FLUORESCENT LIGHT	SIX HOURS RED LIGHT
33	57	10
36	90	60
39	83	53
42	73	30
45	53	0
50	0	

always greater after a fluorescent light period (Figure 23b and Table 7). The timing of the peaks was distinctly shifted compared to the timing in the uninterrupted control plants (Figure 23a), but, as reported in Chapter III, red and fluorescent light behaved in an identical manner for rhythm rephasing.

In Chapter III it was established that a fixed period of exposure to red or fluorescent irradiation induced the same degree of rhythm rephasing. When the red source was running continuously the temperature could only be maintained at 26°C so that intensity and quality differences between the red and fluorescent radiation may have been confounded by effects of temperature. However, it is unlikely that temperature influenced the capacity to reset the phase of the rhythm since, when given at a temperature of 20°C, brief red light interruptions rephased the rhythm (Table 6) in the same manner as did a continuous period of red irradiation given at 26°C. To further ensure that temperature differences could not influence phasing, a single experiment was performed in which a 6 hour fluorescent light period at 600 ft-c intensity was used to interrupt darkness but, during the light period, the temperature was either 26°C or 20°C. Conditions were otherwise identical for the two treatments and the flowering response was measured for dark period lengths between 33 and 45 hours of darkness. The results of this experiment (Table 8) show that rephasing of the rhythm was unaffected by temperature although the amplitude of the response may have been greater at the higher temperature.

Considering only the differences in intensity and light quality between red and fluorescent radiation, since equivalent phase resetting resulted with either light source it can be concluded that the effective wavelengths of the fluorescent light were those in the red region of the

TABLE 8.

Flowering response of C. rubrum given a dark period of varied duration but with a 6 hour light interruption given from hour 9 to hour 15 in darkness. Fluorescent light of 600 ft-c (1037 $\mu w/cm^2$) was given as the light interruption and the temperature was 20°C or 26°C.

DARK PERIOD LENGTH (HOURS)	PERCENTAGE FLOWERING	
	SIX HOURS FLUORESCENT LIGHT	
	TEMPERATURE 20°C	TEMPERATURE 26°C
30	51	76
37	96	98
39	78	89
42	56	60
45	18	22
48	22	22

spectrum. Furthermore, greater rhythm amplitude after 6 hours of the more intense fluorescent source can probably be ascribed to increased photosynthesis at the higher intensity. Other evidence presented in Chapter II also indicated that the degree of photosynthesis might control the level of flowering in C. rubrum.

IV. DISCUSSION.

The results presented in Chapter III and in this Chapter show that low intensity red light and a higher intensity of fluorescent light were equally capable of resetting the phase of the rhythm of flowering in Chenopodium rubrum. In other higher plants red irradiation is known to be most effective for shifting the phase of a rhythm (see Wilkins, 1960 and Table 5). It has also been established that red light given during the photoperiod is most effective for flowering of the short-day plants Chenopodium amaranticolor (Konitz, 1958); Pharbitis nil (Takimoto and Ikeda, 1959; Takimoto and Naito, 1962b; Salisbury, 1965; Takimoto, 1967; Zeevaart and Marushige, 1967) and Xanthium (Salisbury, 1965). Possibly red light is most effective for Lemna perpusilla as well since red light given as a skeleton photoperiod entrains the rhythmic flowering response of this plant (Hillman, 1964a).

Light absorption by chlorophyll might be advanced to explain these observations of effectiveness in the red region of the spectrum. However, in the absence of any affect of increased light intensity, i.e. of increased photosynthesis, on rhythm rephasing (Figure 23 and Table 7) it seems unlikely that photosynthetic products are immediately involved in rephasing the rhythm of flowering in C. rubrum. Indeed, other results presented in Chapters III and VI show that application of glucose did not

influence rhythm phasing and that glucose probably acted at the apex to increase the flowering response. Furthermore, while the rhythm amplitude responded to light intensity (Figure 23 and Table 7) this could be achieved by varying the light intensity either before or after darkness (Ch. II). Hence, floral development rather than dark period time measurement was modified by photosynthetic energy input.

As an alternative to chlorophyll as the photoreceptor for rhythm rephasing, phytochrome could be activated by red irradiations. The involvement of phytochrome is favoured by the finding that a total of 15 to 25 minutes of red light replacing a 6 hour light period could induce rhythm rephasing provided that the irradiations were spread over the 6 hour period (Table 6). Total energy input was not important at the energy levels used. This point is emphasized by the failure of 30 minutes of red light given as two 15 minute pulses to induce any rhythm phase resetting (Figure 21). A capacity to respond to red light given as repeated short exposures and sensitivity to low energies (115 $\mu w/cm^2$, between 600-800 nm) is suggestive of phytochrome action. That a brief far-red irradiation partly reversed the red light induced phase resetting (Figure 22) provides evidence that phytochrome is the photoreceptor controlling phasing of the rhythm of flowering in C. rubrum and probably in other short-day plants.

Previously, in the absence of definitive evidence, some authors have suggested the action of two photoreceptors controlling photoperiodic time measurement (e.g. Bunning, 1967; Halaban and Hillman, 1970b). Phytochrome was considered to act only as a photoreceptor interacting with rhythmic changes in sensitivity to light and this action was equated to the "coincidence effect" of light impinging on the scotophile phase of the rhythm. However, it would appear that for short-day plants any

model of photoperiodic time measurement must incorporate both an action of P_{fr} that determines rhythm phase and an action of P_{fr} that is determined by rhythm phase. Dual responses to P_{fr} need pose no difficulty as in any one organism multiple responses to P_{fr} are not unknown. So it may be that, in C. rubrum, the presence of P_{fr} over prolonged periods of time (greater than 3 hours, Figure 19) controls rhythm phasing whereas sensitivity to the presence of P_{fr} in darkness - the "coincidence effect"- is evident with a 5 minute irradiation (Cumming et al, 1965).

The precise meaning of "coincidence effects" of light on the timer remains somewhat vague. It is possible that both brief and prolonged irradiations induce rhythm rephasing but to different degrees. This explanation is valid for the photoperiodic response of the rhythm of emergence of Drosophila. Brief light interruptions of darkness are completely effective for rhythm rephasing in this organism. In C. rubrum brief light interruptions of darkness had little effect on rhythm phase (Cumming et al, 1965 and Figure 20) and a separate "coincidence effect" of light would appear essential for this plant. In addition to a rhythm in availability of the substrate on which P_{fr} acts, there may also be superimposed on this response an "hourglass" component of P_{fr} action. This will be discussed further in Chapter V .

CHAPTER V

PHYTOCHROME AND PHOTOPERIODIC TIME MEASUREMENT.

I. INTRODUCTION.

In the control of flowering of Chenopodium rubrum the phasing and hence timing of an endogenous rhythm is crucial. It cannot be argued, however, that rhythms are therefore exclusively the "clock" that measures photoperiod duration and, in Chapter III, it was found that there was a fixed lower limit to the length of the critical dark period and this raised the question of an additional timer whose action was limiting in short dark periods.

Such an action is commensurate with the evidence of Takimoto and Hamner (1964) who suggested that rhythms and an hourglass timer measured the dark period length in the short-day plant Pharbitis nil. Phytochrome in its conversion from the active to the inactive form during darkness could measure time in much the same manner as an hourglass and a number of workers have discussed this possibility (see Borthwick et al, 1952; Hendricks, 1960: Lang, 1965). However, a major shortcoming of this concept has been our inability to measure the levels of phytochrome present in green tissue. Cumming et al (1965) developed a method for estimating the proportions of phytochrome in the active (P_{fr}) form in green tissue during darkness and, using this technique Evans and King (1969) were able to follow phytochrome conversions in the cotyledons of Pharbitis nil during the early hours of a short inductive dark period.

The aim of the experiments presented in this Chapter was to examine the possibility that phytochrome reversion from P_{fr} during

darkness might be limiting to rhythmic time measurement in C. rubrum. The method developed by Cumming et al (1965) and applied by them during a long dark period has been used to measure phytochrome conversions during a short dark period. Two approaches have been adopted in an attempt to vary the timing of phytochrome conversion and hence its timing capacity. Firstly, the proportion of P_{fr} present at the start of darkness has been varied by terminating the light period with brief irradiations to set the initial level of P_{fr} high or low. Secondly, since photoperiod light quality may interact with the tolerence to P_{fr} during darkness (Meijer and Van der Veen, 1960; Takimoto, 1967; Evans and King, 1969), light quality has been varied from all red to all far-red over the last 12 hours of the continuous light period given before a dark period. Under each type of preconditioning phytochrome dark conversion has been assessed and time measurement estimated from both the length of the critical dark period and the timing and phasing of the rhythm of flowering. Although light quality has been varied the timing of the light-off signal has been held constant so that rhythm phasing effects could be excluded.

II. MATERIALS AND METHODS.

a. Growing conditions.

The standard procedures for germination and growth of Chenopodium rubrum (origin 60°47'N 137°32'W) were described previously (Chapter II) and were adhered to in all the experiments reported here. The single dark period always commenced 5½ days after sowing and was preceded and followed by fluorescent light of 600 ft-c intensity at

20°C. Dark period temperatures were 20°C. In two experiments (Figure 36), in place of the normal Hoagland's nutrient solution, 0.4 molar glucose in Hoagland's solution was applied once $4\frac{1}{2}$ days after sowing. This solution was not replaced with Hoagland's solution until the end of the dark period.

b. Light treatments.

(i) Broad band irradiation.

In order to vary the proportion of phytochrome in the P_{fr} form at the beginning of darkness and to give prolonged irradiations with red or far-red, broad band red irradiation was obtained from red fluorescent lamps filtered through a No. 14 Ruby Cinemoid filter; far-red was obtained from incandescent tubes filtered through heat absorbing glass and a layer of far-red plastic or from dark ruby red (BCJ) incandescent lamps. Irradiances were measured with an ISCO SR spectroradiometer and total energies as well as the proportions in the red (600 - 700 nm) and far-red (700 - 800 nm) regions are presented in Table 9 together with precise details of the light sources. The spectral distribution of radiation from the light sources is given in Figure 24 and further descriptions are available in publications by Cumming (1969c) and Wagner and Cumming (1970). Photostationary phytochrome settings established by these lamps were checked using differential spectrophotometry to measure the equilibrium proportions of P_{fr}:P_r set on irradiating dark grown oat coleoptiles. Although it is a crude calculation of phytochrome setting, the percentage of red radiation of a light source ($\frac{\text{energy 600-700}}{\text{energy 600-800}} \times 100$, Table 9) compared well with spectrophotometric settings of P_{fr} measured in non-green tissue.

(ii) Narrow band irradiation.

In order to estimate the proportion of phytochrome in the P_{fr} form at different times during a dark period interruptions with mixtures

FIGURE 24.

Spectral composition of radiation from the light sources used (a and b scale 0 to 10 $\mu w/cm^2/nm$; c, d, e, and f scale 0 to 20 $\mu w/cm^2/$ nm).

(a) Westinghouse cool white fluorescent lamps (F20 T12 CW) giving an intensity of 600 ft-c or a total energy of 1037 $\mu w/cm^2$.

(b) Broadband red irradiation; from red fluorescent lamps (G.E. F30 T12 R.RS) plus one layer of No. 14 Ruby Cinemoid.

(c) Narrow band red radiation; from a Sylvania super-tru flector DLR 10 volt 16 mm projector lamp filtered through a Schott 654 nm interference filter plus one cm of water.

(d) Narrow band far-red radiation; from a Sylvania super-tru flector DLR 10 volt 16 mm projector lamp filtered through a Schott 728 nm interference filter plus one cm of water.

(e) Broad band far-red radiation; from incandescent tubes (Westinghouse Lumiline 60W 120V) filtered through heat absorbing glass (KG1) plus one layer of far-red plastic (No. V58015).

(f) BCJ lamps. G.E. 60W BCJ incandescent photographic ruby lamps.

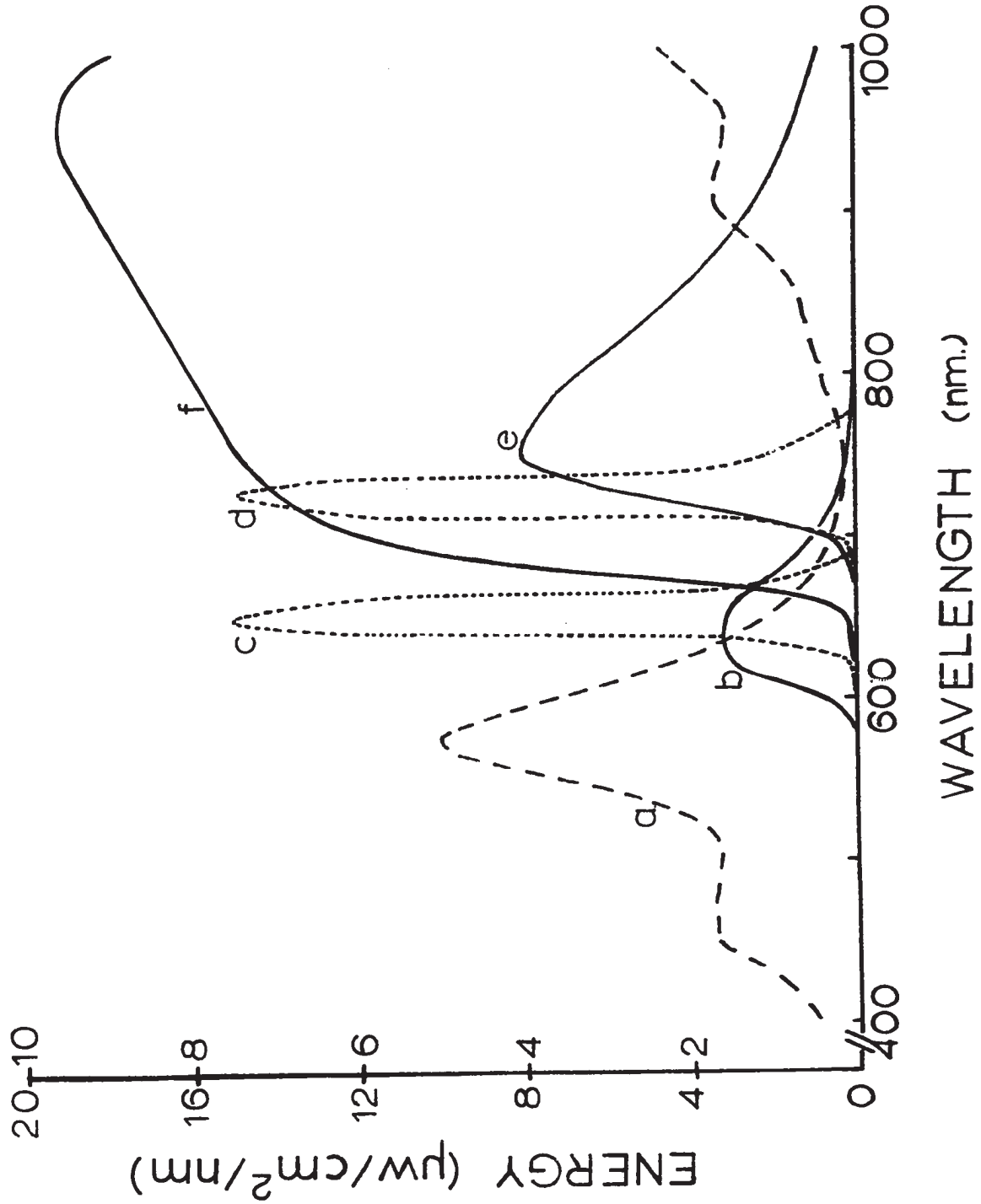

ENERGY (μw/cm²/nm)
20
16
12
8
4
0
10
8
6
4
2
400
600
800
1000
WAVELENGTH (nm.)
a
b
c
d
e
f

TABLE 9.

Energy and spectral composition of light sources. Measurements made at plant height using an ISCO spectroradiometer.

LIGHT SOURCE	ENERGY 654_2nm $\mu w/cm^2/nm$	ENERGY 728_2nm $\mu w/cm^2/nm$	ENERGY 600-700 $\mu w/cm^2$	ENERGY 600-800 $\mu w/cm^2$	% RED ENERGY $\frac{600-700}{600-800}$	TIME TO RADIOSTATIONARY SETTING (SEC.)
a. Fluorescent lamps			283	333	85	
b. Broad band red radiation	1.6		96.6	115	88	240
c. Narrow band red radiation	15.0	0	482	482	100	90
d. Narrow band far-red radiation	0	15.0	10	474	2	80
e. Broad band far-red radiation		6.0	4.0	628	1	50
f. BCJ lamps			280	1668	17	

of red and far-red radiation were given in a light proof box as described by Cumming et al (1965) and Evans and King (1969). Two 16 mm projector lamps were mounted on either side of the box and the light from these lamps, after passing through a 1 cm water filter, was reflected down into the box using stainless steel mirrors placed back to back. Before entering the box all radiation had to pass through a narrow band interference filter. A red (λmax. 654 nm) filter was placed in one light path and a far-red filter (λmax. 728 nm) in the other. The mirrors were placed near the centre of the top of the box so that practically identical cones of radiation could be mixed in the box giving a circle of irradiation of about 6 inches at plant height. Three dishes were placed in the most uniform zone of the mixed beams and, in order to further reduce any inequalities of irradiances the dishes were rotated at 17 rpm on a turntable. Two identical boxes were used routinely so that a series of irradiations was completed in 20 minutes.

Spectral composition and energy distributions of the two narrow band sources are given in Figure 24 and Table 9. The total energies at the wavelength of maximum transmission of the filters was adjusted to 15 $\mu w/cm^2/nm$ (Table 9) using a rheostat connected in series with each projector lamp. This energy has been designated as 100% red or 100% far-red radiation for light passing through the red (654 nm) or far-red (728 nm) filters, respectively. To measure the proportion of phytochrome present in green tissue during darkness by the method of Cumming et al (1965), the proportion of red to far-red radiation in the mixed beams is varied from all red to all far-red. Thus, to obtain a mixture giving 60% red of the red+far-red energy, the lamp voltage of the red source would be reduced to 60% of its maximum energy (100% = 15 $\mu w/cm^2/nm$) and a complementary 40% energy set for the far-red source. Whatever the

composition of red and far-red the total energy of irradiation in the mixed beams always remained at 15 $\mu w/cm^2/nm$.

c. Null response measurements of the proportions of phytochrome in green tissue.

Provided the total energy of an irradiation is sufficient to drive phytochrome interconversions to a photostationary equilibrium, each mixture of red and far-red radiation will set a different photostationary equilibrium of $P_{fr}:P_r$. When different groups of plants are exposed at a particular time in darkness to the various mixtures of red and far-red radiation, the composition of the light interruptions which has no net effect on flowering i.e. which gives a null response indicates the proportion of phytochrome in the P_{fr} form at that time. Excess or insufficient proportions of P_{fr} introduced at that time will alter the flowering response compared to that of the uninterrupted controls. There should be little or no effect on the plant at the null point so that this method will not be influenced by changing sensitivity to phytochrome during darkness but should reflect actual changes in the proportions of P_{fr} in the physiologically active pool.

In vitro there is in fact a fixed relationship between the proportion of phytochrome as P_{fr} at photostationary equilibrium and the proportion of red light in a mixture (see Cumming et al, 1965). In green tissue preferential scattering and screening of red light by chlorophyll will modify the effective proportions of red and far-red penetrating into the tissue. It is therefore only possible to equate the proportion of red light in a mixture to some unknown but relative proportion of P_{fr} set in the tissue. For this reason discussion is confined to the value of the percentage red energy of the red far-red energy as incident on the cotyledons. A high null per cent red reflects

a high proportion of P_{fr} at that time, a low null per cent red reflects a low proportion of P_{fr}. The photostationary equilibrium method yields no information on the amount (level) of phytochrome in the two forms.

III. RESULTS

a. Preliminary experiments.

Three factors were essential for measurements of phytochrome proportions during a dark period. These were:-

(i) That the energy of irradiation whether pure red or far-red or a mixture, should be sufficient to drive the interconversions of P_r and P_{fr} to photostationary equilibrium.

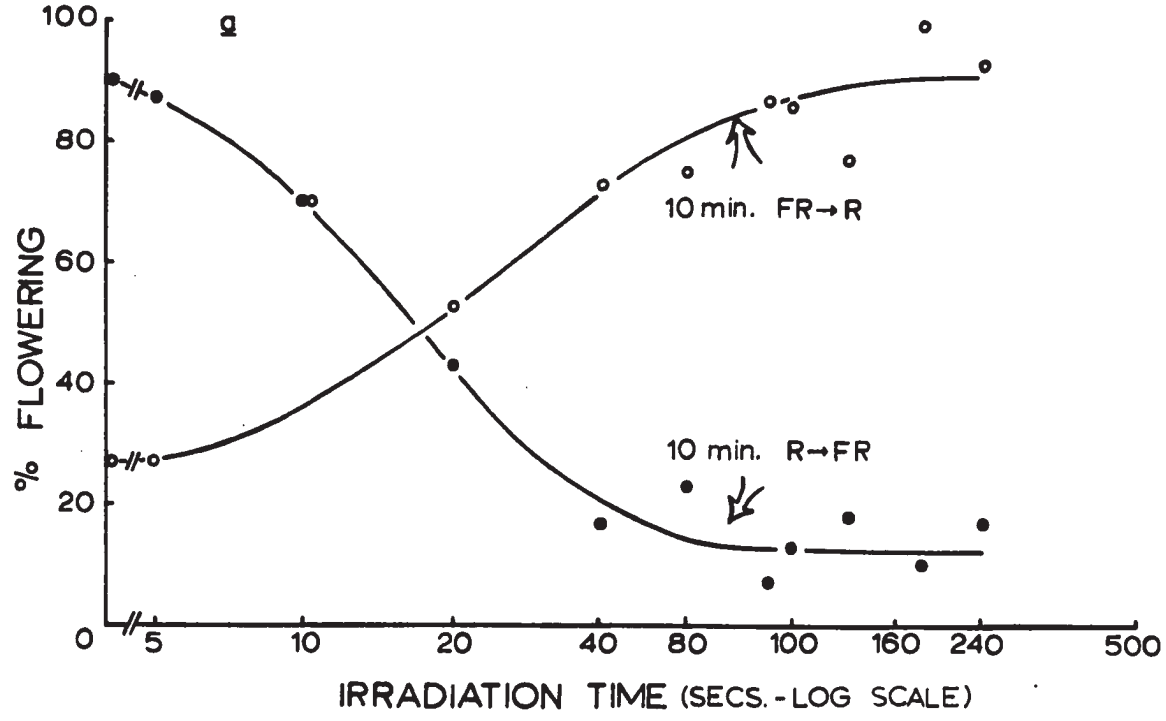
(ii) To give greatest expression to both promotion and inhibition an intermediate level of flowering should be established in the uninterrupted controls. The greater this promotion and inhibition of flowering the greater the accuracy of measurement of the null per cent red value.

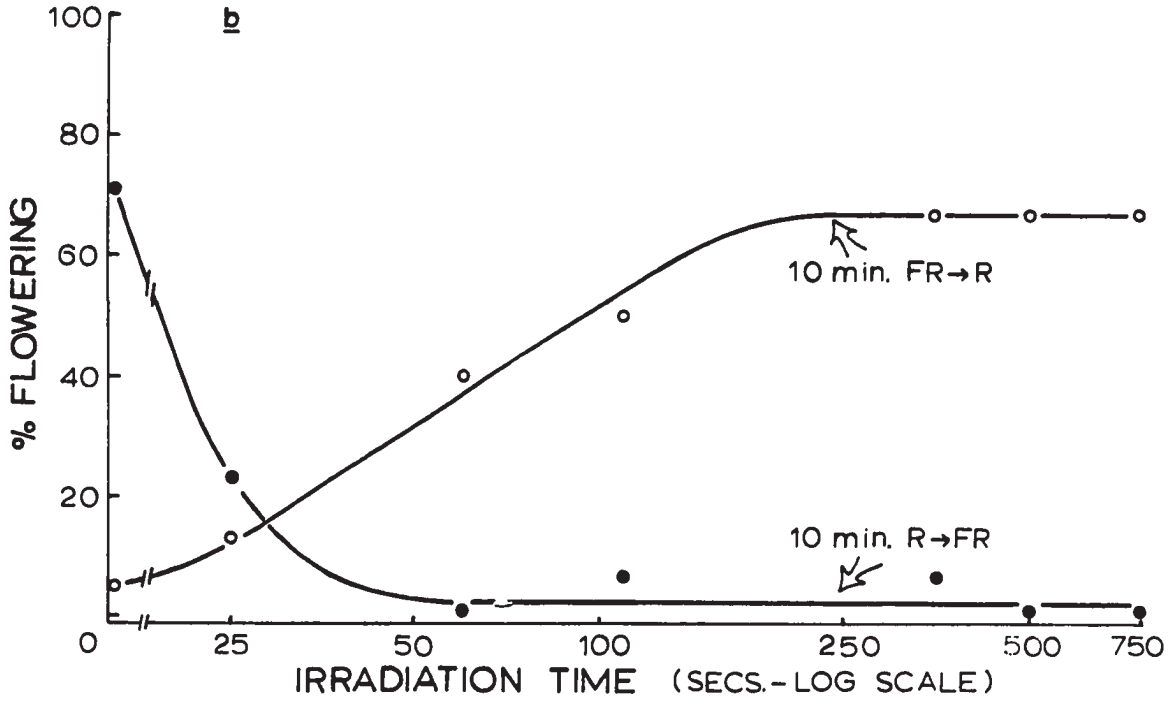
(iii) When comparing tissues set with high or low proportions of P_{fr} such as introduced by irradiations with red or far-red, respectively, then the null per cent red values should differ significantly.

With regard to (i), groups of plants were exposed to 10 minutes of broad band red or far-red radiation at the beginning of darkness to establish initial high or low proportions of P_{fr}. Little flowering resulted when the dark period commenced with a low level of P_{fr}. If plants exposed to 10 minutes of far-red irradiations were immediately exposed to various durations of narrow band red irradiation P_r was reconverted to P_{fr}, and flowering increased (Figure 25a). The longer the irradiation the greater the photoconversion of P_r to P_{fr} and saturation was reached after

FIGURE 25.

