PHYTOCONTROL OF CIRCADIAN RHYTHMS

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by

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SUMMARY

In continuous darkness, at temperatures between 15° and 30°C, detached leaves of Bryophyllum fedtschenkoi displayed a circadian rhythm in the rate of carbon dioxide output into initially carbon-dioxide-free air. At 10° and 35°C the carbon dioxide output was arhythmic. Both the phase and the period of the rhythm were modified by irradiation.

Exposure to 0.25 h of white light in every 24-h cycle entrained the rhythm. Two 0.25-h exposures in every 24-h cycle also entrained the rhythm when applied in schedules including 7.5 or 10.5-h, but not 11.5-h, dark periods. A single 4-h exposure to white light shifted the phase of the rhythm in leaves otherwise maintained in darkness. The nature and magnitude of the phase shift was determined by the time in the circadian cycle at which the leaves were irradiated. Both the period and the transient of the rhythm in leaves at 15°C were shorter in continuous white light than in darkness. After the rhythm had faded out in continuous white light, a transfer to darkness reinitiated the rhythm.

An investigation of the rhythm in leaves exposed to monochromatic radiation from different regions of the spectrum was carried out to elucidate the photoreceptors involved. Radiant energy in a spectral band centred on 660 nm was most effective. The effect of this spectral band was similar to that of white light in all aspects of photocontrol tested. A spectral band centred on 730 nm was also effective in shortening the period and entraining the rhythm, but less so than red light. Longer wavelengths, and those from the blue region of the spectrum were ineffective.

The extent to which red light shortened the period depended on both the ambient temperature and the incident quantum flux density. Between 15° and 25°C, red light significantly reduced the period, while at 30°C no significant reduction was observed. There was a linear relationship between the reduction of the period at 15°C and the logarithm of the quantum flux density of monochromatic (660 nm) radiation to which the leaves were exposed.

Red and far-red radiation interacted to modify the phase shifting and entrainment responses induced by red light alone. Clear red/far-red reversibility was achieved in schedules containing one or two exposures to red light in every 24-h cycle. The spectral dependence of photocontrol and the occurrence of red/far-red reversibility leads to the conclusion that phytochrome is the pigment which mediates photocontrol of the circadian rhythm in Bryophyllum.

ABBREVIATIONS

CAM	-	Crassulacean Acid Metabolism
D	-	Darkness
IRGA	-	Infra-red Gas Analyser
L	-	Light
LD x:y	-	24-h cycles containing a light period of x h and a dark period of y h.
PEP	-	Phosphoenolpyruvate
P fr	-	The far-red absorbing form of phytochrome
P r	-	The red absorbing form of phytochrome
UV	-	Ultra-violet radiation

INTRODUCTION

Under natural conditions nearly all organisms are exposed to an environment in which a number of parameters fluctuate with a periodicity of 24 h. The decrease in radiant flux density experienced as a result of the transition from day to night is accompanied by a drop in temperature and often an increase in relative humidity. Barometric pressure and geophysical factors such as the strength of the Earth's magnetic field and the intensity of cosmic ray bombardment may also vary in 24-h cycles.

Any physiological process with an absolute requirement for a particular set of environmental conditions will clearly show an activity pattern corresponding to the 24-h periodicity encountered in the environment. For example, a light requiring process such as photosynthesis will only be operative during