Flowering response to a dark period of: (a) 12 hours; (b) 11 hours; interrupted at the beginning of darkness with 10 minutes of broad band red or far-red irradiation followed by various durations of narrow band (a) or broad band (b) far-red (●) or red (○) irradiation respectively.


a
100
80
60
40
20
0
% FLOWERING
10 min. FR→R
10 min. R→FR
5
10
20
40
80
100
160
240
500
IRRADIATION TIME (SECS.-LOG SCALE)


b
100
80
60
40
20
0
% FLOWERING
10 min. FR→R
10 min. R→FR
25
50
100
250
500
750
IRRADIATION TIME (SECS.-LOG SCALE)

about 90 seconds of irradiation with an energy of 15 $\mu w/cm^2$ at 554 nm. Conversely, flowering and the level of P_{fr} was high following red irradiation at the start of darkness but both were reduced to a low level after 80 seconds of narrow band far-red irradiation with an energy of 15 $\mu w/cm^2$ at 728 nm (Figure 25a). Thus exposure to 90 seconds of either pure or mixed narrow band irradiation of an energy of 15 $\mu w/cm^2/nm$ should be sufficient to reach a photostationary equilibrium of phytochrome interconversions. Similar results were obtained in other experiments and, in addition to red/far-red reversibility and saturation at low energies, reciprocity was found between the duration and the intensity of the irradiation. All these features leave no doubt that phytochrome was the effective photoreceptor pigment.

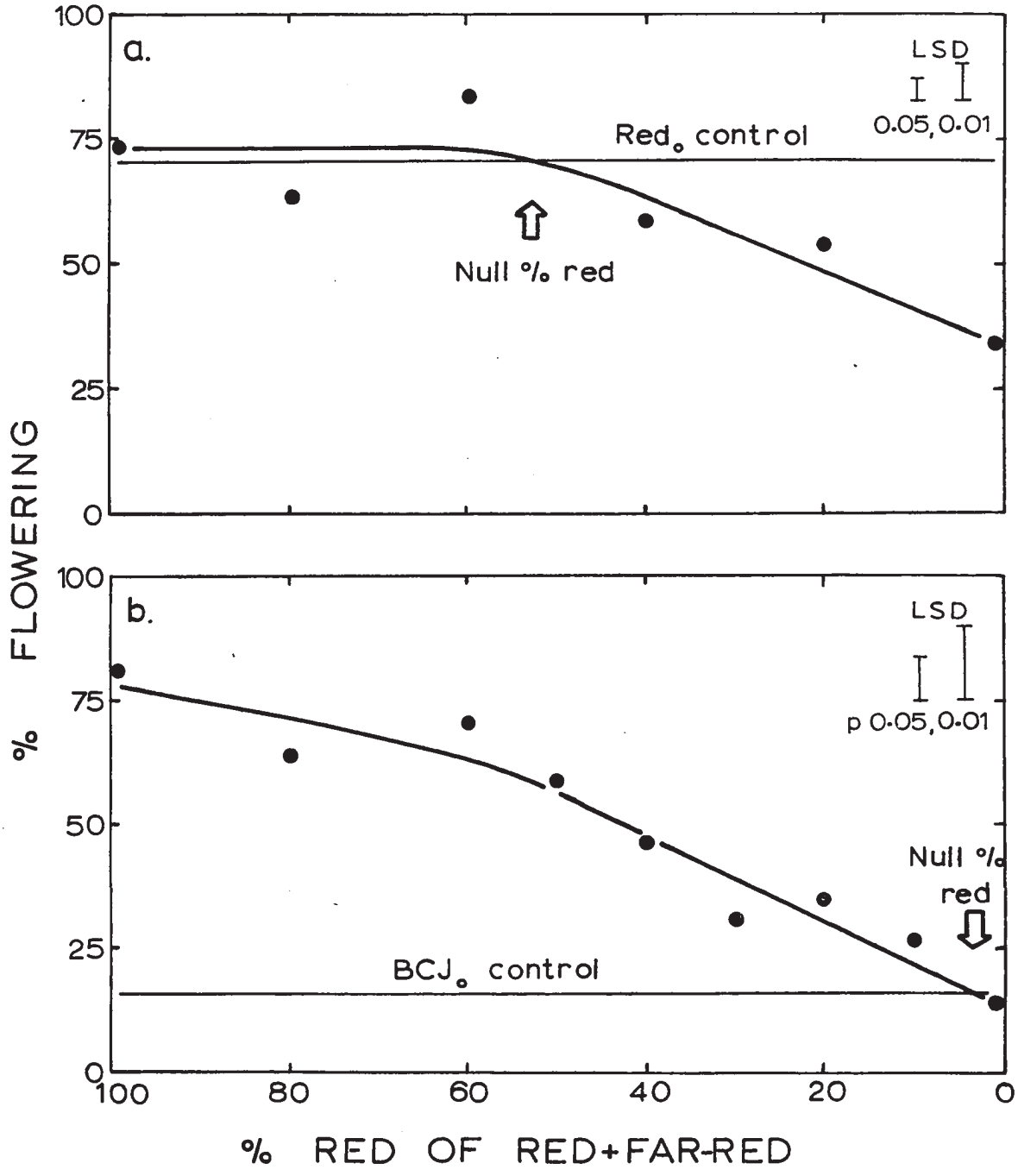
The results presented in Figure 25b established the durations of irradiation required to reach photostationary equilibrium with broad band radiation. Broad band red radiation at an energy of 1.6 $\mu w/cm^2$ at 650 nm achieved photostationary equilibrium after 4 minutes. With broad band far-red radiation (6 $\mu w/cm^2$ at 725 nm) photostationary equilibrium was achieved in less than 50 seconds.

The length of darkness required to give an intermediate level of flowering (ii) was about $9\frac{1}{2}$ hours when darkness commenced with a 5 minute broad band red irradiation (see Figures 36 and 37). If lower proportions of P_{fr} were set at the start of darkness a slightly longer dark period was required to obtain 50% flowering in the control plants (see Figure 37).

With reference to (ii) and (iii) it was evident in Figure 25 that flowering was very sensitive to the proportions of phytochrome P_{fr} set by an irradiation. The results presented in Figure 26 show that for various combinations of red and far-red there was a gradual decline

FIGURE 26.

Effect on flowering of a single 1½ minute exposure to mixtures of red and far-red radiation given after (a) one hour of a 9½ hour dark period or (b) at the beginning of a 10¾ hour dark period. Plants were exposed to either (a) broad band red radiation (Red_o) or (b) BCJ lamps (BCJ_o) for 5 minutes at the beginning of darkness.


a.
LSD
0.05, 0.01
Red$_o$ control
Null % red
b.
LSD
p 0.05, 0.01
Null % red
BCJ$_o$ control
% FLOWERING
100
75
50
25
0
100
80
60
40
20
0
% RED OF RED+FAR-RED

in the flowering response as the per cent red in the mixture decreased and, again, 100% red was distinctly superior to 0% red whether the irradiations were given at the beginning of darkness (Figure 26b) or after one hour of darkness (Figure 26a). All these irradiations were for 90 seconds at an energy of 15 $\mu w/cm^2/nm$ so that the gradual decline in flowering with decreasing per cent red in the mixture reflected changing proportions of P_{fr} established in the cotyledons. There were 5 replicate dishes per point in Figure 26a (a total per point of 75 plants sampled for flowering) and 3 dishes per point in Figure 26b (45 plants sampled for flowering).

In the experiments shown in Figure 26 all plants were given either 5 minutes of red (Figure 26a) or BCJ (Figure 26b) irradiations at the start of the dark period. Thus the phytochrome setting in the uninterrupted control plants can be inferred from the point of intersection of the flowering response for control plants with the curve describing the response to irradiations of various spectral combinations. The point of intersection where the mixtures gave a flowering response no different from the uninterrupted controls (the null per cent red) was about 58% red in Figure 26a and 0% to 2% in Figure 26b. From a number of experiments the null per cent red value at the start of darkness was 82%, 80%, 75%, 67%, 65% and 50% after red irradiation; 35%, 20%, 20% and 2% after BCJ irradiation; and 20%, 0% and 0% after far-red irradiation. These values for the null per cent red are clearly different and correspond with calculated proportions of red to far-red energy in the lamps (Table 9) and hence to the proportions of P_{fr} set by these lamps at the start of darkness. Evans and King (1969) reported similar sensitivity of the null response method in detecting differences in the proportions of phytochrome in the cotyledons of Pharbitis nil.

Unlike their measurements, in C. rubrum after a terminal red irradiation the null per cent red was not 100% at the beginning of a dark period. Cumming et al (1965) also reported that after fluorescent light the null per cent red was 65% at the beginning of a 72 hour dark period. It may be that C. rubrum differs from Pharbitis nil in its sensitivity to high proportions of P_{fr}. These differences could also relate to greater chlorophyll screening in Pharbitis nil than in C. rubrum (Kasperbauer et al, 1963). On the other hand, it was not possible to assess accurately whether the null per cent red value was at 60% or 100% red. Fortunately these difficulties do not distort the overall response pattern although for this reason experiments have been repeated a number of times. Since the flowering response was generally low after far-red, to establish a low level of P_{fr} at the start of darkness BCJ rather than far-red lamps were used.

b. Null per cent red during a short dark period.

The results presented in Figures 27 and 28 illustrate some of the raw data obtained when the proportions of phytochrome in the cotyledons were tested at various times during the dark period. These data are identical to those presented in Figure 26 in which flowering was related to the spectral composition of a light interruption. So that comparisons could be made between the shape of curves and the time of measurement of the null per cent red values the curves in Figures 27 and 28 have been rotated 90° relative to those in Figure 26. The upper curves in both Figures 27 and 28 are taken from single experiments (C47 and C42) whereas the lower curves show a representative sample of the responses in a number of other experiments. The null per cent red value is at the point of intersection of the flowering response of the uninterrupted controls (vertical line) with the curve for the response to different

FIGURE 27.

Effect on flowering of a single 1½ minute exposure to mixtures of red and far-red irradiation given to different groups of plants every hour during a dark period of 9 hours 30 minutes. All plants were exposed to broad band red irradiation for 5 minutes at the beginning of the dark period. Vertical lines represent the flowering of the uninterrupted controls. Upper curves from experiment C46. Data in lower curves from experiments C17, C66, C17, C66, C17, C60 and C60 for the respective times of interruption.

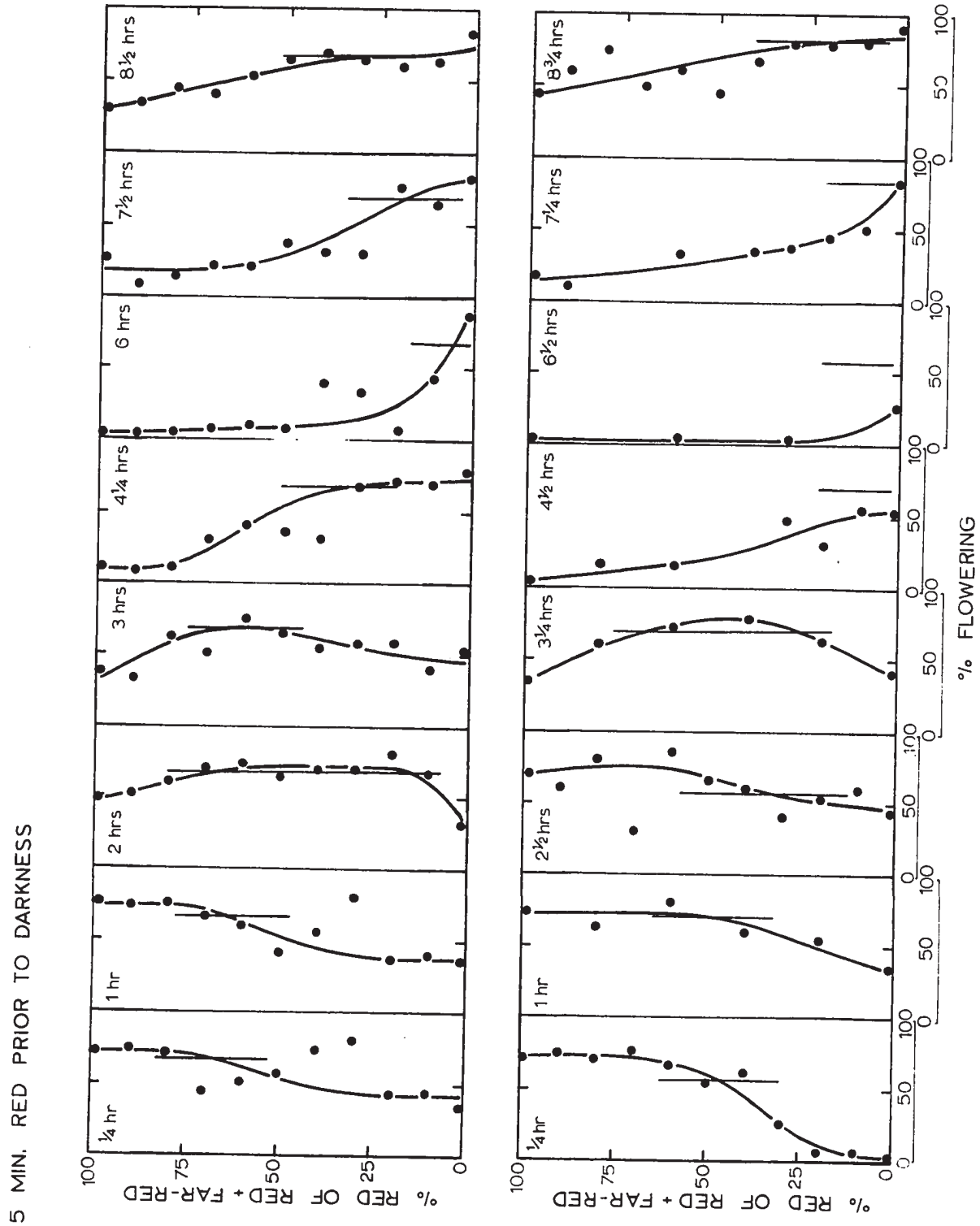

5 MIN. RED PRIOR TO DARKNESS
¼ hr
1 hr
2 hrs
3 hrs
4¼ hrs
6 hrs
7½ hrs
8½ hrs
2½ hrs
3¼ hrs
4½ hrs
6½ hrs
7¼ hrs
8¾ hrs
% RED OF RED + FAR-RED
% FLOWERING
0
25
50
75
100

FIGURE 28.

Effect on flowering of a single 1½ minute exposure to mixtures of red and far-red irradiation given at various times during a dark period of 10 to 10¾ hours. All plants were exposed to broad band BCJ irradiation for 5 minutes at the beginning of the dark period. Independent variable plotted on the ordinate scale. Vertical lines represent flowering of the uninterrupted controls. Upper curves from experiment C42 (10 hour dark period). Lower curves from experiments C47, C51, C51, C44, C44, C61, C61 and C61 for the respective times of interruption. Dark period length 10 hours (C44); 10½ hours (C47); 10¾ hours (C51) and 10½ hours (C61).

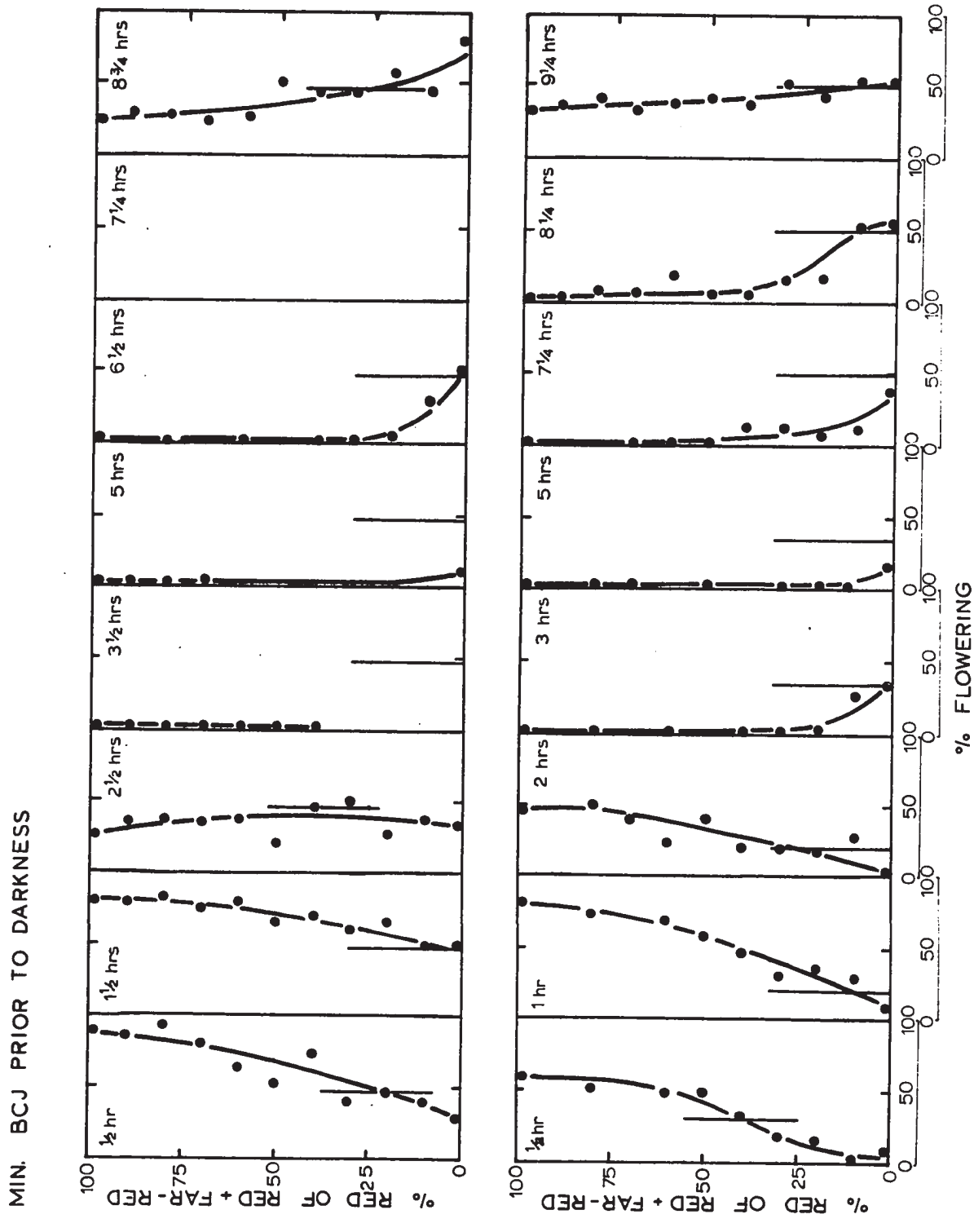

5 MIN. BCJ PRIOR TO DARKNESS
½ hr
1½ hrs
2½ hrs
3½ hrs
5 hrs
6½ hrs
7¼ hrs
8¾ hrs
½hr
1 hr
2 hrs
3 hrs
5 hrs
7¼ hrs
8¼ hrs
9¼ hrs
% RED OF RED + FAR-RED
% FLOWERING
0
25
50
75
100

TABLE 10.

Effect on flowering of a single 1½ minute exposure to mixtures of red and far-red irradiation given at various times during the early hours of a dark period of 10½ to 10¾ hours. All plants were exposed to broad band irradiation for 5 minutes at the beginning of the dark period.

Percentage red of red + far-red mixture	Percentage Flowering										
	Experiment C44 Time in darkness				Experiment C47 Time in darkness			Experiment C51 Time in darkness			
	½ h	1¼ h	2½ h	3¼ h	½ h	1¼ h	3¼ h	½ h	1¼ h	2¼ h	3½ h
100	60	40	0	0	60	37	0	80	47	18	0
90			0				0				
80	56	27	0	0	51	37	0	72	51	20	
70		47	0				0		40	22	7
60	42	41	7	0	47	30	0	70	22	18	
50	13	20*	7		49	33	0	58	40	16*	
40	44	20	13	0	33	20*		47	20	11	0
30	42	10	16	0	18*	20	0	31	16*	9	3
20	36	22	24*	5	16	10		36	29	2	7
10	3*	27	7	27	4	3	0	30	4	18	0
0	7	26	18	33*	9	10	0	9*	0	9	9*
control	34	34	34	34	30	30	30	19	19	19	19

* Values closest to null response.

mixtures of red and far-red. As discussed above, when the light period was terminated with red light there was some ambiguity about the position of the null per cent red at the beginning of darkness (Figure 27). After two hours in darkness (Figure 27) the null per cent red may have been 65% or 15% but the 65% value must be accepted as being in best agreement with earlier and later values. The value of the null per cent red at 3¼ hours (Figure 27) was taken as the average of the two points of intersection (i.e. $\frac{30 + 65}{2} = 47\%$).

The null per cent red and hence the proportion of phytochrome P_{fr} was high at the start of a dark period following red irradiation that terminated the light period (Figure 27) but as expected the null per cent value was low following terminal BCJ irradiation (Figure 28). In either case no or very low null response was apparent after 3 to 4 hours of darkness suggesting that P_{fr} had reverted by this time.

Figure 29b,c summarize the data for the null per cent values at different times in darkness as derived from Figures 27 and 28 and from a number of other experiments. The percentage of red light in a mixture that gave the highest level of flowering, i.e. the optimum per cent red, has also been presented in Figure 29b,d for dark periods commencing with a brief initial red or BCJ irradiation. All curves were fitted by eye.

Following an initial red irradiation the null per cent red values remained high during the early hours of darkness (Figure 29) but fell dramatically after the third to fourth hour of darkness suggesting that P_{fr} reverted to P_r at this time. In some experiments far-red alone (0% red) was inhibitory to flowering if given between 4 and 6½ hours in darkness but this was not always true and in experiment C47 the null per cent red did not go below 5% (see Figure 27). Most far-red sources in

FIGURE 29 a and c.

The percentage of red light (% red of red + far-red energy) in interruptions having no net effect, i.e. a null response, on flowering when given at different times during a single dark period preceded by either red (a) or BCJ (c) light for 5 minutes.

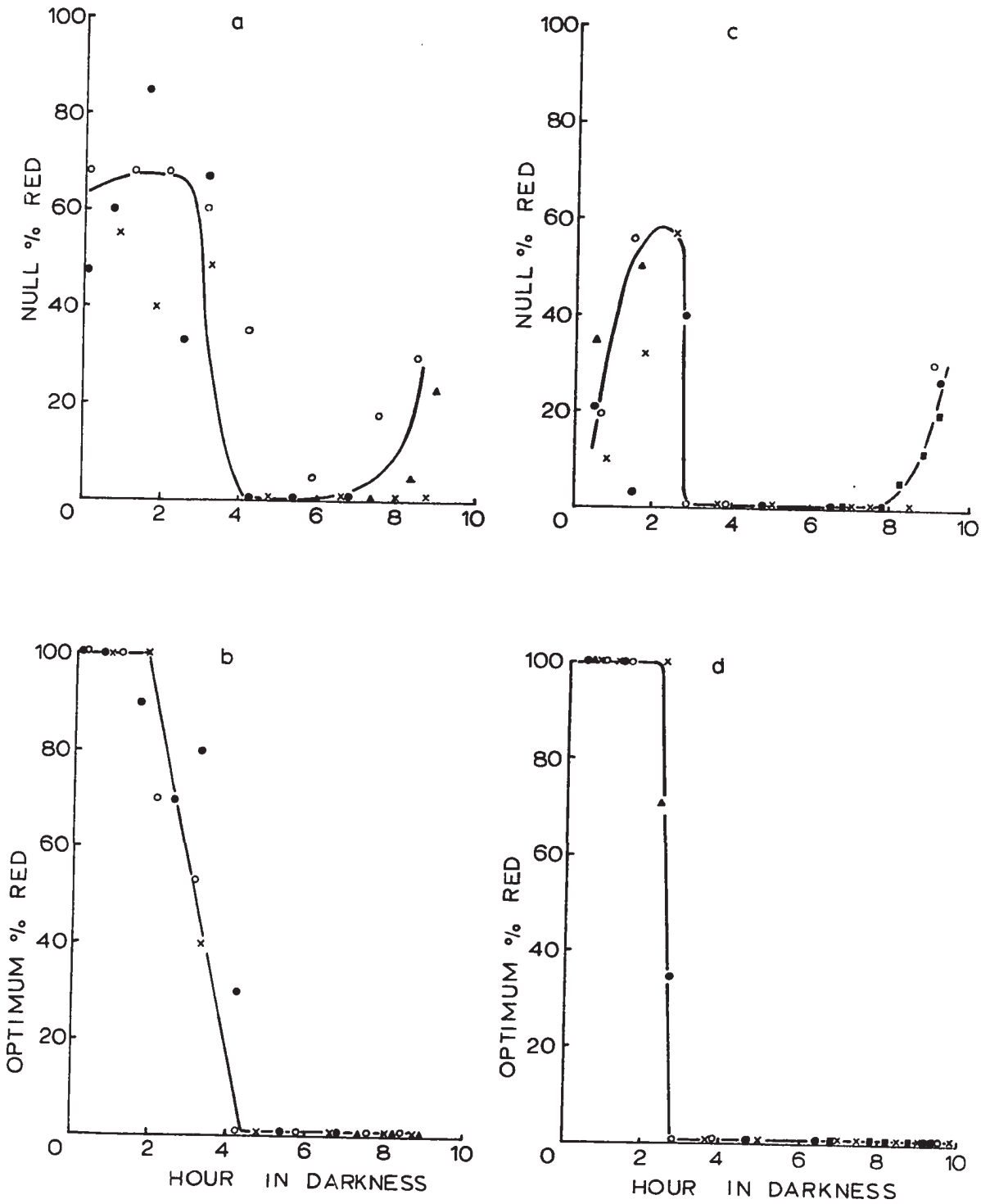
FIGURE 29 b and d.

The percentage of red light in interruptions that gave the highest flowering response, (optimum per cent red), for interruptions given at different times during a single dark period preceded by either red (b) or BCJ (d) light for 5 minutes.

Null and optimum per cent red values derived from experiments C17 (●); C46 (○); C66 (X) and C60 (▲), and C42 (●); C44 (○); C47 (▲); C51 (×) and C61 (■).

5 MIN. RED PRIOR TO DARK

5 MIN. BCJ PRIOR TO DARK



reality set a finite but very low level of P_{fr} in dark grown tissue (see Butler et al, 1963) so that a 0% red mixture may introduce P_{fr} into the system and hence reduce the flowering response below that of an uninterrupted control in which, presumably, the proportion of P_{fr} was actually zero. Inhibition of flowering of C. rubrum by far-red interruptions was also reported by Cumming et al (1965). The apparent rise in the null per cent red late in darkness will be discussed later.

When BCJ irradiation was given prior to darkness the proportion of phytochrome was low at the start of the dark period (Figure 29c). The null per cent red was about 20% at this time but rose within 1 to 1½ hours to a value of 40% to 60%, falling to values below a zero null per cent red by the third hour of darkness. As a supplement to the raw data presented in Figure 28, Table 10 presents results obtained during the early hours of darkness in experiments C44,47 and 51. There is an unmistakable increase in the null per cent red value (Figure 29 and Table 10) although it is somewhat complicated by differences between experiments. A similar response was reported for Pharbitis nil by Evans and King (1969).

The degree of inhibition of flowering by far-red interruptions given after 3 to 4 hours in darkness varied between experiments (Figure 28). There was less tolerance to far-red interruptions in the middle of the dark period for plants commencing darkness with an initial low proportion of P_{fr} (BCJ_o) than for those with an initial high P_{fr} proportion (Red_o).

In the results presented in Figure 29a,c there is a suggestion of a rise in the null per cent red after 8 or more hours of darkness. When the null response irradiations were given less than an hour from the end of the dark period (Figures 27 and 28) flowering was always reduced below the control level the more red there was in a mixture.

Inhibition by red light occurred at progressively greater values of the null per cent red the later the measurements were made in darkness. However, the later the irradiation was given the less the response to changing spectral composition in an irradiation. Consequently the slope of the curve for the response to mixtures of red and far-red approached that of the control curve and it became more difficult to estimate accurately the null per cent red value. On this basis the rise in the null per cent red values could be an artifact. On the other hand, while the evidence is slim at present, in some experiments (e.g. C46; Figure 27, C42; Figure 28 and see C68 and C50; Figure 32) towards the end of the dark period flowering was promoted above the control level by low proportions of red in the mixture and inhibited by higher levels of red. Such a response could only be taken to indicate that the null per cent red values do actually rise towards the end of the dark period.

The optimum per cent red (Figure 29band d) changed in time in a manner comparable to the null per cent red values. Greatest flowering resulted if P_{fr} was high during the early hours of darkness and this was particularly clear when BCJ light preceded darkness; flowering of the uninterrupted controls was only 20 to 40% in most instances but red interruptions even after two hours of darkness, enhanced flowering (Figure 28). As found in earlier experiments with Pharbitis nil (Evans and King, 1969) a high optimum per cent red over the early hours of darkness suggests P_{fr} may be needed and acting over this period. After 3 hours in darkness low levels of P_{fr} were optimal and remained so even when P_{fr} may have been reappearing towards the end of darkness.

c. Photoperiod light quality and changes in phytochrome during a dark period.

The light quality of the photoperiod affects the flowering response of short-day plants and this response has been related to the tolerance to P_{fr} during darkness. Here an attempt is made to relate these effects to phytochrome changes during darkness.

Initial experiments indicated that low intensity red irradiation could be used to replace the 600 ft-c fluorescent light normally given for 24 hours prior to darkness. When a 12 hour dark period commenced $5\frac{1}{2}$ days after sowing, 9 hours of low intensity red light immediately prior to darkness did not reduce the flowering response at all (Figure 30). A slight reduction resulted with 12 to 15 hours of red light replacing fluorescent light and flowering decreased progressively with longer periods given as red rather than as fluorescent light.

If, during the hours immediately prior to darkness red or fluorescent light were replaced with pulses of far-red radiation there was a changing pattern of sensitivity of the flowering response to a subsequent dark period. Figure 31 summarizes the results of two experiments in which 3 hour pulses of far-red were used to replace red or fluorescent light at different times over the 12 hours prior to a 12 hour dark period. When far-red interrupted red light 9 to 12 hours before darkness there was some promotion above the control level of flowering but far-red pulses commencing 6 or 3 hours before darkness were clearly inhibitory. Flowering was about 50% when the far-red light pulse was given from 6 to 3 hours before darkness yet there was a further 3 hours of red light given before the dark period commenced and this should have been adequate to reestablish a high proportion of phytochrome as P_{fr}. Since far-red was equally effective when interrupting high or low

FIGURE 30.

Flowering in response to red irradiation (115 $\mu w/cm^2$ ▨) replacing, for various periods, the 24 hours of fluorescent light (600 ft-c ▭) normally givrn before a dark period of 12 hours that commenced 5½ days after sowing (■).

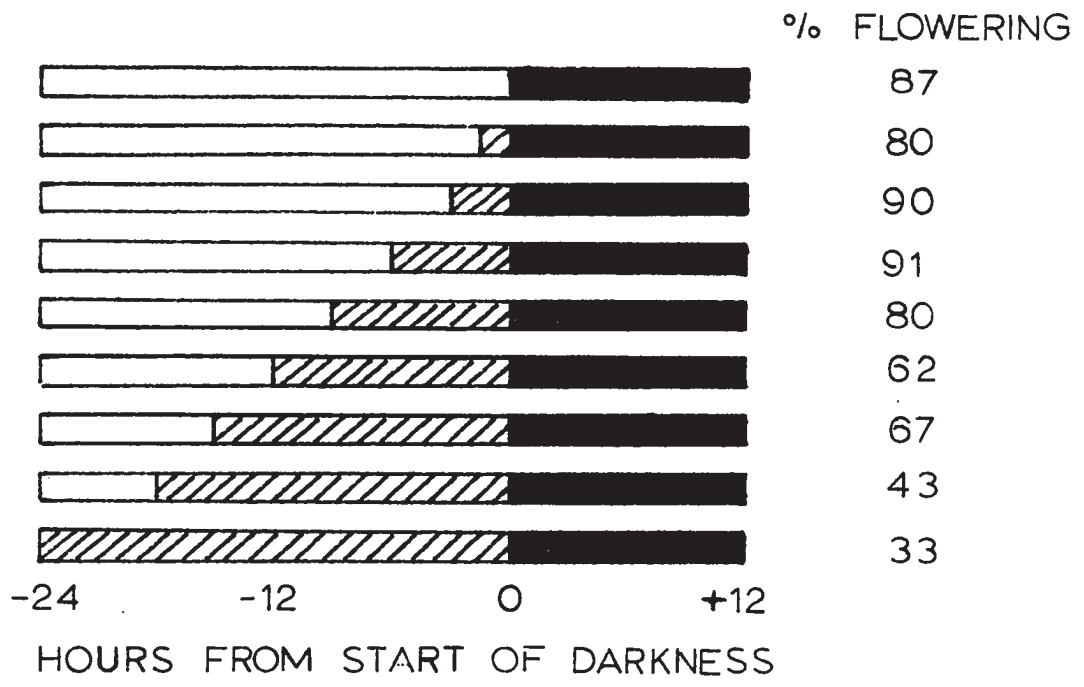
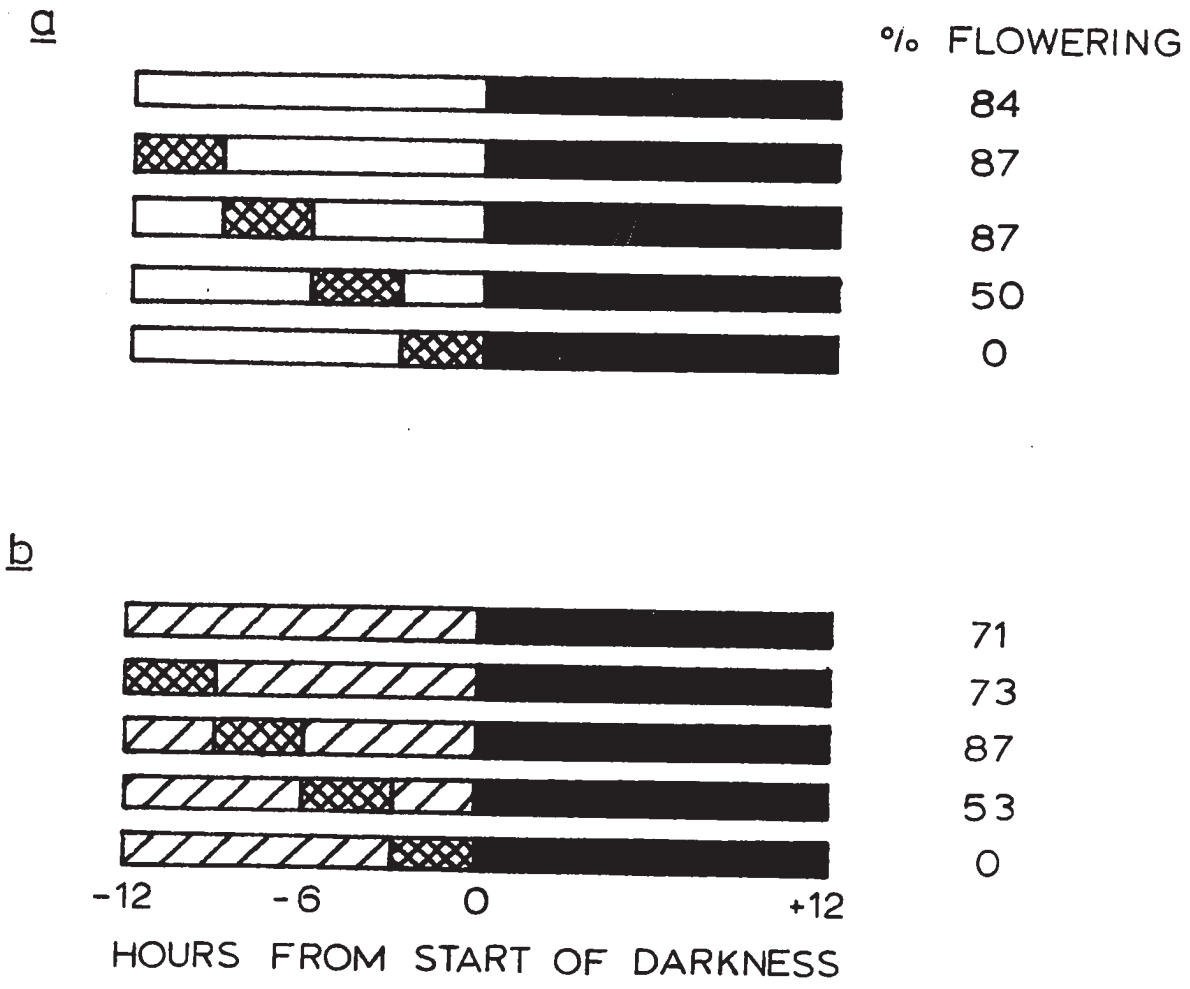


FIGURE 31.

Flowering in response to a 3 hour pulse of far-red radiation (▩) inserted at various times in a fluorescent (□) or red light (▨) period given prior to a 12 hour dark period (■) that commenced 5½ days after sowing.



intensity light these responses appear not to reflect a shortage of photosynthate and, as shown later, they were in fact phytochrome mediated. It was not possible to maintain the temperatures under the red source at 20°C. However, under far-red or fluorescent light the temperature was 20°C and therefore a temperature differential could not account for the response to far-red interruptions.

Following on from these results two types of experiments were designed to study possible interactions with phytochrome conversions during darkness. In one series of experiments 12 hours of red light replaced the last 12 hours of fluorescent light before darkness; in the other a 12 hour red light period was interrupted with $1\frac{1}{2}$ hours of far-red irradiation that commenced three hours before darkness and hence was followed by $1\frac{1}{2}$ hours of red radiation prior to darkness. Dark periods were 10 to 11 hours in duration and at various times during darkness interruptions were given to different groups of plants with mixtures of red and far-red radiation for estimation of the timing of changes in the proportions of P_{fr}. Figure 32 (a and b) presents representative results for flowering in response to interruptions with mixtures of different spectral composition given at various hours in darkness. Changes in the null per cent red and the optimum per cent red are summarized in Figure 33. As expected the value of the null per cent red was initially high (70 - 80%) following exposure to red irradiation prior to darkness. It remained high for about $1\frac{1}{2}$ hours and then fell sharply to a value at or below a zero level of detection by 2 to $2\frac{1}{2}$ hours of darkness. At later times the tolerance to phytochrome was well below that set even by any interruption with pure far-red radiation. After 9 to 10 hours there was some evidence of P_{fr} "reappearance" but as discussed for Figure 29 this response requires further substantiation.

FIGURE 32.

Effect on flowering of a single 1½ minute exposure to mixtures of red and far-red irradiation given at various times during a dark period of 10 to 11¾ hours (a) or of 11 to 11¾ hours (b). In (a) all plants were exposed to broad band irradiation for 12 hours prior to darkness in (b) plants were exposed to 9 hours of broad band red irradiation followed by 1½ hours of broad band far-red irradiation that was terminated by 1½ hours of red irradiation prior to darkness. Vertical lines represent the flowering of the uninterrupted controls.

Curves in (a) from experiments C68, C68, C72, C75, C60, C68, C68, C68; in (b) C54, C48, C48, C48, C50, C69, C69, C50 for the respective times of interruptions.

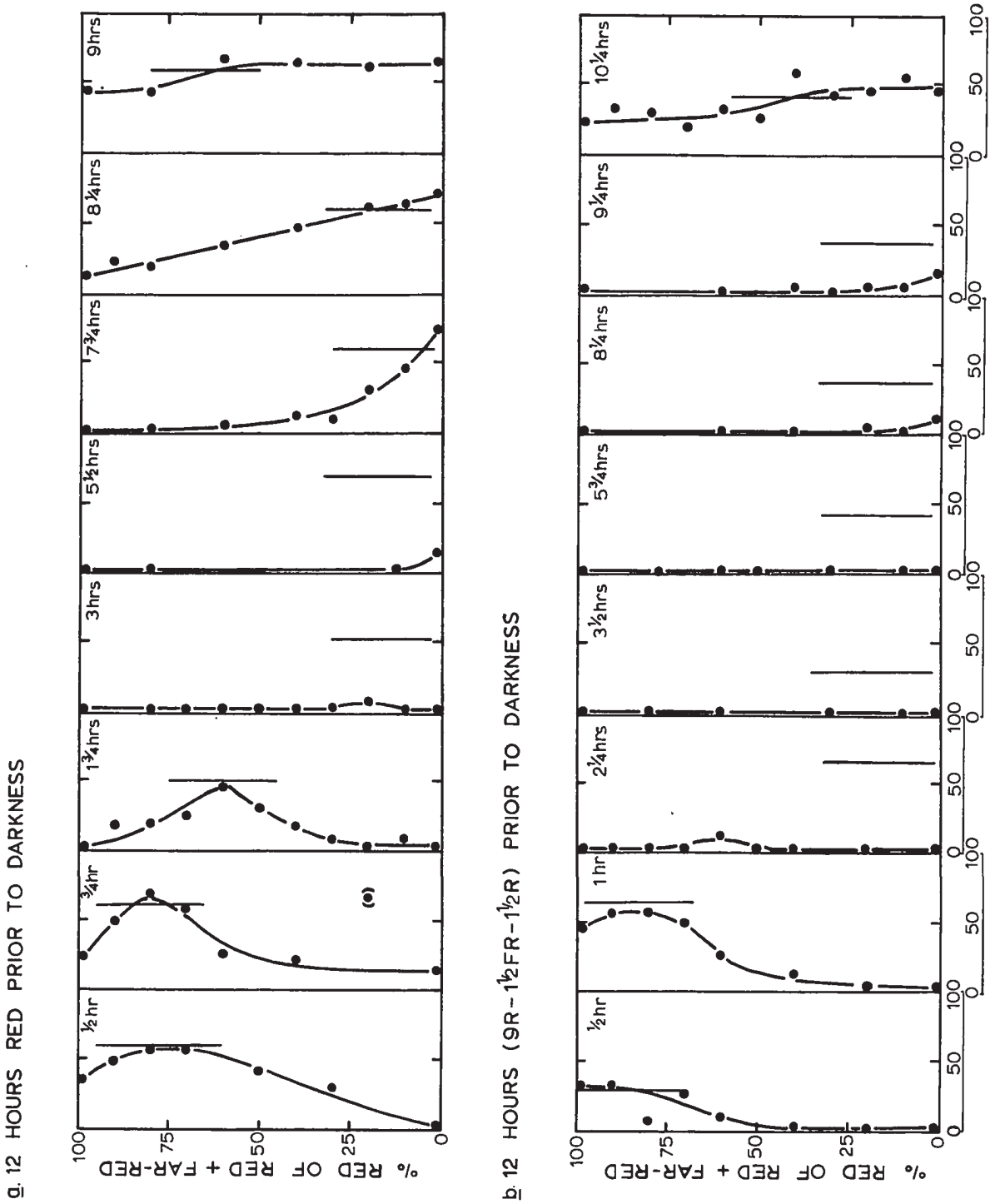

a. 12 HOURS RED PRIOR TO DARKNESS
½hr
¾hr
1¾hrs
3hrs
5½hrs
7¾hrs
8¼hrs
9hrs
% RED OF RED + FAR-RED
100
75
50
25
0
b. 12 HOURS (9R-1½FR-1½R) PRIOR TO DARKNESS
½hr
1hr
2¼hrs
3½hrs
5¾hrs
8¼hrs
9¼hrs
10¼hrs
% RED OF RED + FAR-RED
% FLOWERING
0
50
100

FIGURE 33 a and c.

The percentage of red light (% red of red + far-red energy) in interruptions having no net effect , i.e. a null response, on flowering when given at different times during a single dark period preceded either by 12 hours of broad band red irradiation (a) or by 12 hours red interrupted with 1½ hours of far-red (c).

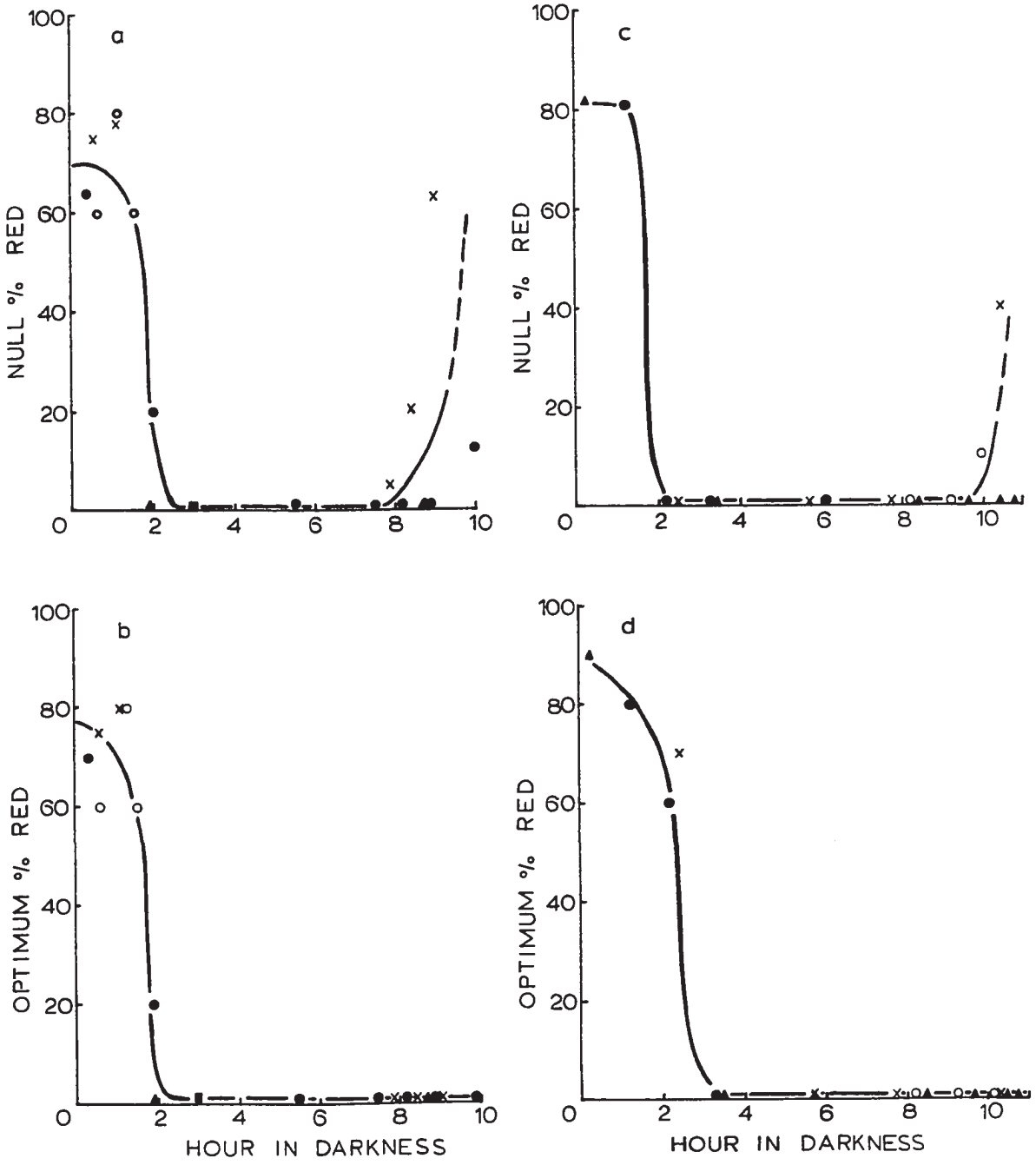
FIGURE 33 b and d.

The percentage of red light in interruptions that gave the highest flowering response, ie. optimum per cent red, for interruptions given at different times during a single dark period preceded by either 12 hours of broad band red irradiation (b) or by 12 hours of red interrupted with 1½ hours of far-red (d).

Null and optimum per cent red values derived from experiments C60 (●), C72 (○), C68 (×), C69 (▲), C75 (■) and C48 (●), C69 (○), C50 (×), C54 (▲).

12 HRS. RED PRIOR TO DARK

"9R–1½FR–1½R" HRS. PRIOR TO DARK



In comparison to the response to brief red irradiation (Figure 29), when treatments were with 12 hours of low intensity red light prior to darkness, phytochrome reverted one to two hours earlier and there was less tolerance to even low levels of P_{fr} in the middle of the dark period (Figure 33).

The time of P_{fr} reversion was identical for plants given a 12 hour exposure to red light or $1\frac{1}{2}$ hours of far-red interrupting the red light. Thus the reduction in flowering that resulted with far-red interruptions was not related to an affect on the timing of P_{fr} reversion. Again, as shown in Figure 29, the optimum per cent red was high during the early hours of darkness, fell to a low value at the same time as the null per cent red values and remained low when phytochrome may have reappeared towards the end of darkness.

d. Phytochrome action during the light period.

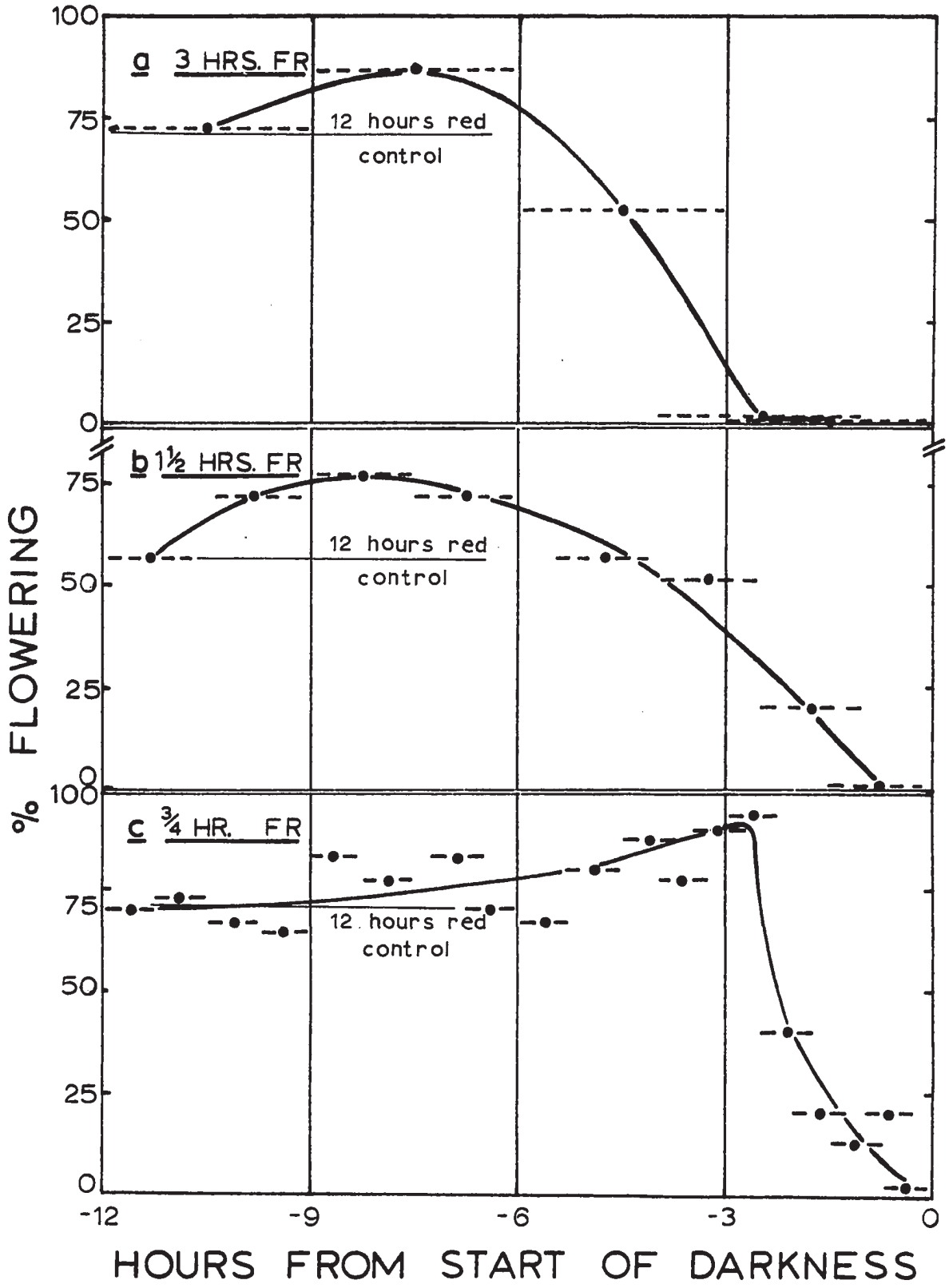
In Figure 31 it was shown that far-red interruptions of the photoperiod reduced the effectiveness for induction by a subsequent dark period. The timing of phytochrome reversion during darkness could not account for this effect (Figure 33) and as will be shown later rhythm timing was also unaltered. That far-red was active suggested that the level of P_{fr} might be important during the photoperiod.

In extending the observations reported in Figure 31, single pulses of broad band far-red radiation of 3 or $1\frac{1}{2}$ hours or 45 minutes in duration were inserted at different times during a 12 hour period of red irradiation that preceded a 12 hour dark period. As shown in Figure 34a (and see Figure 31) a three hour pulse of far-red irradiation reduced flowering below the control level if given in the last 6 hours before a dark period. However, there may have been promotion above the uninterrupted control level of flowering when the 3 hour far-red pulse

FIGURE 34.

Flowering in response to 3, 1½ or ¾ hour of far-red irradiation inserted at various times in a 12 hour period of broad band red irradiation that preceded a 12 hour dark period. Experiments in (a) and (c) from C59, in (b) from C53. Seedlings 5½ days from sowing at the start of the dark period.

Broken horizontal lines represent the period of the irradiation with far-red. Solid points indicate the mid point of an irradiation.


a 3 HRS. FR
12 hours red
control
b 1½ HRS. FR
12 hours red
control
c ¾ HR. FR
12 hours red
control
% FLOWERING
100
75
50
25
0
-12
-9
-6
-3
0
HOURS FROM START OF DARKNESS

was given from hour 9 to hour 6 before the dark period. Using shorter light pulses greater detail was obtained but the far-red was still inhibitory if given close to the dark period and clearly promoted flowering at earlier times i.e. further away from the dark period. The precise pattern of this response has been verified in a number of other experiments.

If the timing of sensitivity to far-red was controlled by the timing of some type of cellular oscillation then, irrespective of duration, promotory and inhibitory times of all far-red pulses would have been superimposed. This is not an explanation for these results since the shorter the pulse, the later it expressed both its promotory and inhibitory effect on flowering. This is shown most clearly when comparing a 45 minute and an $1\frac{1}{2}$ hour pulse (Figure 34b,c). When spanning a period 2 to 3 hours before darkness a 45 minute far-red pulse promoted flowering above the control level but any $1\frac{1}{2}$ hour pulse closer than 4 hours to darkness inhibited flowering. Significant promotion of flowering only resulted for a $1\frac{1}{2}$ hour pulse of far-red radiation given 7 to 9 hours before darkness. Infrequent application of 3 hour pulses of far-red precludes any precise comparison to the timing of sensitivity to $1\frac{1}{2}$ hour far-red interruptions. However, taken together, these results can only suggest that the far-red pulses interacted with the timing of commencement of the dark period.

In the experiments reported in Figure 34 the energy of irradiation was fixed at 6 $\mu w/cm^2$ at 725 nm and, therefore, the total energy of the 3 hour pulse was 4 times that of a 45 minute pulse. To examine the response at equal energies 45 minute, $1\frac{1}{2}$ hour or 3 hour pulses of far-red were given interrupting red light. The mid point of each pulse coincided in time and the energy in a pulse was varied by a factor of as much as 100. That energy alone was not important is

TABLE 11.

Energies required to saturate the effect on flowering of 3 hours, 1½ hours, or 45 minutes of far-red when interrupting a 12 hour red light period. A 12 hour dark period commenced at the end of the 12 hour period of red irradiation. The far-red pulses interrupted the red irradiation to give pulses whose mid points coincided, i.e. 2 hours from the start of the dark period. Values underlined for comparison of equal energy flux to equal total energy.

Far-red Energy	Percentage Flowering		
($\mu w/cm^2$ at 725 nm)	3 hours	1½ hours	45 minutes
0	4	62	53
0.05	8	46	51
0.5	11	34	56
1.5	15.5	41	52
2.0	15	42	67
3.0	12	42	84
4.5	9	42	74
6.0	11	42	68
9.0	8	56	79
continuous red control	59	59	59
LSD p 0. 05	13	13	7

shown by the results in Table 11 - the values underlined. For a 45 minute, $1\frac{1}{2}$ or 3 hour far-red interruption at a constant radiant flux density of 6 $\mu w/cm^2$ at 725 nm flowering was 68%, 42% and 11% for each of the respective durations. When the total energies were equal the flowering response remained unchanged, i.e. 68%, 42% and 15.5% for 45 minute, $1\frac{1}{2}$ or 3 hour pulses given at energies of 6, 3 and 1.5 $\mu w/cm^2$ at 725 nm.

As discussed for Figure 34 there was a time dependence to the application of the far-red light and it might be argued that a 45 minute far-red pulse ending at the same time as the 3 hour pulse and of the same energy would inhibit flowering to the same degree. In a further experiment the response to pulses of equivalent energy but different durations was examined. When a 45 minute far-red pulse was given at an energy of 11 $\mu w/cm^2$ at 725 nm and commenced $4\frac{1}{2}$ hours before darkness, flowering was 40%. A similar pulse that ended $1\frac{1}{2}$ hours before darkness gave 27% flowering. However, following a 3 hour pulse that commenced $4\frac{1}{2}$ hours and terminated $1\frac{1}{2}$ hours before darkness, flowering was 10%, 10% and 7% at energies of 11.0, 3.0 and 1.0 $\mu w/cm^2$. In other words the same or even less total energy, i.e. 1.0 to 3.0 $\mu w/cm^2$, given in a 3 hour pulse did not give the degree of flowering obtained with the same total energy (11 $\mu w/cm^2$) given as a 45 minute pulse. Therefore only the timing and duration of the pulse not its total energy was important in the experiments reported in Figure 34.

Since inhibition of flowering resulted with a 3 hour period of darkness (Table 11, 0 $\mu w/cm^2$ far-red) it could be suggested that there was no actual response to far-red. With the shortest pulse there was, however, an energy dependence; saturating energies of far-red were only reached at about 2 $\mu w/cm^2$ and darkness no longer gave the same response. Since it was shown previously (Figure 33) that phytochrome P_{fr} may revert

very rapidly in darkness, the apparent differences between 3 hours or 45 minutes of darkness or far-red (Table 11) could be explained if with relatively short periods, e.g. 45 minutes, photoconversion of P_{fr} to P_r was more important than P_{fr} reversion whereas both dark reversion and photoconversion by far-red might give similar responses over a 3 hour period.

To examine whether phytochrome was indeed the photoreceptor for these responses to far-red and red irradiation, a normal 12 hour period of red irradiation was interrupted with 45 minutes of far-red irradiation or with 45 minutes of darkness from 1¾ to 1 hour before the start of darkness. As expected from the results in Figure 34 and Table 11, a 45 minute far-red pulse at this time inhibited flowering compared to plants kept in red light or given darkness (Table 12). When 3 brief pulses of 5 minutes of red light (Table 12c) interrupted the 45 minute period of darkness flowering was identical to that of the controls kept in continuous red light (Table 12a). Inhibition of flowering resulted when 3 equally spaced 3 minute pulses of far-red irradiation interrupted the 45 minute dark period (Table 12d). However, the inhibition by far-red could be completely reversed by 5 minutes of red light given after the far-red (Table 12f). Red/far-red reversibility of the light interruptions establishes beyond any doubt that phytochrome was the photoreceptor for the response to far-red interrupting red light.

Invariably, when a one minute or longer period of far-red radiation preceded darkness flowering was low when following fluorescent light (see Figures 25, 36, 37 and 38) or non-existent when far-red followed low intensity red light (Figure 35 and Table 13 b to d). When increasing periods of red light terminated decreasing periods of far-red light in a total light period of 12 hours, the longer the red light the

TABLE 12.

Red/far-red reversibility of the effect on flowering of a 45 minute pulse of far-red irradiation interrupting a 12 hour period of red radiation prior to a 12 hour dark period.

Treatments (a) continuous red, (b) darkness or (d) far-red, were given for the forty-five minute period. In treatments (c), (e) and (f) a forty-five minute period of darkness was interrupted three times at 0, 15 and 30 minutes with brief pulses of red and/or far-red radiation.

IRRADIATION SEQUENCE DURING HOUR $-1\frac{3}{4}$ to -1 OF A 12 HOUR RED IRRADIATION.	PERCENTAGE FLOWERING		
	EXPT. C71	EXPT. C72	EXPT. C74
a. Control 45 min. red	58	-	68
b. control 45 min. darkness	-	55	75
c. (3 min. dark + 5 min R + 7 min. D) x 3	-	62	76
d. Control 45 min far-red	27	36	49
e.† (3 min. FR + 12 min. D) X 3	22	37*	48**
f. (3 min. FR + 5 min.R + 7 min. D) x 3	62	55	69

R = red FR = far-red D = darkness

† Treatment (e) significantly different from treatments (c) and (f) at *p 0.05 **p 0.01 by the method of linear comparisons.

FIGURE 35.

Flowering response to red (▨) or far-red (▩) irradiation given for various intervals over a 12 hour period prior to a 12 hour dark period (■) that commenced 5½ days after sowing.

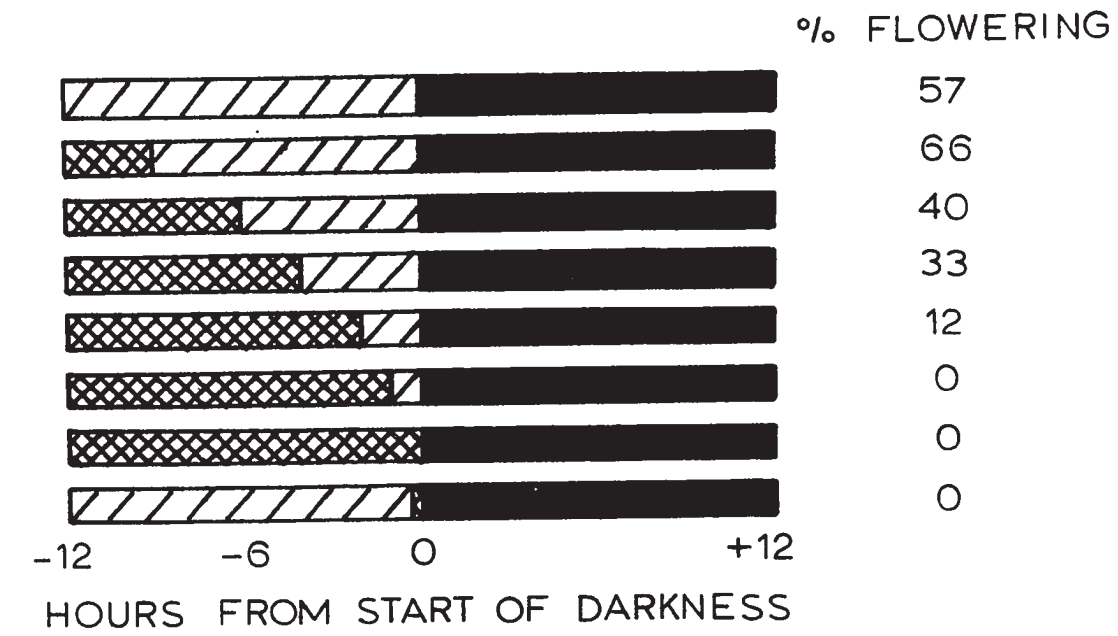
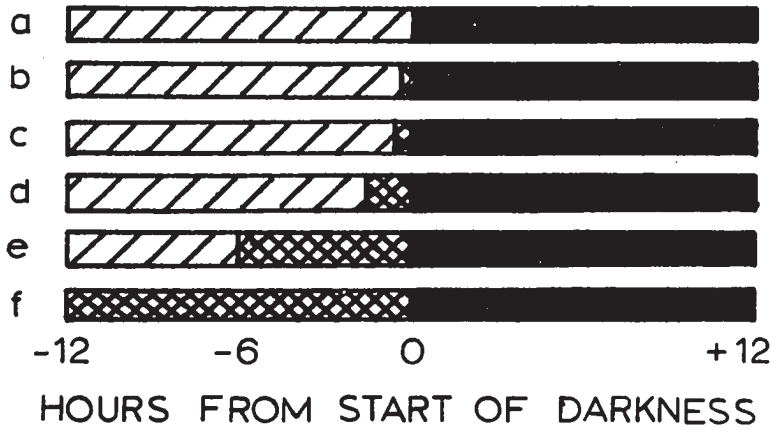


TABLE 13.

Flowering response to red irradiation (115 uw/cm^2) given for 12 hours prior to a 12 hour dark period commencing 5½ days after sowing. (i) 12 hours red irradiation replaced by a terminal far-red irradiation of: a 0 minutes; b 5 minutes; c 15 minutes; d 1½ hours; e 6 hours; f 12 hours. (ii) 12 hours red irradiation replaced by far-red irradiation of various durations and terminated by a 10 minute red irradiation prior to darkness. Duration of far-red irradiation: a 0 minutes; b 15 minutes; c 55 minutes; d 45 minutes; e 6 hours; f 11 hours 50 minutes.



(i)	Treatment					
	a	b	c	d	e	f
12 hour total (red + far-red)	12hR	11h55mR +5mFR	11 hR + hFR	10½hR +1½hFR	6hR+ 6hFR	12hFR
% Flowering	57	0	0	0	0	0

(ii)	a	b	c	d	e	f
12 hour total (red + far-red +10 min R)	12hR	11h35mR +15mFR +10mR	10h55mR +55mFR +10mR	10h5mR +1h45mFR +10mR	5h50mR +6hFR +10mR	11h50mFR +10mR
% Flowering	57	60	7	0	0	0

greater the flowering (Figure 35). However, not even 6 hours of red light could completely reverse the inhibitory action of a prior 6 hours of far-red light. These experiments suggest it was not the presence or absence of P_{fr} that was limiting because photostationary photoconversions between P_{fr} and P_r are attained in less than 5 minutes (Figure 25). By terminating all treatments with 10 minutes of red irradiation just prior to darkness any inhibitory effect of far-red during the photoperiod can be isolated from effects on the level of P_{fr} at the beginning of darkness. Thus a 15 minute far-red pulse given prior to darkness inhibited flowering (Table 13(i)c) but terminating this pulse with 10 minutes of red light gave complete reversal of the inhibition of flowering (Table 13 (ii)b). However, when only an additional 40 minutes of far-red irradiation was given and this was also terminated by 10 minutes of red irradiation, flowering was completely inhibited (Table 13(ii)c). Longer periods of far-red irradiation showed no reversal at all by the terminal red irradiation (Table 13ii). Clearly duration and quality of light preceding darkness are extremely important in the control of flowering and from the measurements of the changes in the value of the null per cent red response (Figure 33), the effects of photoperiod light quality were not dependent on changes in P_{fr} during darkness.

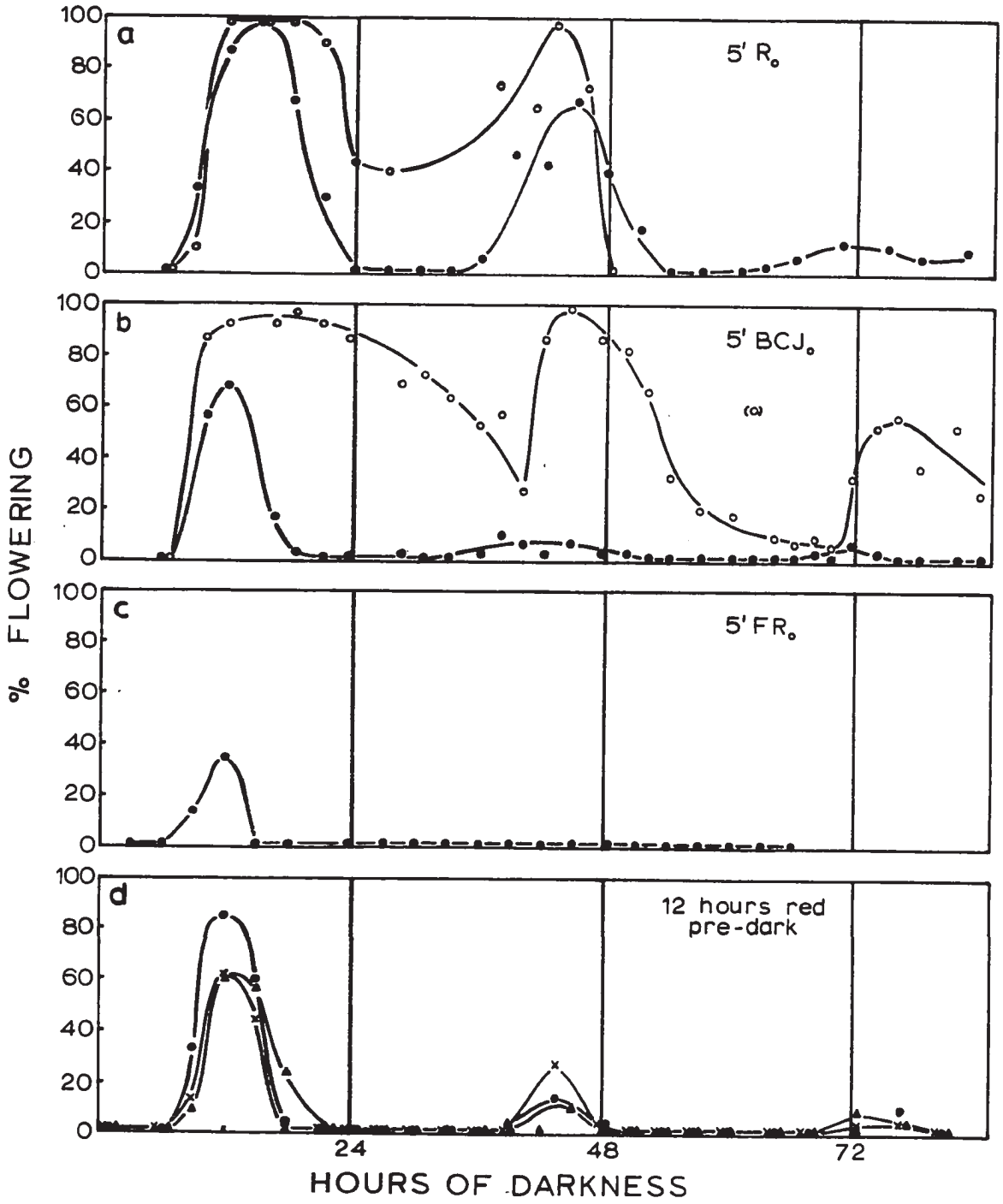
e. Photoperiod light quality and flowering in dark periods of varied duration.

The results presented in Figure 36 illustrate the flowering response to dark periods of various lengths when preceded by a terminal 5 minute irradiation with red, BCJ or far-red radiation or by 12 hours of red irradiation. In two of the experiments 12 hours of red light was interrupted by 1½ hours of far-red commencing either 10 or 3 hours before the dark period. The timing of the peaks of the flowering response and

FIGURE 36.

Flowering response to dark periods of various lengths that commenced $5\frac{1}{2}$ days after sowing. The fluorescent light, prior to darkness, was terminated by 5 minutes of (a) red, (b) BCJ, (c) far-red or (d) by 12 hours of red irradiation (×) or 12 hours red irradiation interrupted with $1\frac{1}{2}$ hours of far-red from $10\frac{1}{2}$ to 8 hours (●) or from 3 to $1\frac{1}{2}$ hours (▲) before the dark period commenced.

In some instances, in place of Hoagland's solution, a solution of 0.4 molar glucose in Hoagland's solution (o) was applied $4\frac{1}{2}$ days after sowing.


a
5' R$_o$
b
5' BCJ$_o$
(ω)
c
5' FR$_o$
d
12 hours red
pre-dark
100
80
60
40
20
0
% FLOWERING
24
48
72
HOURS OF DARKNESS

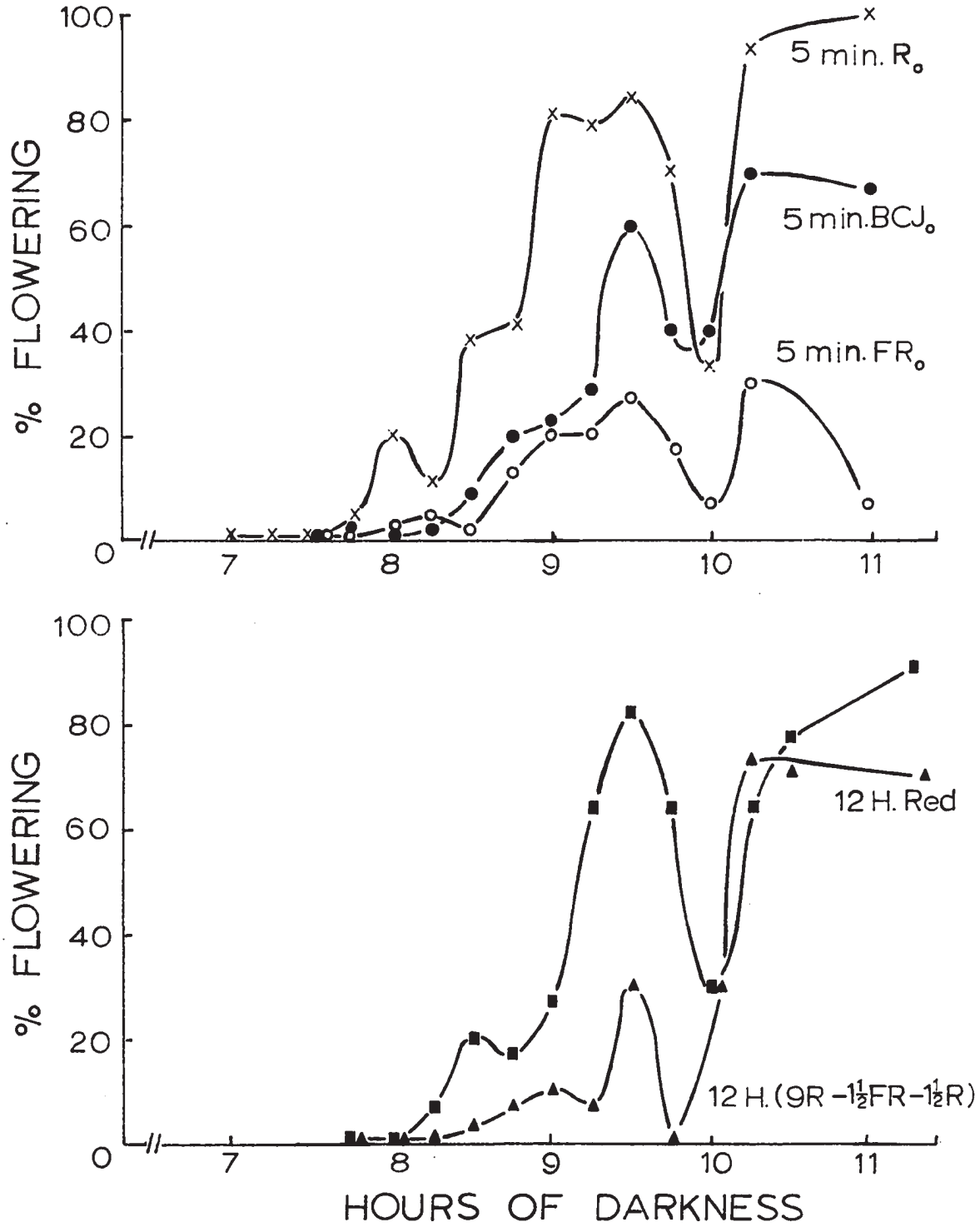
hence the timing of the rhythm was unaffected by all these treatments and the peak times are identical to those reported earlier for plants moved directly from fluorescent light (Figure 2). Glucose solutions (0.4 molar) were applied in two instances so that rhythm peak times could be obtained more readily. Here, as reported earlier (Figure 9) glucose enhanced amplitude without affecting peak timing. Although the lack of flowering at 24 to 35 hours could be overcome by glucose application, flowering was not induced during the early hours of darkness.

The amplitude of the flowering response was strongly reduced if low levels of P_{fr} were set by BCJ or far-red irradiations at the beginning of darkness. Amplitude may also have been reduced by giving 12 hours of red light prior to darkness. In terms of rhythmic phasing, however, the light-off signal after the red light was just as effective as that after fluorescent light. Neither BCJ nor far-red appeared to be effective in reducing the length of the critical dark period compared to plants given red light at the start of darkness. Even when glucose was applied to enhance the amplitude of the flowering response a terminal BCJ irradiation did not reduce the critical dark period length.

In order to define more precisely the effect of various light pre-treatments on the critical dark period length, detailed studies were carried out in which the dark period length was increased by 15 minute intervals (the flowering response was estimated from the average flowering in at least 3 dishes). The results of these experiments (Figure 37) verify that, when phytochrome P_{fr} was set low at the beginning of darkness, the amplitude of the response decreased but the length of the critical dark period was not shortened. There were no differences between the response to 5 minutes of red light terminating fluorescent light (Figure 37) and results for plants moved directly from fluorescent

FIGURE 37.

Flowering response to dark periods of various lengths that commenced $5\frac{1}{2}$ days after sowing. The fluorescent light prior to darkness was terminated by 5 minutes of red (×), BCJ (●) or far-red (○) irradiations or by 12 hours of red (■) or 12 hours of red interrupted by $1\frac{1}{2}$ hours of far-red from 3 to $1\frac{1}{2}$ hours before the dark period commenced (▲).


% FLOWERING
100
80
60
40
20
0
7
8
9
10
11
5 min. R$_o$
5 min. BCJ$_o$
5 min. FR$_o$
12 H. Red
12 H. (9R – 1½FR – 1½R)
HOURS OF DARKNESS

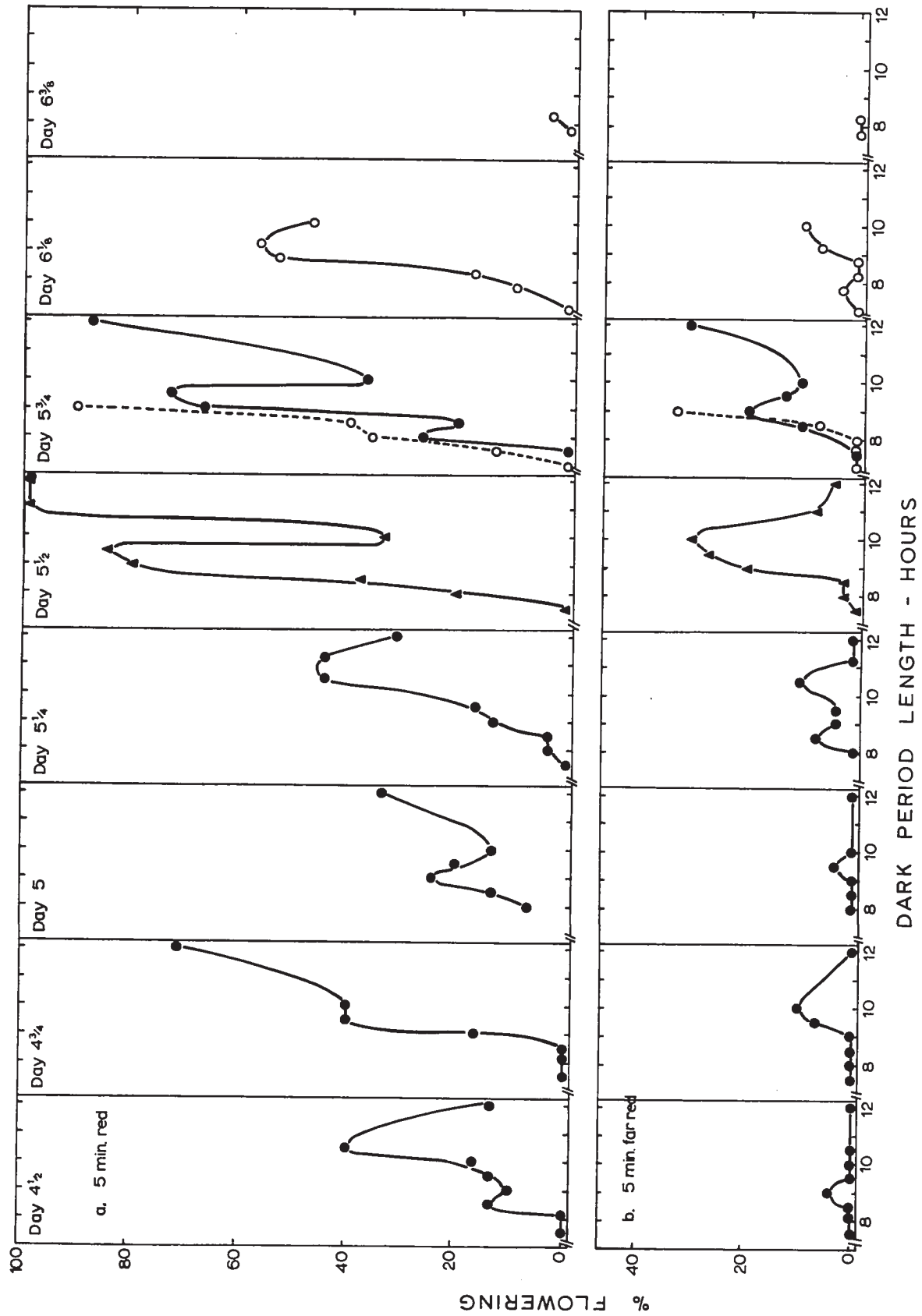
light into darkness (Figure 2). Compared to continuous red irradiation, 1½ hours of far-red inserted just prior to darkness reduced the amplitude of the flowering response.

A striking feature of these results was the rapid and sometimes very large short-term fluctuations in capacity to flower as the dark period length increased. These fluctuations were most marked for dark periods 10 to 10¼ hours in length and may have occurred also at 8¼ hours of darkness. At least 3 replicate dishes were sampled at each harvest, the same trends were obtained in repeat experiments and, in any one experiment, the same changes occurred consistently despite the application of a variety of prior light conditions. Previous sampling at hourly or longer intervals (Figure 2) was sufficiently infrequent to pick up these rapid fluctuations Although there is an indication of rapid fluctuations in experiments of Cumming (1967a, 1969c) neither replication of sampling nor a repeat of the experiment has been attempted to date and comment must be reserved on the presence of these rapid fluctuations in other selections of C. rubrum. In one further experiment (see Figure 38) the timing of the free running 30 hour rhythm and of these rapid fluctuations was delayed when the timing of the light-off signal at the start of darkness was delayed. Thus the light-off signal initiated or rephased the timing of the short term fluctuations when dark periods commenced at 5, 5½ and 5¾ days (Figure 38a).

In Pharbitis nil, Nakayama (1958) reported that relative to a brief red irradiation given prior to darkness, a brief terminal far-red irradiation could give more or less flowering depending on the age of the seedlings. The results presented in Figure 38a for flowering after a brief red irradiation and in Figure 38b after a brief far-red irradiation prior to darkness show that the relative effectiveness of red to far-red

FIGURE 38.

Flowering in response to a dark period of varied durations that commenced $4\frac{1}{2}$, $4\frac{3}{4}$, 5, $5\frac{1}{4}$, $5\frac{1}{2}$, $5\frac{3}{4}$, $6\frac{1}{8}$, or $6\frac{3}{8}$ days after sowing of the seed. Three different experiments reported (●,▲,○), (a) 5 minutes of red irradiation or (b) 5 minutes of far-red irradiation given prior to darkness.


a. 5 min. red
b. 5 min. far red
Day 4½
Day 4¾
Day 5
Day 5¼
Day 5½
Day 5¾
Day 6⅛
Day 6⅜
% FLOWERING
DARK PERIOD LENGTH - HOURS

did not change with seedling age. The critical dark period was never shorter following terminal far-red irradiations and the amplitude of the response was reduced. Both the critical dark period length and the degree of flowering varied with seedling age and seedlings were most sensitive $5\frac{1}{2}$ to $5\frac{3}{4}$ days after sowing. Results presented in Chapter VI highlight the changing sensitivity to a single dark period given to plants of different ages.

IV. DISCUSSION.

a. Phytochrome changes during darkness.

In Chenopodium rubrum phytochrome reverts from the P_{fr} form during the early hours of darkness. If P_{fr} was high at the beginning of darkness it remained high for two to three hours before falling rapidly to a low level by the fourth hour. Setting a low level of P_{fr} at the start of darkness had only a small effect on the time of reversion (Figure 29).

High levels of P_{fr} should have been set by giving either 12 hours of fluorescent light terminated with 5 minutes of red light or by giving 12 hours of red light. Earlier P_{fr} reversion following the latter treatment (Figures 29 and 33) may indicate that light period intensity interacted with dark period time measurement. In Pharbitis nil (Fredericq, 1964) and Xanthium (Borthwick and Downs, 1964) light intensity was particularly important during very short photoperiods and may have interacted with phytochrome dark reversion.

Also working with Chenopodium rubrum, Kasperbauer et al (1964) estimated the rate of P_{fr} reversion by comparing the effect on flowering of interrupting darkness with brief red irradiations or with far-red

irradiation of an hour or more in duration. They estimated dark reversion rates of P_{fr} of about 0.8%/minute but such values have little meaning since it cannot be assumed, as they did, that reversion proceeds at a constant rate in darkness. In another study of C. rubrum, Cumming et al (1965) generally obtained little or no flowering when mixtures of red and far-red light were imposed at the fifth hour of a 72 hour dark period. However, as shown in Figures 29 and 33, P_{fr} reversion would have occurred before the fifth hour of darkness so that introduction of any P_{fr} at this time might inhibit flowering.

The term "reversion" has been used rather loosely here since measurements of the null response cannot distinguish P_{fr} reversion from P_{fr} decay. However, decay and reversion proceed at constant rates in aetiolated tissue (see Hillman, 1967a)and such measurements would appear to have little relevance to changes during darkness in the physiologically active pool of phytochrome in a green leaf. Decay would alter the ratios of P_{fr} to P_r and thus the sensitivity of the null response, but , the direction and timing of null response changes would not be distorted by P_{fr} decay.

Any dramatic changes in chlorophyll content during darkness would alter the spectral composition of light reaching phytochrome and could invalidate the use of the null response method. In fact, during the course of this work it was shown by A. S. Chia-Looi (unpublished results) that the level of extractable chlorophyll increased over the first 9 hours of darkness. However, hourly increases were at the most 4 % of the total chlorophyll content and there could have been no dramatic changes in screening of red radiation by chlorophyll. Any increase in chlorophyll screening that did alter the spectral composition inside the tissue would cause only apparent increases in the value of the

null per cent red.

Synthesis of P_r proceeds during darkness in aetiolated (Clarkson and Hillman, 1967) or far-red grown plants (Marme, 1969); in greening cotyledons (Grill and Vince, 1969); or in light grown callus tissue (Wetherell, 1969). P_r synthesis during an inductive dark period would induce an apparent decline in the null per cent red value. The rate of P_r synthesis is slow in darkness; i.e. from the above references 1 to 10% per hour; and would not contribute significantly to rapid and large decreases in the null response as detailed in these experiments with C. rubrum. Therefore, despite these qualifications, the null per cent red values will reflect the gross changes in the physiologically important pool of P_{fr} in the tissue.

Whether the level of P_{fr} was set low or high at the beginning of darkness the proportion of P_{fr} that gave most flowering (the optimum per cent P_{fr}) was high over the first hours of darkness and fell at about the same time that the null per cent red values fell (Figures 29 and 33). In C. rubrum a requirement for the presence of P_{fr} during the early hours of darkness was postulated previously by Cumming (1963) and photoperiod duration and light quality may determine whether an optimal level of P_{fr} is established in darkness (Cumming, 1963; Figures 29 and 33). That the optimum level of P_{fr} was high over the early hours of darkness implies that P_{fr} may have been acting over this time. Therefore by setting the proportion of P_{fr} low rather than high at the start of darkness, it might be expected that the optimum requirement for P_{fr} would remain high for a longer period of time. Such a response was evident in Pharbitis nil (Evans and King, 1969) but not in C. rubrum (Figures 29 and 33). However, in C. rubrum, P_{fr} reappeared in darkness far more rapidly than in Pharbitis nil and the null response values fell soon

afterwards. P_{fr} could well act in C. rubrum during the early hours of darkness but rates of change of P_{fr} may have been too rapid to allow detection of any differences in the timing of changes of the optimum per cent red values.

The rise in the proportion of red light giving a null response over the first two hours of darkness (Figure 29) was found in all experiments in which the level of P_{fr} was set low by exposure to BCJ light at the start of darkness. A similar increase in the proportion of P_{fr} was reported previously in experiments in which seedlings of Pharbitis nil were given brief irradiations with incandescent, BCJ or far-red light at the start of darkness (Evans and King, 1969). Cumming et al (1965) also noted that the proportion of P_{fr} may have increased between the twenty-third to twenty-ninth and forty-seventh to fifty-ninth hours of a 72 hour dark period. Spectrophotometric studies on imbibed seeds have shown the presence of a small pool of phytochrome that undergoes what has been termed "inverse reversion" from P_r to P_{fr} in darkness (Boisard et al, 1968; Spruit and Mancinelli, 1969; Kendrick et al, 1969). The rate of P_{fr} reappearance was of the same order of magnitude as that indicated by the changes in the null response (Figure 29).

While not disputing the reality of this phenomenon, an equally plausible explanation of "inverse reversion" is that P_{fr} itself is synthesized or released from a light stable form of phytochrome. A current hypothesis developed by Borthwick et al (1969) postulates that a form of phytochrome absorbing at 650 nm, termed $P_{fr}H$ by Anderson et al (1969), may provide the source of P_{fr} during its reappearance in darkness. Borthwick and coworkers also suggested that in C. rubrum appreciable and non-photoreversible formation of $P_{fr}H$ might result from a one hour irradiation with far-red light although with irradiations shorter than 10 minutes

complete red/far-red reversibility was still possible.

Regardless of the interpretation placed on P_{fr} reappearance, the null responses explain why far-red or BCJ irradiations at the beginning of darkness did not inhibit flowering completely. Subsequent phytochrome changes reestablished the same pattern as found in plants entering darkness with a high level of P_{fr}. Reappearance of P_{fr} towards the end of the dark period, if it occurs (Figures 29 and 33), would fit with earlier evidence of Cumming et al (1965) who reported that the proportion of P_{fr} was high in C. rubrum at the eleventh hour of darkness. However, that the optimum per cent red was still low at the end of darkness suggests P_{fr} reappearance could limit flowering at this time.

b. Phytochrome and time measurement in the photoperiodic control of flowering.

In C. rubrum after 3 hours in darkness P_{fr} was found to revert rapidly (Figure 29). Similar changes were evident in earlier experiments of Evans and King (1969) with Pharbitis nil and it appears that the timing of reversion may provide at least partial control over photoperiodic time measurement. This is suggested by the relationship between the time of reversion and the length of the dark period that was required for flowering. In both C. rubrum and Pharbitis nil reversion was sudden and occurred 4 to 5 hours before the critical dark period length was reached. This resulted despite the fact that only $7\frac{3}{4}$ hours of darkness were required for flowering of C. rubrum (Figure 37) but $11\frac{1}{2}$ hours of darkness were required in Pharbitis nil (Evans and King, 1969).

In both plants if P_{fr} was set low at the beginning of darkness the proportion of P_{fr} rose progressively until reversion occurred. An initial low setting of P_{fr} resulted in slightly earlier P_{fr} reversion

but only in Pharbitis nil was the critical dark period length shortened to the same degree. It is not known what initiates these changes in the null per cent red values but in estimating the proportions of P_{fr} present, the use of a null response technique avoids complications that may be introduced by changes in sensitivity to P_{fr}. Therefore these relationships between P_{fr} reversion and dark period time measurement are very suggestive of a clock function of phytochrome reversion in both of these short-day plants.

The experimental conditions in which Pharbitis nil is grown can determine whether the flowering response is rhythmical as in C. rubrum or whether it shows a linear response to increasing dark period length (Takimoto and Hamner, 1964). These latter observations could explain why in contrast to Pharbitis nil hastening P_{fr} reversion in C. rubrum had no influence on dark period time measurement (Figures 33 and 36). It is suggested that in C. rubrum rhythmic changes play a dominant role in time measurement. Therefore, apart from setting a lower limit to the length of the critical dark period (see Chapter III) and possibly acting as a preparatory reaction for rhythm initiation, phytochrome reversion would be of little significance to time measurement in C. rubrum. In Pharbitis nil, on the other hand, Evans and King (1969) found evidence of a time measurement function of phytochrome reversion under conditions involving the least interaction with rhythms.

Another approach in studies of phytochrome control over time measurement is to reintroduce P_{fr} after its reversion. In Pharbitis nil (Takimoto and Ikeda, 1960; Takimoto and Hamner, 1964; Evans and King, 1969) and probably Xanthium strumarium (Papenfuss and Salisbury, 1967) the reintroduction of P_{fr} after its reversion has a dramatic effect on dark period time measurement. The critical dark period and hence time

measurement may be delayed by 9 to 12 hours by such treatments but, as might be expected, the introduction of P_{fr} before its reversion has no effect on time measurement. The results of a similar series of experiments with C. rubrum were presented in Figure 20. Brief red light interruptions of darkness had no influence on phasing of the second and third rhythm peaks of flowering and hence failed to influence rhythm phasing as was also suggested in Chapters III and IV and by Cumming et al (1965). On the other hand, the introduction of P_{fr} after, but not before, its reversion, delayed the time of the first peak of the rhythm and increased the length of the critical dark period. The simplest interpretation of these results is that, within the limits set by the rhythm, P_{fr} reversion can control time measurement in C. rubrum in the same fashion as it controls time measurement in Pharbitis nil. Results such as those illustrated in Figure 20 also suggest that phytochrome and rhythms function as separate clocks.

The possibility of multiple clocks in the photoperiodic control of flowering was advanced previously by Takimoto and Hamner (1964) to explain their results with Pharbitis nil. They proposed an hourglass component which it would now appear is equivalent to P_{fr} reversion as studied by Evans and King (1969). Two rhythmic clocks were also proposed by Takimoto and Hamner but it is clear from the evidence presented in Chapter III that only one rhythmic clock need be advanced to explain changing dependence on dawn and dusk signals.

The model presented here of the action of dual photoperiodic clocks need not be in conflict with but may extend Bunning's hypothesis of time measurement in photoperiodism. For flowering at least, the dual responses to light suggested by Bunning's model could be mediated by dual timers. Probably phytochrome and the rhythm interact directly, and,

depending on the plant and its prior treatment, undue emphasis should not be placed on the action of a rhythm or an hourglass timer alone.

c. Light quality preceding darkness.

For some time it has been known that flowering of short-day plants often depends on the degree of photosynthetic input over the period prior to an inductive dark period (see Hamner, 1940; Parker and Borthwick, 1940; Liverman and Bonner, 1953). However, it is now generally accepted that photosynthetic supply is of no immediate importance to the following dark period processes although photoperiod light quality may be critical (Takimoto and Ikeda, 1959, 1960; Takimoto and Naito, 1962b; Salisbury, 1965; Zeevaart and Marushige, 1967; and see Salisbury, & Ross, 1969). A similar conclusion can be drawn from the results presented here for C. rubrum. Flowering was little affected when low intensity red irradiation replaced higher intensity fluorescent light during the light period prior to darkness (Figures 30, 36 and 37) but even short far-red interruptions (one hour) during a light period could reduce flowering considerably (Figures 31 and 34). That photoperiod quality and not intensity was so important to flowering in a subsequent dark period suggested the action of a photoreceptor other than chlorophyll. In fact the demonstration of red/far-red reversible control over the response to photoperiod quality established beyond doubt that phytochrome was the effective photoreceptor pigment.

Not only was P_{fr} required during the photoperiod but it had to be present over prolonged periods of time. A distinction is drawn here between inhibition of flowering by a brief period of far-red light terminating a light period and inhibition by more than 45 minutes of far-red light. In the former situation photoreversibility of the inhibition of flowering was achieved with 5 minutes or less red irradiation (Figure25,

Table 13). Thus only photointerconversions of P_{fr} and P_r were important with brief irradiations. On the other hand, inhibition of flowering by longer periods of far-red irradiation was only reversed by quite long periods of red light (Figures 34 and 35, and Table 13). Reconverting P_r to P_{fr} immediately prior to darkness was to no avail if preceded by as little as 55 minutes of far-red light during the photoperiod. If intermittent red light interruptions were given every 15 minutes over a 45 minute period, flowering was equivalent to that for plants kept in continuous red light (Table 12). Since P_{fr} reversion took longer than two hours (Figure 33) continuous or intermittent red light could be replaced equally well by 45 minutes of darkness (Table 12) but, as might be expected with longer periods and associated P_{fr} reversion, darkness acted more like far-red than red light (Table 12). Clearly, for flowering, the presence of P_{fr} was required over prolonged periods of time prior to darkness.

In contrast to the much studied high energy reaction (HER) of photomorphogenesis the response of C. rubrum to prolonged irradiations was a function of the duration rather than of the energy of the light. Almost as though a clock-spring had run down and was being re-wound, the longer P_{fr} was absent during the photoperiod the longer its presence was subsequently required for a dark period to be fully inductive. An explanation is not apparent at this time for the promotive effect of far-red irradiation given some time before the start of darkness (Figure 34) but the maintenance of P_{fr} over excessively long periods may shift the balance of the P_{fr} requiring reactions to a supraoptimal level.

In Chapter IV it was shown that the presence of P_{fr} was required for a 6 or 12 hour light period to shift rhythm phase. Prolonged P_{fr} action during the photoperiod (see above) may be a manifestation of the

same reaction that controls rhythm phasing. However, in all the experiments reported in this chapter photoperiod light quality was manipulated without introducing the complication of effects on rhythm phasing. Rhythm phasing was determined solely by the timing of the light-off signal which was always given 5½ days after sowing. Changing the light quality during the photoperiod had no effect on rhythm phasing in the subsequent dark period (Figures 36 and 37).

That photoperiod light quality did not alter the timing of P_{fr} dark reversion is illustrated by the results given in Figure 33. P_{fr} reversion occurred at the same time after a 12 hour period of red irradiation or after a period of red irradiation interrupted with 90 minutes of far-red light. On the other hand, photoperiod light quality influenced the amplitude of the flowering response. This effect on amplitude resulted whether P_{fr} was set low at the beginning of darkness (Figures 36 and 37), hence limiting P_{fr} action during the early hours of darkness, or whether P_{fr} was set high at the beginning of darkness but its action during the photoperiod limited by interrupting the photoperiod with long periods of far-red irradiation (Figures 36 and 37). It would appear from these results that light period quality does not influence any clock function in C. rubrum. However, expression of the timer is affected by the presence of P_{fr} during the photoperiod and its absence in darkness.

From their experiments with Pharbitis nil, Evans and King (1969) postulated that a high P_{fr} reaction occurring in the light period and during the early hours of darkness interacted with a low P_{fr} reaction that could only occur after P_{fr} reversion. The results presented above for C. rubrum appear to provide detailed confirmation of their deductions about a high P_{fr} reaction. However, in C. rubrum, only the amplitude of flowering responded to the high P_{fr} reaction whereas in Pharbitis nil

both amplitude and to a lesser degree the timing of P_{fr} reversion were influenced by the balance set between the high and low P_{fr} reactions.

The nature of the interaction between the two reactions is not known but could reflect differences in availability of metabloites between light and darkness that induces sequential or cooperative interactions between high and low P_{fr} reactions. Rhythmic changes in substrate supply for P_{fr} action could confer on the plant such an interacting pattern of high and low P_{fr} reactions. In Chenopodium amaranticolor, Konitz (1958) obtained evidence suggesting that changes in sensitivity to P_{fr} were linked to an endogenous rhythm and in similar experiments with the long-day plant Lolium temulentum, Vince (1965) also found evidence suggestive of a rhythmic change in sensitivity to P_{fr} that could relate to a rhythm of substrate supply during the photoperiod. On the other hand, in experiments similar to those of Konitz, when far-red interruptions were given to C. rubrum during the light period it was shown that sensitivity to far-red interruptions related only to the timing of the pulse relative to the timing of the commencement of darkness (Figure 34). For long-day plants the timing of sensitivity to light interruptions of the photoperiod has also been shown to vary between species and within the one species given different light qualities during the photoperiod (Lane et al, 1965). Therefore there appears to be no support at present for the conclusion that rhythmic changes in substrate supply during the photoperiod confer on a plant a specific pattern of sensitivity to P_{fr}. Likewise, there is no definitive evidence for the concept of a high P_{fr} reaction leading to an accumulation of product for the low P_{fr} reaction.

Another possible explanation of the high P_{fr} - low P_{fr} interactions could be that photodestruction of P_{fr} during the light period

and decay during the early hours of darkness determine the absolute P_{fr} levels present at the time of P_{fr} reversion and hence the degree of response to the low P_{fr} reaction. Extreme sensitivity to absolute but low levels of P_{fr} has been advanced as an explanation of the inhibition of synthesis of the enzyme lipoxygenase in Sinapis alba (Oelze-Karrow et al, 1970). In these experiments destruction of 60% of the total phytochrome, by giving one hour of red irradiation induced a greater tolerance to subsequent far-red irradiation than was evident for plants maintained with higher total levels of phytochrome. In C. rubrum evidence for the importance of P_{fr} destruction can be adduced from the inverse relationship found between P_{fr} levels in the light (high P_{fr} reaction) and those tolerated during darkness (low P_{fr} reaction). For example, from Figures 27, 28, and 32, tolerance to P_{fr} in the middle of the dark period was greater under conditions in which higher levels of P_{fr} were maintained during the light period and early hours of darkness. Such conditions would be expected to lead to greater P_{fr} decay and lower absolute levels of P_{fr} would then be introduced by dark period interruptions. Conversely, tolerance would be reduced if P_{fr} decay was reduced. Comparable changes in the tolerance to P_{fr} have also been reported for other short-day plants (Meijer and Van der Veen, 1960; Takimoto, 1967; Evans and King, 1969). A similar suggestion was advanced by Mancinelli and Downs (1967) to explain the influence of photoperiod length on the sensitivity of flowering to P_{fr}. The shorter the photoperiod the less P_{fr} destruction and the greater the sensitivity to P_{fr} introduced in darkness.

Threshold levels for P_{fr} could well differ between long-day and short-day plants and P_{fr} destruction, reversion and resynthesis would vary in their importance between species. A long-day plant given continuous irradiation might attain the same absolute level of P_{fr} under

continuous light as a short-day plant given a dark period. There are, however, difficulties associated with this hypothesis. Apparently contradictory results have been obtained in Lemna perpusilla by Hillman (1967b) but judgement must be reserved on Hillman's results since it was not clear whether his different treatments influenced rhythm phasing and hence the timing of sensitivity to phytochrome. Another complication is that continuous light may not only influence P_{fr} photodestruction but may induce a number of other P_{fr} interconversions (see Borthwick et al, 1969). Detailed spectrophotometric measurements of phytochrome changes in green tissue would be extremely valuable in this regard, particularly as an adjunct to physiological evidence provided by null response measurements. Needless to say, substrate availability could also play a crucial role.

CHAPTER VI

RHYTHMICITY AT THE APEX AND TIMING OF EVENTS OF FLORAL DEVELOPMENT.

I. INTRODUCTION.

The events which occur in the course of photoperiodic induction of flowering can be separated into a number of sequential steps (cf. Lang, 1965) which start with the perception of the daily cycle of light and darkness and culminate in the formation of a flower. In both short- and long-day plants the leaf is the site of photoperiodic perception (see Lang, 1965) and produces a stimulus to flowering which is translocated to the apex where it evokes flowering. Grafting and translocation experiments (Zeevaart, 1958; Lang, 1965; Evans, 1969) have provided much support for the concept of a floral stimulus, and a number of workers have succeeded in recent years in extracting an inductive principle from leaves of Xanthium (Lincoln et al, 1961; Carr, 1967; Cleland and Zeevaart, 1968; Hodson and Hamner, 1970). The identity of this factor is as yet unknown but it has been obtained only from induced leaves and causes flowering in non-induced plants.

The floral stimulus can be translocated very rapidly to the apex, and biochemical and ultrastructional changes are evident 18 to 24 hours after the beginning of a single inductive period. Studies of the incorporation into apices of ^{32}P and ^{35}S (Rijven and Evans, 1967b; Evans and Rijven, 1967) and of labelled uridine (Gressel et al, 1970; Bernier et al, 1970) have shown that early responses involve RNA and protein synthesis. Ultrastructural changes also occur at this time; the number of ribosomes and dictyosomes increasing (Healey, 1964) and the distribution

of smooth and rough endoplasmic reticulum may change (Gifford and Stewart, 1965). Histochemical changes are less marked initially but greater staining for RNA and protein is apparent during the early days after induction (Knox and Evans, 1966). The term floral evocation is used here, as suggested by Evans (1969), to describe the early changes at the apex following the arrival of the floral stimulus. These processes are thus separated from the succeeding events of floral differentiation.

Soon after the arrival of the floral stimulus at the apex, DNA synthesis and the percentage of dividing cells increase sharply (Gifford and Tepper, 1961; Thomas, 1963; Lance-Nougarède and Bronchart, 1965; Bernier et al, 1967). Often mitotic activation is most striking in the central zone of the apex (Healey, 1964) but stimulation of division may also result in the flank regions of the apex (Bernier et al, 1967). In the light of these results it is important, obviously, to know if there is any rhythmicity of cell division at the apex.

In animals and unicellular organisms, rhythms of cell division have been reported widely (see Bruce, 1965). There have also been a number of studies in plants of diurnal changes in cell division at the apex, and Bunning (1952b) was one of the first to draw attention to this phenomenon in the shoot apex of Tradescantia zebrina. Others have found evidence of diurnal changes in the mitotic index in vegetative shoot apices of Rudbeckia bicolor (Jacqmard, 1970b), Samolus (Sachs et al, 1959), Trifolium repens (Denne, 1966a) and Tradescantia fluminensis (Denne, 1966b). During the early stages of floral evocation of Sinapis alba, Bernier and his associates found diurnal changes in cell division. Diurnal fluctuations in metabolism may also result following inductive treatments and Rijven and Evans (1967a and b) and Evans and Rijven (1967) reported oscillations in the level of soluble amino nitrogen and the incorporation

of ^{32}P and ^{35}S into RNA and Protein in the shoot apex of Lolium temulentum

Control over the synchrony of events at the apex may be possible using chemical stimuli such as gibberellic acid (Sachs et al, 1959; Jacqmard, 1970b) or by the arrival of the floral stimulus at the apex (Bernier et al, 1967) and daily fluctuations in temperature and in the length of the daily photoperiod can control the timing of diurnal oscillations of cell division (Denne, 1966b).

Since cell division is an integral part of flowering, evidence that diurnal fluctuations in cell division and metabolism occur at the apex suggests that rhythmic changes in the meristem could have a significant influence on the capacity of the apex to flower. The floral stimulus might not be nearly as effective if it were to arrive at the apex at a wrong phase of a rhythm of synthetic capacity. As a first approach to examining the possibility that rhythms at the apex limit flowering, the present study explores the timing of export of the floral stimulus from the cotyledons of Chenopodium rubrum after a single inductive dark period. Early responses of the apex have been estimated from changes in the mitotic index. Since temperature fluctuations might synchronize mitosis at the apex, seedlings were germinated in constant conditions and also in fluctuating temperatures and then their capacity to flower and their mitotic activity was estimated after various periods in constant conditions. As a further test of rhythmicity at the apex, plants were germinated in fluctuating conditions but placed in constant conditions after floral induction. Solutions of gibberellin, glucose or ethanol were then applied to the plants at different times during floral evocation and differentiation. From measurement of the timing of export of the floral stimulus and of mitotic activation of the apex it is argued that at least some rhythmicity in C. rubrum resides in the sensitivity of the apex. The bearing of these

results on time measurement and on the nature of floral evocation is considered in the discussion.

II. MATERIALS AND METHODS.

a. Seed source.

Chenopodium rubrum (origin 60°47'N 137°32'W) was used exclusively in these investigations but seed was grown in 1966, 1968 or 1969. There were no differences in the flowering response between batches of seed as had been established previously by Cumming *et al* (1965), Cumming (1967a) and Cumming (1969c) for seed grown in 1963, 1964, 1965 and 1966. Immersion for 90 seconds in concentrated sulphuric acid broke the hard seededness of 1966 seed without any deleterious effects on the embryo. This seed was used for experiments in which constant germination conditions (20°C; 600 ft-c) were employed. Because of their larger size, seeds grown in 1968 were preferred for studies of cell division at the apex. Seed harvested in 1969 was used for those experiments in which alternating conditions of temperature were applied during germination.

b. Growing conditions and treatments.

Standard germination conditions were the same as described in Chapter II. After $4\frac{1}{2}$ days germination under alternating temperatures (32.5°C for 12 hours ; 10°C for 12 hours), Hoagland's No. 1 nutrient solution was applied to the dishes and they were placed in fluorescent light of 600 ft-c at 20°C. The single dark period at 20°C normally began one day later although,in one series of experiments (Figures 44 and 45) a dark period of 12 hours was begun at various times after the $4\frac{1}{2}$ day period of germination. After the dark period, plants were always returned to white fluorescent light of 600 ft-c at 20°C. Seed treated

with sulphuric acid germinated in constant conditions as rapidly as seeds germinated in alternating temperatures.

For the 6 hour applications of gibberellic acid (GA_3), glucose or alcohol, the compounds were dissolved in Hoagland's No. 1 nutrient solution. The glucose, in the Hoagland's solution, was filter sterilised prior to use. Washing with a total of 10 mls of solution at the time of application gave reproducible results. Excess Hoagland's solution (15 - 20 mls) was used to wash solutions off the filter paper at the end of the 6 hour period of application.

Plants were dissected for flowering no sooner than 7 days after the end of the dark period. Values of the least significant difference (LSD) between the flowering responses for all treatments were calculated from the pooled error variance of an analysis of variance. This calculation was only applied where 3 or more replicate dishes were sampled for flowering (15 plants per dish). All analysis was carried out on arcsin transformed values of the percentage of plants flowering. Since the LSD provides a confidence interval, the transformed LSD value and each average of the transformed flowering response could be retransformed to a percentage scale. When a square root transformation was applied it was not possible to apply any retransformation back to the natural scale. The statistical methods follow those in Steel and Torrie (1960).

c. Measurements of cell division.

Seedlings grown in alternating or constant conditions were harvested at various times, fixed in cold ethanol : acetic acid (3 : 1) for 24 hours and then placed in a solution of 70% ethanol in which they could be held indefinitely provided all the fixative was removed. Harvests were made at approximately two hourly intervals, commencing,

at the earliest, $5\frac{1}{2}$ days after germination (0 hours on the time scale). Normally, to make samples over a 24 hour period plants would be reared in two cabinets, germination in each commencing 12 hours apart. However, in these studies of cell division all harvests were made on a single batch of plants grown in one cabinet only. To induce a 100% flowering response plants were given a $13\frac{1}{2}$ hour dark period that commenced $5\frac{1}{2}$ days after sowing. After darkness they were returned to fluorescent light of 600 ft-c at a temperature of 20°C. Control plants were either held continuously in constant light (600 ft-c, 20°C) - subsequently designated "vegetative"-or were given a $13\frac{1}{2}$ hour dark period interrupted at the sixth hour of darkness with 15 minutes of red light - subsequently designated "non-induced." Both these latter treatments prevented flowering completely.

To prepare the apex for the counting of cell divisions, instead of embedding and sectioning, the whole plant was acid hydrolysed in 5N hydrochloric acid at room temperature for 40 - 45 minutes prior to staining with Feulgen solution for two to three hours, this method was adopted from Fox (1969). The apex with its small cells appeared relatively deeply stained and was clearly distinct from the larger pith cells immediately below it. Also, after the acid hydrolysis, the apical cells disintegrated less readily than the subapical pith cells so that it was possible to tease out the apex from the associated pith cells using 50X binocular magnification. The first and only visible pair of leaves were removed before the apex was squashed. There was never any contamination by vascular tissue and few if any of the large pith cells were carried over with the apical dome. If, after squashing, either a single layer of cells was not obtained or the cells were indistinct, those apices were not counted.

After squashing, dividing cells were clearly visible at a magnification of 400x under a light microscope. Generally the whole apex could be seen in the field of the microscope. The total number of cells and the number of dividing cells were counted for the whole apex. To facilitate counting, the field of the microscope was divided into 9 squares using a grid placed in one ocular. The number of cells was counted systematically in each square of the grid. When counting cell divisions only metaphase, anaphase and telophase stages were included in the first experiment (C19), but subsequently late prophase stages of division were counted as well. Most counts were of 8 replicate apices, however, replication was increased to 16 apices per harvest for the two or three very early harvests on very young plants - fewer than 160 cells / apex. With older apices, particularly of flowering plants, the average number of cells exceeded 1200 cells / apex and only 6 replicates were counted (the last 4 harvests). All counting was completed before values of the percentage of dividing cells were calculated and counting was carried out "blind" - without knowledge of the time of harvest. There was less than 10% variation when recounts were made of the total number of cells present in any one apex. The results for experiment C91 were obtained by S. Woo, and in order to avoid any complication of differences in counting between observers all apices for any one experiment were counted by the same person. A one-way analysis of variance was applied to the transformed values ($\sqrt{X + \frac{1}{2}}$) of the per cent mitosis (X represents the value of the per cent mitosis).

III. RESULTS.

a. Generation and export of the floral stimulus.

As was evident in earlier experiments (Figure 2) 100% flowering of Chenopodium rubrum resulted when plants were given a dark period of about 13½ hours in duration. If the cotyledons were removed at any time during the 7 hours after the dark period, little or no flowering resulted (Figure 39), but delaying leaf removal a further 9 hours (i.e. to 16 hours after darkness) resulted in almost maximal flowering. Thus, the cotyledons had fulfilled their essential role for flowering within 30 to 35 hours after the beginning of a 13½ hour dark period. When fewer replicates were used, variability was greater (Figure 39X)but the pattern of the response was comparable in this and three other experiments. The similarity of these results to those obtained with the short-day plants Xanthium (Salisbury, 1963) and Pharbitis nil (Zeevaart, 1963) allow the conclusion that measurements assayed the export of the floral stimulus out of the cotyledons. At the latest, stimulus export and hence its arrival at the shoot apex occurred 20 hours after the start of darkness.

When various portions of the cotyledons were removed, flowering was reduced proportionately to the reduction in area (Table 14). No flowering resulted if a dark period was given to plants without cotyledons or if the cotyledons were removed at the end of darkness. Maximal induction was achieved only when both cotyledons were intact or, in other words, with a total cotyledon area of about 0.3 mm^2. A requirement for the presence of the cotyledons for induction and the relationship between cotyledon area and flowering further substantiate the concept of a floral stimulus. Induction here could not be

FIGURE 39.

Flowering response of C. rubrum as affected by removal of the cotyledons at various times after the beginning of a dark period of 13½ hours. Experiment C5 (X), C79 (●).

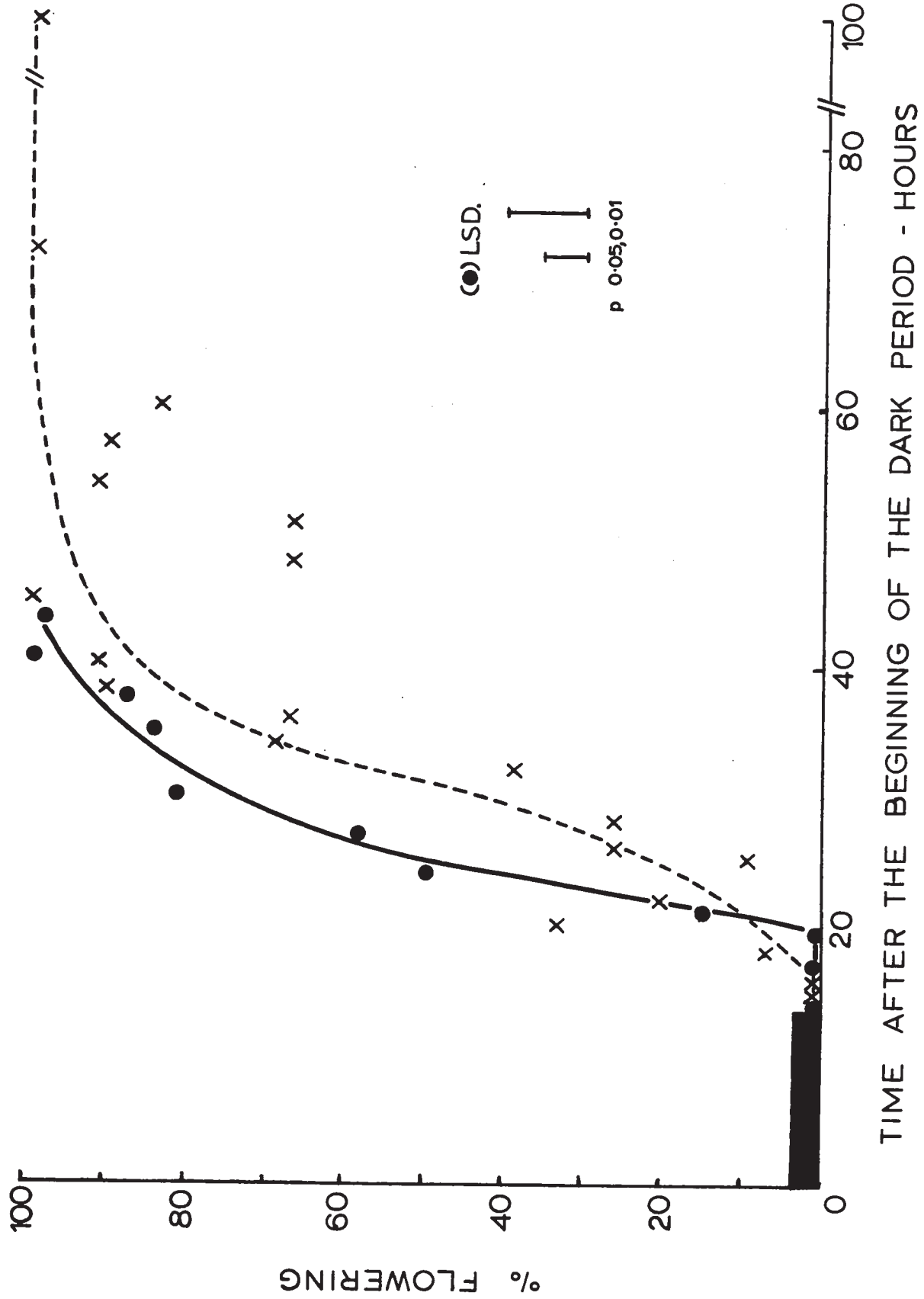

% FLOWERING
0
20
40
60
80
100
TIME AFTER THE BEGINNING OF THE DARK PERIOD - HOURS
20
40
60
80
100
(●) L.S.D.
p 0·05,0·01

TABLE 14.

Flowering in response to a 12 hour dark period given to plants with various proportions of their cotyledons removed before or after darkness. Percentage flowering of a total of 20 plants sampled from two dishes.

PERCENTAGE OF COTYLEDON AREA RETAINED	PERCENTAGE FLOWERING	
	REMOVAL PRIOR TO DARKNESS	REMOVAL AT THE END OF DARKNESS
0	0	0
25	33	35
50	43	56
75	56	76
100	88	88

considered as a removal by short days of a long-day inhibition. Far from allowing flowering, removal of what would be viewed as an inhibitory organ actually prevented flowering. This is not to say that floral induction might not involve an interaction between inhibitors and stimuli but, in the absence of other evidence, the action of daylength will be considered subsequently only in terms of a floral stimulus that is produced in the cotyledons and exported to the apex.

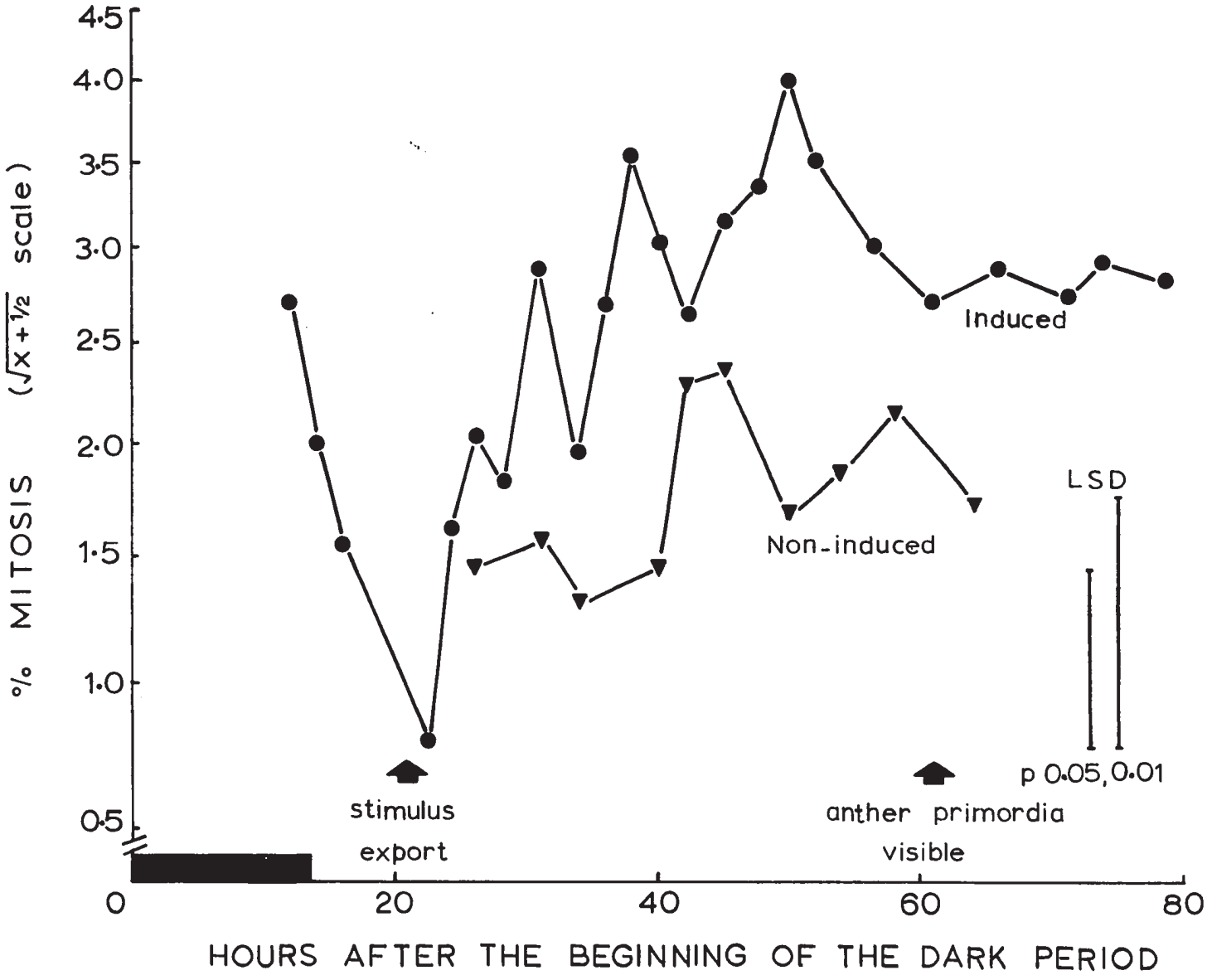
b. Effect of the floral stimulus on the mitotic index at the apex.

As shown in Figure 40, the mitotic index at the apex (percentage cells in mitosis) increased sharply 6 to 10 hours after the time of arrival of the floral stimulus at the apex. The greatest value of the mitotic index resulted about 10 hours before anther primordia were visible at hour 60. Fully differentiated flowers were visible on all plants at 120 hours after the beginning of darkness. This timing for the appearance of floral primordia is in accordance with results presented before (Figure 1). If plants were given a dark period but were prevented from flowering by inserting a 15 minute light interruption at the sixth hour of darkness the mitotic index was as much as one half of that of the induced plants (Figure 40).

In the results in Figure 40 there is some indication of periodic changes in the mitotic index although the timing of changes differed for induced and non-induced plants. More definitive evidence for the existence of rhythmic cell division at the apex was obtained when plants were germinated either under normal temperature alternations or in constant conditions (i.e. acid treated seed) and were then kept in continuous light until harvested. Changes of the mitotic index with the time of harvest are presented in Figure 41. For seeds germinated in alternating temperatures the results in Figure 41a provide clear evidence

FIGURE 40.

Mitotic index ($\frac{\text{number of cells in mitosis}}{\text{total number of cells}} \times 100$) at the shoot apex of plants harvested at various times in the light after the start of a dark period given $5\frac{1}{2}$ days after sowing. Germination in alternating temperatures ($32^{\circ}/10^{\circ}C$) for $4\frac{1}{2}$ days. (●) induced plants given a $13\frac{1}{2}$ hour dark period, (▼) non-induced plants given a 15 minute red light interruption at hour 6 of a $13\frac{1}{2}$ hour dark period. Experiment C58.


4·5
4·0
3·5
3·0
2·5
2·0
1·5
1·0
0·5
% MITOSIS ($\sqrt{x+1/2}$ scale)
Induced
Non-induced
LSD
p 0.05, 0.01
stimulus export
anther primordia visible
0
20
40
60
80
HOURS AFTER THE BEGINNING OF THE DARK PERIOD

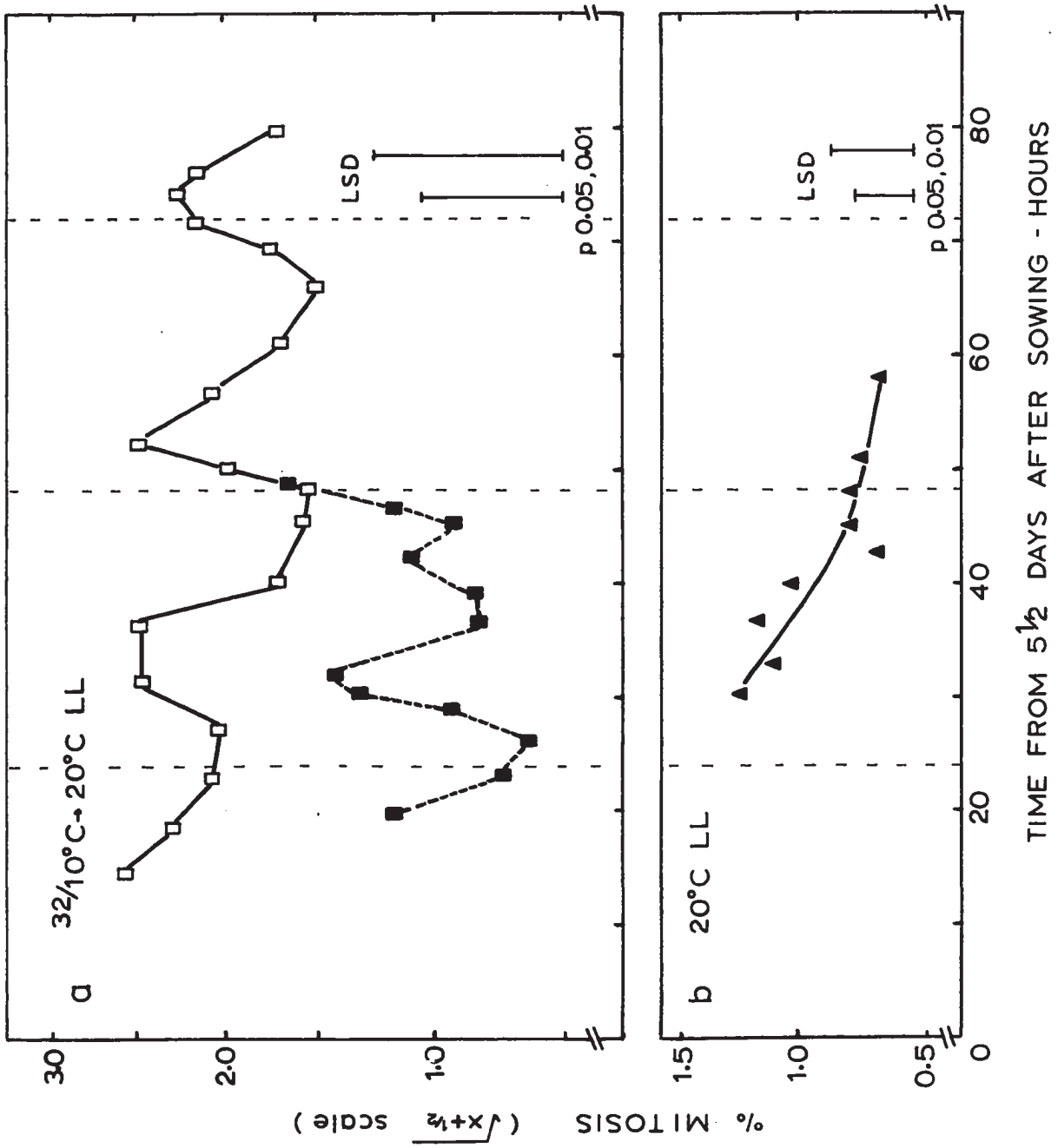
of a circadian rhythm of cell division at the apex. The oscillation continued in constant conditions for at least three cycles with a period of 20 hours. Peaks of the rhythm fell at about 33½, 52 and 74 hours and the troughs at 27, 48 and 66 hours and there were significant differences between the values at successive peaks and troughs. Similar peak timing was evident in experiments carried out nine months apart. The plastochron interval was greater than three days, therefore the rhythm of cell division did not reflect periodicity of leaf initiation. By contrast to the results presented in Figure 41a, when seedlings were germinated in constant conditions, there was no evidence of a circadian rhythm of cell division at the apex (Figure 41b).

The timing of the peaks of greatest cell division in vegetative plants held in continuous light (Figure 41a) differed from that of plants given a non-inductive dark period (Figure 40). Giving a dark period alone rephased the timing of the rhythm of cell division and when the dark period was also inductive for flowering the magnitude, period and phasing of the rhythm of the mitotic index apparently changed.

For vegetative plants there appeared to be a gradual reduction in the mitotic index with time (Figure 41a and b). If for some reason total cell number changed in its relationship to the number of dividing cells, determinations of the mitotic index might be ambiguous and give an apparent age trend. To examine this point the total number of cells in an apex was compared with the number of mitotic cells in the same apex. As shown in Figures 42 and 43 for large or small apices there was clearly a correlation between the number of mitotic cells and the number of cells in the apex. Only with very large floral apices (more than 1200 cells) was there any suggestion of a curvilinear relationship

FIGURE 41.

Mitotic index at the shoot apex after various times in continuous light (600 ft-c at 20°C) for plants germinated in alternating temperature cycles for 4½ days - experiment C19 (■), C58(□); or germinated at a constant temperature of 20°C - experiment C91 (▲).


a 32/10°C→20°C LL
b 20°C LL
LSD
p 0.05, 0.01
% MITOSIS (√x+½ scale)
TIME FROM 5½ DAYS AFTER SOWING - HOURS
0
20
40
60
80
0.5
1.0
1.5
2.0
3.0

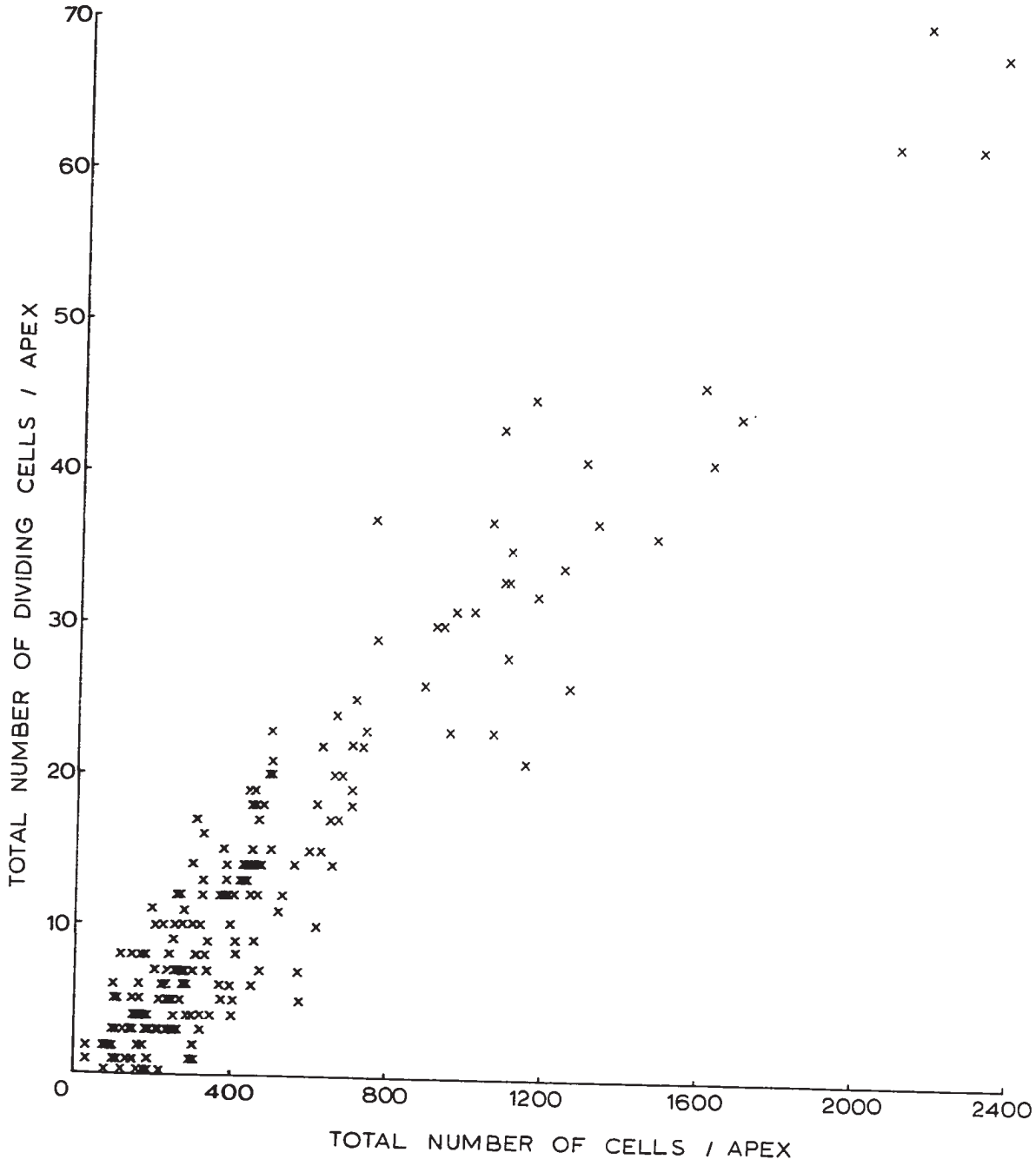
FIGURES 42 AND 43.

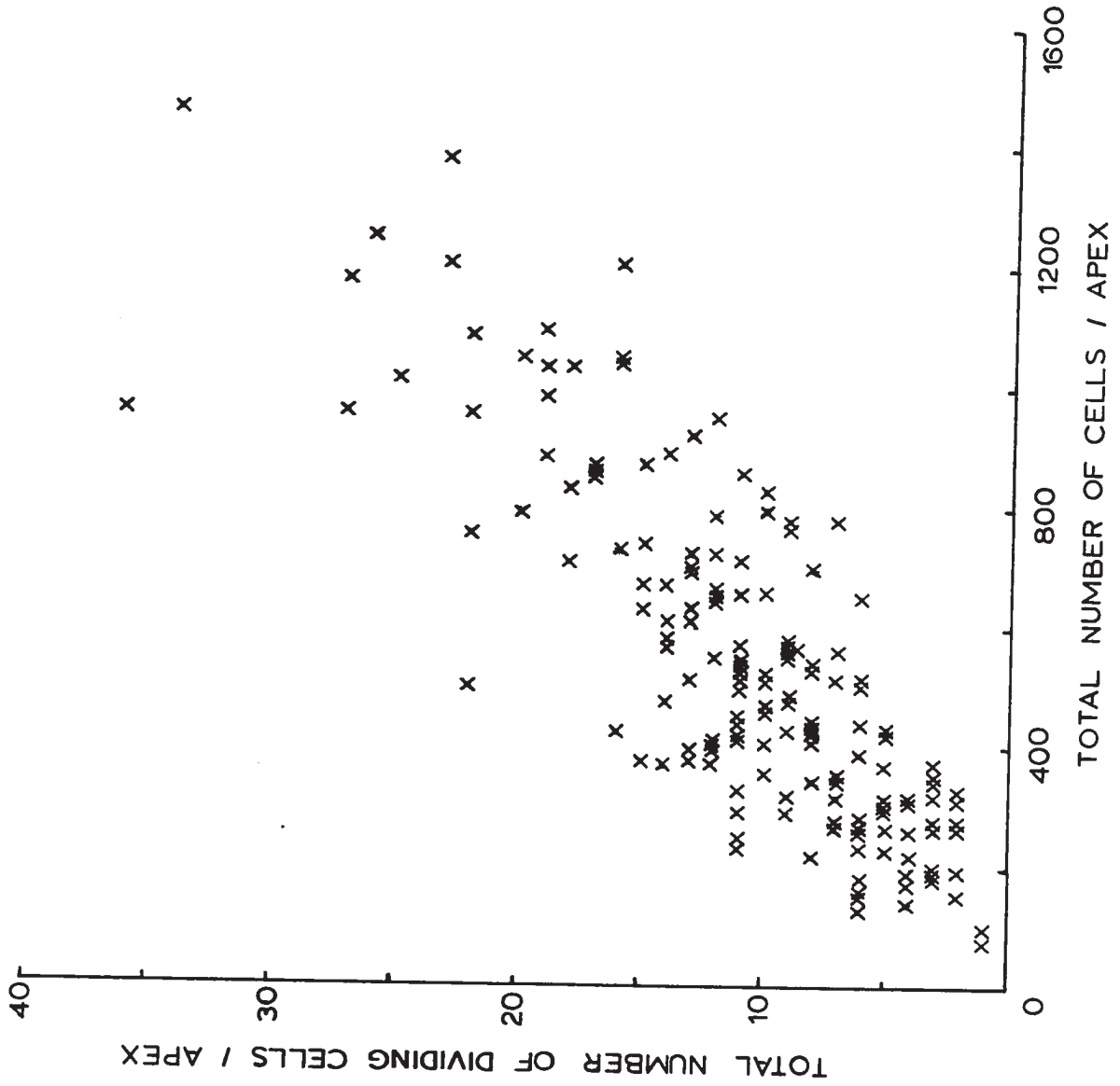
Change in the number of mitotic cells in an apex in relation to the total number of cells in the same apex.

Figure 42 - apices harvested from induced plants.

Figure 43 - apices harvested from plants kept in continuous light.

Germination in alternating temperature cycles.


70
60
50
40
30
20
10
0
400
800
1200
1600
2000
2400
TOTAL NUMBER OF DIVIDING CELLS / APEX
TOTAL NUMBER OF CELLS / APEX


TOTAL NUMBER OF DIVIDING CELLS / APEX
40
30
20
10
0
0
400
800
1200
1600
TOTAL NUMBER OF CELLS / APEX

(Figure 42). At this time floral primordia were evident (after more than 60 hours, Figure 40) and active cell division might be expected only in specific regions of the apex so that the mitotic index might decline and reflect less sensitively the rate of cell division. A similar explanation might be advanced for the decline in the mitotic index with age in vegetative plants but this conclusion would in no way invalidate the results in Figures 40 and 41.

c. Effects of environmental pretreatment on the capacity to flower.

Since prior environmental conditions determined whether cell division at the apex was rhythmical or not it was of interest to know whether environmental conditions prior to darkness also influenced the capacity to flower. In order to study this question plants were germinated either in alternating or constant temperatures for $4\frac{1}{2}$ days. Capacity to flower was assayed by giving a single 12 hour dark period at various times to different groups of plants.

When plants were germinated in constant conditions optimal sensitivity for flowering was achieved 5 to $5\frac{1}{2}$ days after sowing (Figure 44). There was no indication of any daily fluctuation in the capacity to flower and the overall change in response to seedling ontogenetic age probably reflects changes in the sensitivity of the cotyledons for induction. A similar response has been reported previously for seedlings of Pharbitis nil (Marushige and Takimoto, 1967; King and Evans, 1969).

If seeds were germinated in alternating temperatures, optimal sensitivity occurred about $5\frac{1}{2}$ days after sowing (Figure 45). The trend with seedling age was similar to that illustrated in Figure 44 for seedlings germinated at 20°C, however, superimposed on this trend, there was now evidence of daily changes in the capacity to

FIGURE 44.

Effect of seedling age at the time of exposure to a single dark period on the flowering response of seedlings germinated in constant conditions (600 ft-c at 20°C).

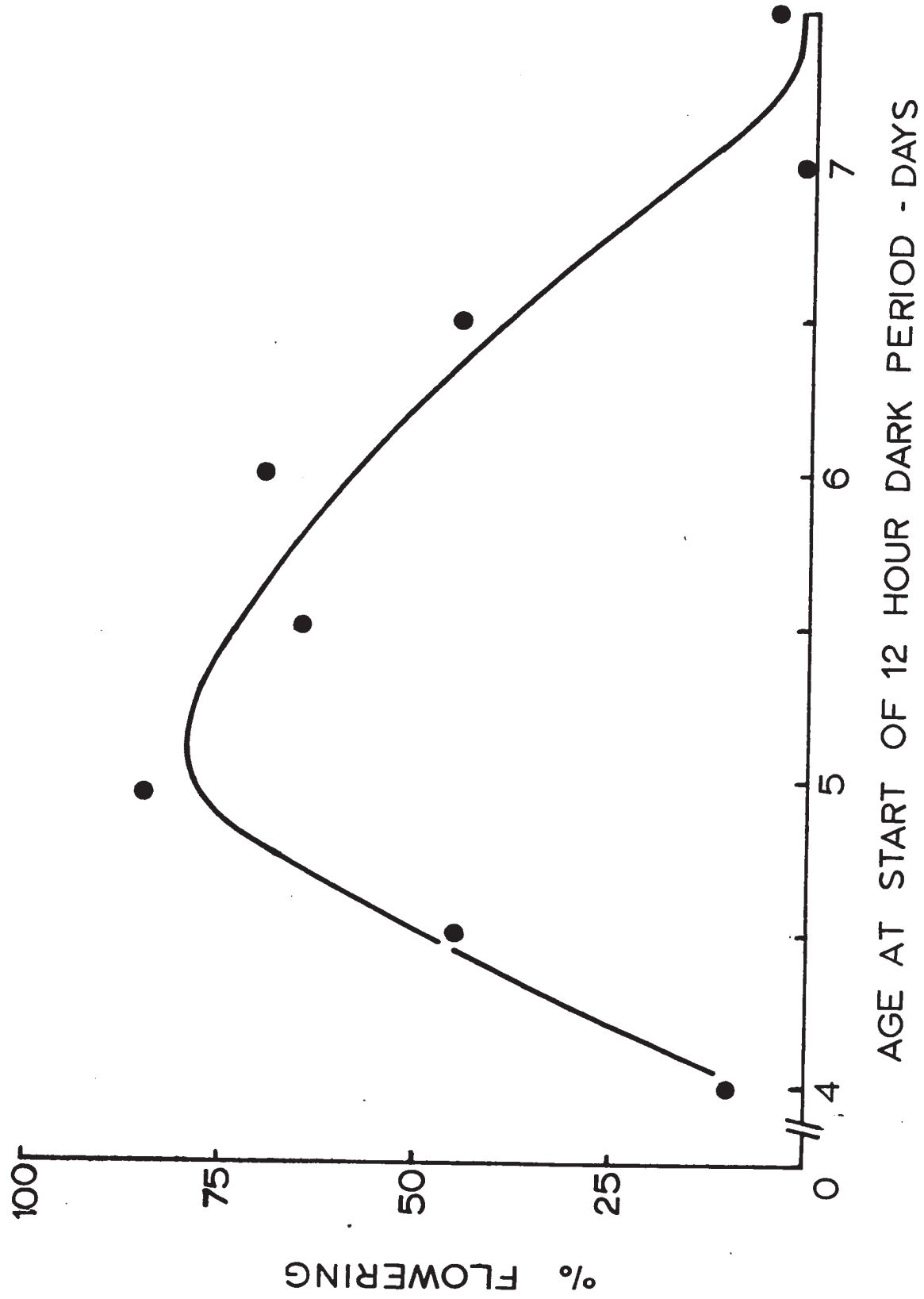
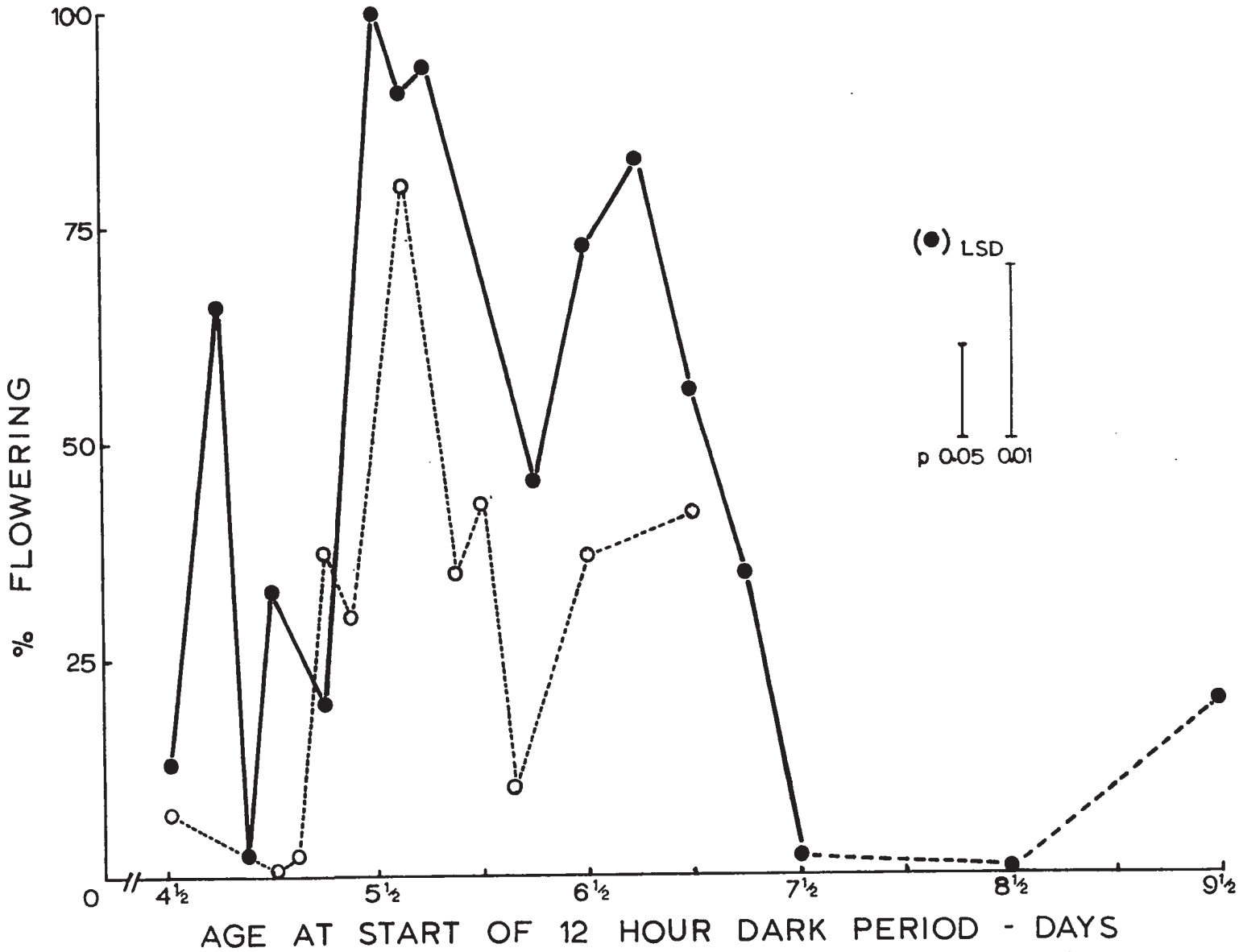

% FLOWERING
100
75
50
25
0
4
5
6
7
AGE AT START OF 12 HOUR DARK PERIOD - DAYS

FIGURE 45.

Effect of seedling age at the time of exposure to a single dark period on the flowering response of seedlings germinated under alternating temperature cycles ($32^0/10^0$C at 600 ft-c) given for $4\frac{1}{2}$ days. Values for two different experiments.


100
75
50
25
0
% FLOWERING
4½
5½
6½
7½
8½
9½
AGE AT START OF 12 HOUR DARK PERIOD - DAYS
(●) LSD
p 0.05 0.01

flower. For the two experiments reported in Figure 45 and in two further experiments, similar daily trends were observed. The period of the oscillation was about 24 hours.

It was shown in earlier experiments of Cumming (1967a) that a normal rhythm of flowering was observed in response to varied lengths of a dark period that commenced $4\frac{1}{2}$, $5\frac{1}{2}$, $6\frac{1}{2}$, $7\frac{1}{2}$ and $8\frac{1}{2}$ days after germination. An age trend was evident in his experiments although sampling intervals were not frequent enough to indicate the superimposed rhythm of daily changes in capacity to flower of the type indicated by the present results.

While there was no daily oscillation in the capacity to flower when seedlings were germinated in constant conditions, there was normal rhythmicity of flowering in response to dark periods of varied durations (Figure 46). This observation highlights the distinction between rhythmicity that may be inductive , i.e. the response to varied lengths of darkness (Figures 2 and 46) and the superimposed 24 hour rhythmicity in capacity to flower i.e. the daily changes in sensitivityt to a 12 hour dark period (Figure 45). In passing it should also be noted in Figure 46 that a brief far-red irradiation at the start of the dark period reduced the amplitude of the rhythm and lengthened the critical dark period and similar results were presented elsewhere in this thesis for plants germinated under conditions of alternating temperatures (Figure 38).

d. Sensitivity of floral development to applications of various solutions.

In the results presented in Figure 47 it is evident that, following a single dark period, there were rhythmic changes in sensitivity of flowering to the application of a 6 hour pulse of Hoagland's solution containing gibberellic acid, alcohol or glucose or

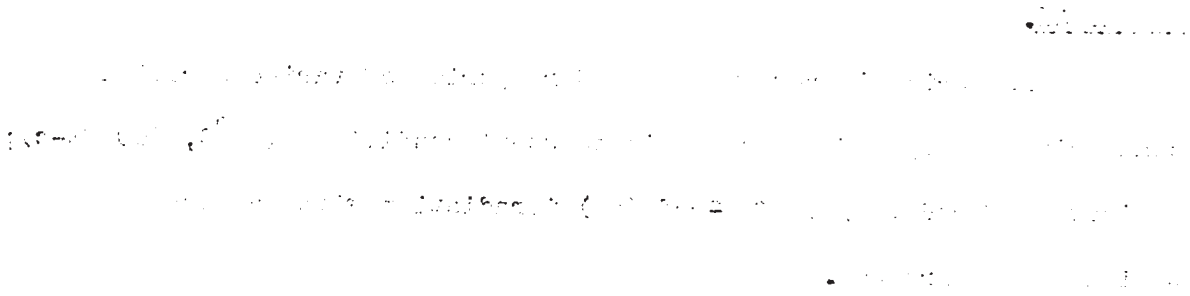


FIGURE 46.

Flowering in response to a dark period of varied duration commencing $5\frac{1}{2}$ days after sowing in constant conditions (20°C, 600 ft-c); 5 minutes of red (●) or far-red (○) irradiation given at the beginning of darkness.

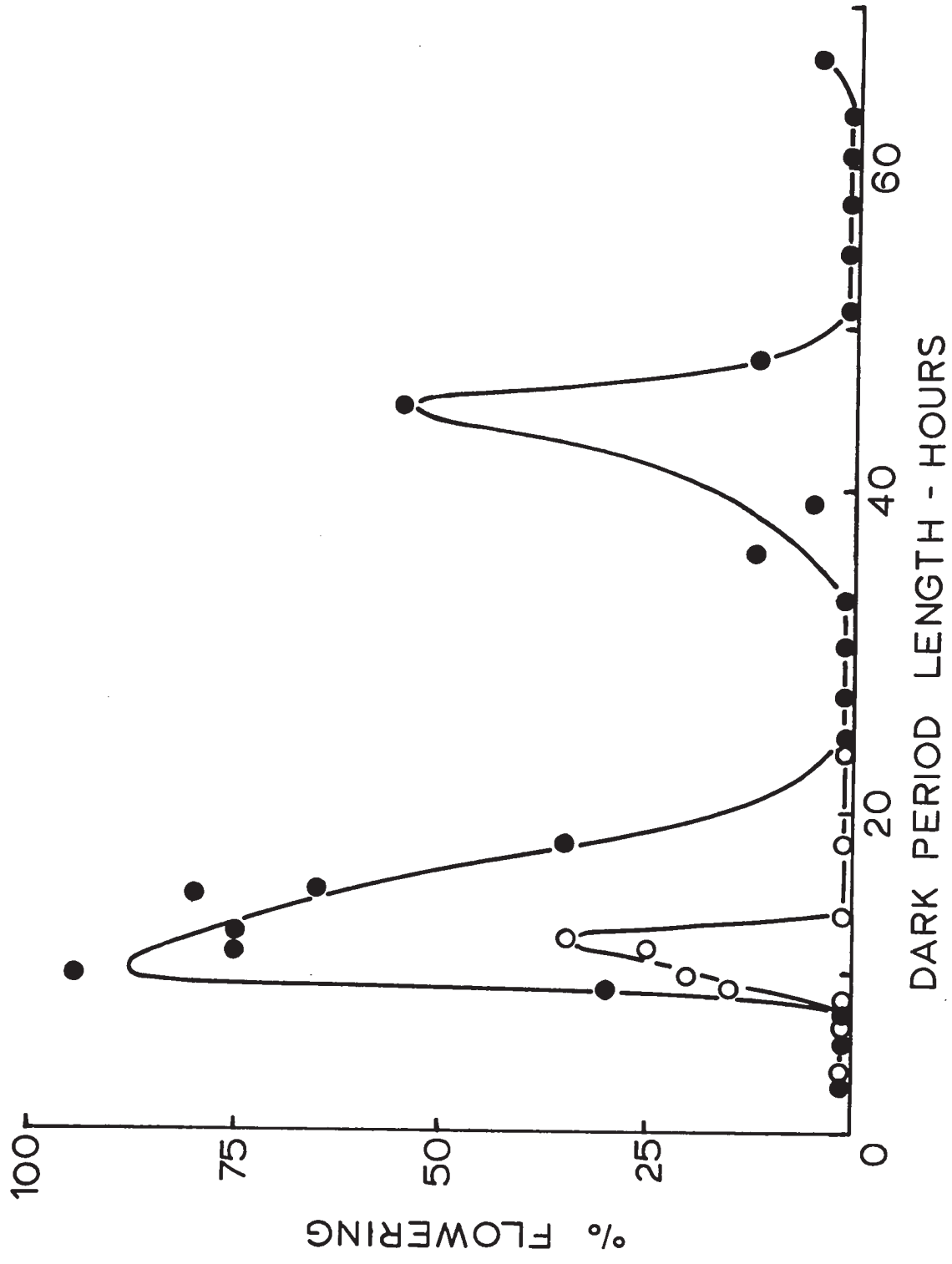
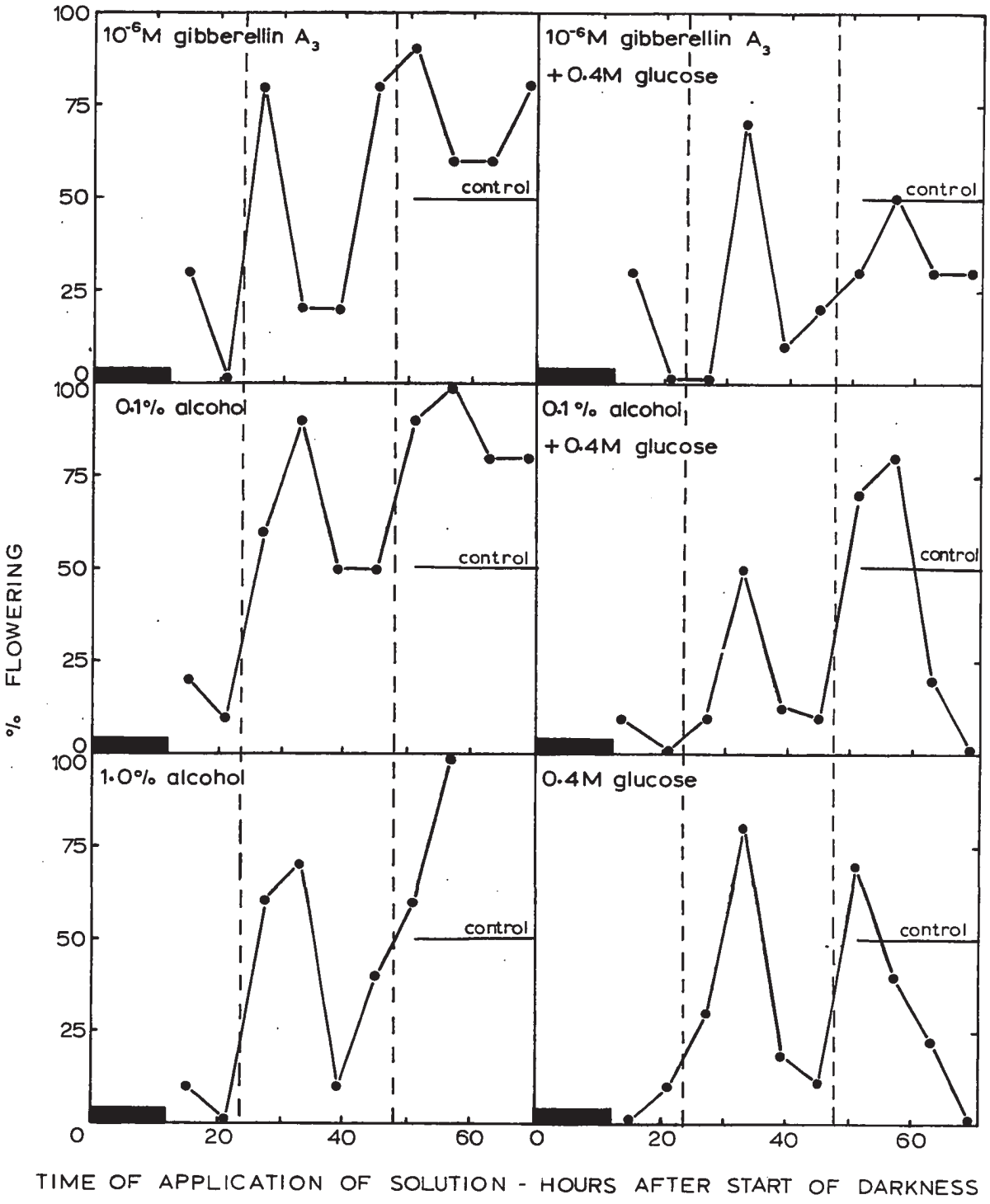

% FLOWERING
100
75
50
25
0
0
20
40
60
DARK PERIOD LENGTH - HOURS

FIGURE 47.

Flowering response to various compounds applied in Hoagland's solution for 6 hours at different times during the light period after a 12 hour inductive dark period. Points mid values of the times of each 6 hour application.

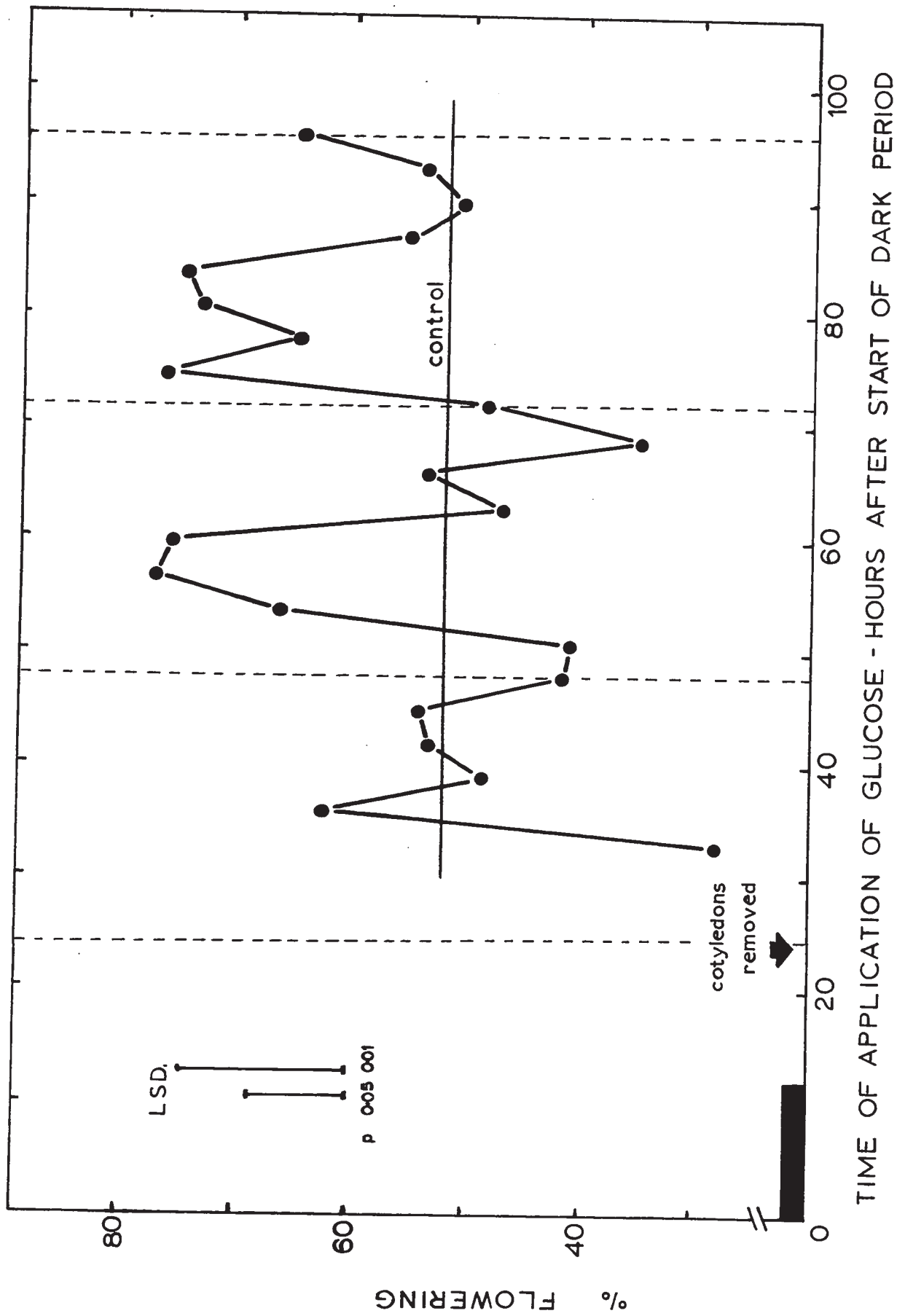

10⁻⁶M gibberellin A₃
10⁻⁶M gibberellin A₃ + 0.4M glucose
0.1% alcohol
0.1% alcohol + 0.4M glucose
1.0% alcohol
0.4M glucose
control
% FLOWERING
TIME OF APPLICATION OF SOLUTION - HOURS AFTER START OF DARKNESS
0
25
50
75
100
20
40
60

to mixtures of glucose and gibberellic acid or glucose and alcohol. The solutions were washed off the filter paper after 6 hours using Hoagland's solution. Flowering of untreated control plants was close to 50% and application of a solution enhanced or reduced flowering relative to the controls. In plotting the data as a curve rather than as an histogram the midpoint of the time of application has been used. Greatest promotion of flowering above that of the untreated controls resulted for pulses applied during the light period at 33 and 57 hours after the beginning of the dark period. The one exception was for the solution of Hoagland's and gibberellic acid in which case peaks were about 6 hours earlier. The average values of the period length of the rhythm of sensitivity was about 22 hours - from the peak and trough times for all of the data. In experiments in which 0.4 molar glucose was applied in Hoagland's solution for 6 hours the period length was 18, 18 and 21 hours.

On returning plants to the light after an inductive dark period export and early action of the floral stimulus occurs within 30 hours of the beginning of darkness (Figures 39 and 40). Nevertheless sensitivity to 6 hour pulses of solutions was evident until at least 60 hours after the beginning of the dark period (Figure 47). By this time photoperiodic induction and evocation must have been completed. In another experiment, sensitivity of flowering to a 6 hour pulse of glucose (0.4 molar in Hoagland's solution) was evident for at least 80 hours after the beginning of darkness (Figure 48). Rhythm period length was about 21 hours and times of the peaks and troughs were close to those reported in Figure 47. In the experiment reported in Figure 48 the cotyledons on all plants were removed 12 hours after the end of the 12 hour dark period and hence before application of the glucose

FIGURE 48.

Flowering response to a solution of 0.4 molar glucose in Hoagland's solution applied for 6 hours at different times during the light period after a 12 hour inductive dark period. Cotyledons on all plants removed 12 hours after the end of the dark period. Points mid values of the times of each 6 hour application.


% FLOWERING
80
60
40
0
20
40
60
80
100
TIME OF APPLICATION OF GLUCOSE - HOURS AFTER START OF DARK PERIOD
control
cotyledons removed
LSD.
p 005 001

pulses. Cotyledon removal excludes all possibility of any action of the sugar pulses on photoperiodic induction which occurs in the cotyledons. Therefore the action of the glucose pulses was directly on floral differentiation at the apex.

Other than evidence for changing sensitivity with the time of application of the various solutions, the basis of the action of glucose, alcohol or gibberellin has not been thoroughly examined. Osmotic effects may have been important at the concentrations of glucose and alcohol applied and there was no indication of response at lower concentrations that were closer to physiological levels. For instance, 6 hour pulses of 0.2 molar glucose or of 0.05 % alcohol had no effect on flowering nor did sensitivity change appreciably to a treatment in which Hoagland's solution alone was applied. If it were truly an osmotic effect it is possible that there might be some interaction with the uptake of a component of the Hoagland's solution. This proposition is supported by a recent report of Halaban and Hillman (1970a) who found that flowering of Lemna perpusilla underwent diurnal changes in sensitivity to replacement of their standard culture solution with distilled water.

IV. DISCUSSION.

In view of the many reports of diurnal changes in the mitotic index of shoot apices (Bunning, 1952b; Sachs et al, 1959; Denne, 1966a,b; Bernier et al, 1967; Jacqmard, 1970b) it is not surprising that a rhythm was found in the mitotic index at the shoot apex of Chenopodium rubrum (Figure 41). There appears to be little variation in the length of the cell cycle in shoot apices (Saint-Côme, 1969; Jacqmard, 1970c) and

it therefore seems highly unlikely that the oscillations in the mitotic index reflected repeated but chance concurrence of mitosis in a population of cells with differing cycle lengths. The interpretation favoured in this thesis is that fluctuations in the mitotic index indicated synchrony of cell division. Furthermore, because the oscillation in the mitotic index persisted for at least three cycles under constant conditions and maintained a fixed period length, it can be designated as an endogenous circadian rhythm.

It cannot be established from these results (Figure 41) whether changes in the mitotic index reflected the rate of passage of cells through interphase or through mitosis itself. However, other studies of the control of cell division in root and shoot apices have established that most variation in the rate of cell division relates to the time spent in interphase (see Bernier *et al*, 1967; Van't Hof, 1968). On the basis of this latter evidence; changes in the mitotic index whether rhythmical (Figure 41), or such as occur following the action of the floral stimulus (Figure 40), should reflect, directly, actual increases or decreases in the rate of cell division.

In root and shoot apices the progression of cells through interphase can be controlled by many factors, including: temperature fluctuations (Evans and Savage, 1959; Bodson, 1970); photosynthesis and the supply of sugars (Ballard and Wildman, 1964; Van't Hof, 1965; Bodson, 1970), the application of growth substances (Sachs *et al*, 1959; Van't Hof, 1967) and the supply of energy metabolites (Webster and Van't Hof, 1969). Therefore, as found in unicellular organisms (see Bruce, 1965), synchrony and hence rhythmicity of cell division might be induced by almost any environmental changes that control the progression of cells through the cell cycle. For *C. rubrum* it appears

that at least temperature and light : dark cycles control synchrony of cell division at the apex. Rhythmicity in the mitotic index only resulted when plants had been exposed previously to alternating temperature cycles (Figure 41a), while giving a non-inductive dark period altered the phase of the rhythm of mitosis compared to that of plants kept in continuous light (Figure 40 compared to Figure 41a). Periodicity in other environmental factors or in the supply of metabolites and hormones could also impose synchrony on cell division at the apex and it is suggested that the effect on flowering of 6 hour applications of solutions of glucose, alcohol or gibberellic acid may be related in part to changes in the synchrony of cell division at the apex.

Considering the possible influence of cell division on floral initiation, it is almost axiomatic that the formation of a flower requires an increase in cell number. Indeed, by the use of nucleic acid antimetabolites Krekule *et al* (1969) have established that DNA synthesis and hence cell division is essential for flowering of *C. rubrum*. Their observations give added significance to the rise in the mitotic index that was associated with the arrival of the floral stimulus at the apex (Figures 39 and 40). However, for flowering not only the presence of cell division but its rate might be important.

Relevant to this latter suggestion is the observation of Tomkins *et al* (1969) that hormonal induction of the enzyme tyrosine aminotransferase was possible only during a specific phase of the cell cycle in a synchronous culture of rat hepatoma cells. Similarly, in plants the action of the floral stimulus may require the passage of cells through the cell cycle (Zeevaart, 1962). Thus, particularly if the floral stimulus is labile as it appears to be in some plants (Nakayama, 1958; King and Evans, 1969), the rate of cell division

could limit the action of the floral stimulus. This suggestion explains the association found between induction by temperature cycles of rhythmic changes in both the rate of cell division and the capacity to flower in response to a 12 hour dark period (Figures 41a and 45 compared to Figures 41b and 44). The effectiveness of the floral stimulus depended on its timing of arrival and action at the apex relative to the temperature induced rhythmic changes in the rate of cell division. The suggestion that rhythmicity in the rate of cell division controlled the capacity to flower also explains the similarity in the period lengths of the rhythm of the mitotic index (about 20 hours, Figure 41), and the period lengths of the rhythms of sensitivity to the application of various solutions (about 21 hours, Figures 47 and 48) and of sensitivity to the action of the floral stimulus (approximately 24 hours, Figure 45).

While this discussion has inferred an essential role of cell division, Haber (1962) found that morphogenesis could occur sometimes in the absence of cell division. It is unlikely that this is true for floral morphogenesis but the evidence presented here for rhythmicity at the shoot apex might not reflect a simple causal relationship between cell division and flowering. Rhythmicity of cell division and of sensitivity of flowering to pulses of solutions might be controlled separately at the apex but by the same master rhythm. No matter what the type of coupling between rhythms at the apex and flowering, there is no doubt that the apex does confer some rhythmicity on the capacity of C. rubrum to flower.

The foregoing discussion raises questions about the nature of the rhythm of induction of flowering when plants of C. rubrum are exposed to dark periods of varied duration. The cotyledons in responding

to the dark period produce a stimulus to flowering (Figure 39) and, at least in Xanthium, the leaf is the site of rhythmic induction of flowering as well (Mitchell, 1964). However temperature fluctuations could induce a rhythmic change in the capacity to flower and also induce a rhythm of mitosis at the apex (Figures 40,41 and 45). Is rhythmicity of flowering therefore the superimposition at the apex of environmentally controlled rhythms that limit the expression of the floral stimulus? At present an unequivocal answer cannot be given to this question but a number of lines of evidence suggest that an additional rhythm(s) may be responsible for rhythmicity of floral induction in response to long dark periods. Firstly, whether rhythms were present or absent at the apex before darkness (Figure 41), in either condition there was a similar rhythmic response to dark periods of varied lengths (Figure 46 and see Figure 2). The period length of this rhythm induced by the dark period was 30 hours, whereas those rhythms controlled at the apex showed periods 20 to 24 hours in length. The former rhythm was suspended in continuous light (Figure 19) but rhythms controlled at the apex were manifested in continuous light and possibly in darkness. When a single dark period commenced at different times the amount of flowering was dependent on plant age at that time (Figures 16, 38), but, rhythm peak times and hence actual time measurement in darkness was basically independent of plant age. Thus, while the capacity for expression of the timer changed rhythmically, the actual dark period time measurement remained unaltered. It appears, therefore, that there are at least two rhythms controlling flowering of C. rubrum and since these rhythms respond to environmental conditions any model of environmental control of flowering must take into account complex interactions between individual "clocks."

The presence of rhythmicity at the apex complicates experiments involving applications of sugars and other compounds to C. rubrum. This is emphasised by the finding that pulses of glucose, when applied in the light after an inductive dark period, affected flowering after export of the floral stimulus from the leaf and acted even in the absence of the cotyledons (Figures 47 and 48). The basic control for the rhythmic sensitivity to applied glucose probably resided in the apex and was definitely not an effect on induction in the cotyledon. A similar conclusion was reached in Chapters II and IV when discussing the influence of varying light intensity (i.e. photosynthetic input) on flowering. Action of glucose and photosynthetic products at the apex provides an adequate explanation of the observations of Cumming (1967a) of an influence on flowering of glucose pulses applied at various times during a long dark period that was given to intact seedlings of C. rubrum. It therefore remains to be established that there is a more direct interaction of glucose with dark period induction of flowering. Even the application of glucose before induction might not distinguish between responses of floral induction and floral development. Treatment with a glucose solution 24 hours before darkness (Figures 9 and 36) apparently influenced floral development alone since the timing of rhythm peaks remained unchanged although the amount of flowering was increased.

CHAPTER VII

GENERAL DISCUSSION.

Previously it has only been possible to indirectly infer the action of a biological oscillator in time measurement in the photoperiodic control of flowering. However, the evidence presented here of a relationship between the timing, i.e. phasing, of the rhythm controlling flowering and the photoperiodic response of Chenopodium rubrum (Table 4) establishes beyond doubt that a biological oscillation is one effective photoperiodic timer.

The measurements of changes in the proportions of P_{fr} present during darkness (Chapter V) also indicate a potential of P_{fr} reversion to act as an hourglass clock in C. rubrum. The timing of phytochrome reversion probably sets a lower limit to the length of the critical dark period, i.e. to expression of the rhythmic clock. However, as the rhythm timer functions even when plants are grown in continuous light and constant temperatures prior to darkness, dominance of the rhythm can be suggested for C. rubrum. This suggestion could explain why treatments influencing the functioning of the hourglass clock had only a slight influence on dark period time measurement in this plant. For instance, only the peak time of the first peak of the rhythm was shifted by reintroducing P_{fr} after its reversion in darkness. The peak time of the second and third peaks of the rhythm were not altered and the rhythm appeared to set a limit to the expression of affects of the hourglass clock at the first peak of the rhythm (Figure 20). Conversely, attempts to hasten P_{fr} reversion failed to shorten the length of the critical dark period in C. rubrum (Chapter V).

In contrast to C. rubrum with its apparently dominant rhythmic

clock, in Pharbitis nil and possibly Xanthium there is little evidence of a rhythmic response to dark period length. Now, reintroducing P_{fr} after its reversion or hastening P_{fr} reversion can alter dark period time measurement (see Discussion in Chapter V). Rhythmicity in the response of Pharbitis nil can also be superimposed on its hourglass response if the plants are subjected to a cycle of dark and light prior to an inductive dark period (Takimoto and Hamner, 1964).

The operation of dual photoperiodic timers is suggested to reconcile the evidence of dark period time measurement that is controlled by the timing of phytochrome reversion - the hourglass - with the evidence of dark period time measurement under the control of a rhythmic timer. A similar proposition was advanced by Takimoto and Hamner in 1964.

The concept of dual photoperiodic timers as applied here also explains some of the anomolies previously reported in the literature. For instance, action of a dominant hourglass clock might obscure any relationship between a rhythm such as that of leaf movement and the flowering response. Dominance of an hourglass clock in Xanthium explains why Denney and Salisbury (1970) found that rhythms of leaf movement are not valid indicators of the photoperiodic clock controlling flowering in that plant. Likewise, earlier experiments of Papenfuss and Salisbury (1967) with Xanthium can be interpreted as indicating an hourglass clock measuring dark period duration (see discussion in Evans and King, 1969). The postulation of a dominant hourglass clock in Pharbitis nil and Xanthium is also consistent with the absence of a requirement for a light signal terminating darkness in these two species. Generation and export of a stimulus to flowering occurs equally well in darkness or in light (Searle, 1961; Zeevaart, 1963; King et al, 1968). In C. rubrum, however, expression of the rhythm requires the action of the light-on signal terminating darkness. Action of the hourglass timer is all

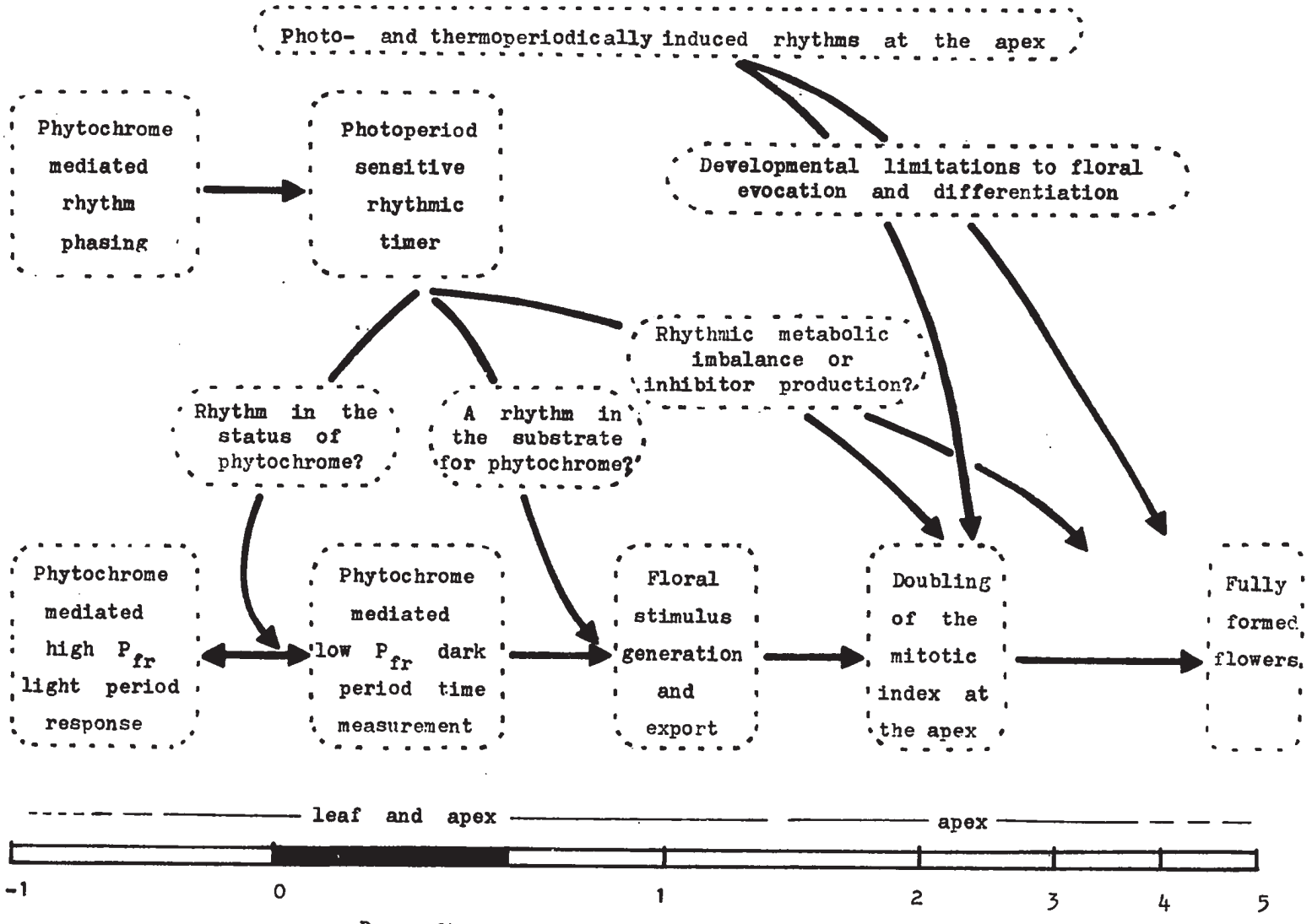
that is required for stimulus production and a rhythmic timer apparently superimposes on the hourglass in C. rubrum.

Considering that an hourglass timer operates in Pharbitis nil in the presence or absence of rhythms (Takimoto and Hamner, 1964), phytochrome reversion must therefore be central to the photoperiodic induction of flowering. Are rhythms also controlling the production of the floral stimulus in the leaf? Do they control production of a separate "inhibitor" of flowering? Or, is rhythmicity only controlling later steps of floral evocation and development and hence providing a rhythm of flowering superimposed on the inductive action of the floral stimulus?

Figure 49 presents a summary of the possible interactions between various photoperiodically controlled phenomena in C. rubrum and suggests ways these clock functions might control flowering. The action of phytochrome in time measurement and a dependence on both a high P_{fr} and low P_{fr} reaction was discussed in detail in Chapter V. Evidence of the production of a floral stimulus was given in Chapter VI.

Turning to the question of rhythmic control directly over floral evocation and development, the results presented in Chapter VI indicated that both thermo- and photoperiodic cycles could influence flowering. The rhythm that resulted, although important to time measurement, was not inductive but modulated the response at the apex and was therefore superimposed on induction. In Xanthium, results obtained by Mitchell (1964) also suggest the action of a rhythm that was superimposed on induction. Photosynthesis was found to be essential for this rhythm but it is not essential for floral induction in Xanthium (Salisbury and Ross, 1969). Thus rhythmicity probably resulted from metabolic imbalances in the supply of photosynthate to the apex. Probably in Lemna gibba, too, rhythmicity is superimposed on induction. In this

FIGURE 49. A MODEL OF THE INTERACTIONS BETWEEN HOURGLASS AND RHYTHMIC CLOCKS IN C. RUBRUM.



latter plant conditions that induce a rhythm in flowering (Nakashima, 1966) also induce an identical but inverse rhythm in frond production (Oota, 1970). There is, however, an inverse relationship between frond production and flowering of Lemna gibba (Cleland and Briggs, 1967) and hence it can be suggested that rhythmic frond production superimposed a rhythm on floral induction.

On the other hand, not all rhythms need act indirectly and on the apex. In C. rubrum, despite the evidence for a rhythm that was superimposed on flowering at the apex (Chapter VI), the rhythmic response to dark period length was distinct from the thermoperiodically induced rhythm. This dark period rhythm played an important role in time measurement during photoperiodic induction of flowering (Chapter III). Its control over flowering by production of an inhibitor or generation of a metabolic imbalance cannot be excluded but, since rhythm phasing emphasizes the first trough and peak of the rhythm (Table 4) it appears likely that the rhythm would interact with phytochrome controlled production of the floral stimulus. From the evidence of Cumming et al (1965) of rhythmic changes in the sensitivity to P_{fr} during darkness it follows that rhythmic supply of substrate for P_{fr} action could modulate stimulus generation. At present, there is no further evidence for this concept and although not excluding this possibility the results discussed in Chapters II, IV and VI provide no support for the suggestion of Cumming (1967a) of a rhythm in the supply of sugar for P_{fr} to act upon.

An alternative explanation could be that the rhythm, by altering the energy charge of the cell and hence membrane structure, might induce conformational changes in phytochrome which appears to be membrane bound (see Wagner and Cumming, 1970). That the status of P_{fr} itself might change rhythmically in C. rubrum is suggested by the measurements of the

null response proportions of phytochrome present during a long dark period (Cumming et al, 1965). Possibly only at specific phases does the rhythm control P_{fr} reversion and hence stimulus generation. This would lead to oscillations in what is otherwise a "tension-relaxation" response of the hourglass. A similar interpretation of the interaction of phytochrome and rhythmicity was suggested by Konitz in 1958; however, in his experiments it was not possible to distinguish between changes in the status of phytochrome and changes in the status of a substrate on which phytochrome acted. The postulation of a rhythm in the status of phytochrome might also provide the simplest explanation of the action of phytochrome to rephase the rhythm of flowering and to inhibit the response if P_{fr} is introduced during darkness.

Further answers to questions of the types of clocks involved in flowering might result from comparisons of ecotypic variants of a species that show differences in their responses to photoperiod. One such study with Pharbitis nil (Imamura et al, 1966) has shown that there are differences between ecotypes both in the ability of leaves to generate the floral stimulus and in the capacity of the shoot apices to respond to it. Similar experiments are not available for C. rubrum but at least for the selection 62°46'N, the response to dark periods of varied duration is distinctly different from that of the selection that originated at 60°47'N (Cumming, 1969a). Either the hourglass and/or rhythm clocks might function in a different fashion in selection 62°46'N. On the other hand, the apex might be limiting to induction in selection 49°58'N. In this latter ecotype the rhythm in darkness has the same phase and period length as found in selection 60°47'N but flowering requires more photoperiodic cycles or the application of sugar solutions (Cumming, 1969a).

A study of ecotypic differences might not only help in building models of photoperiodic time measurement but might also highlight adaptive advantages of the different types of limitations to flowering.

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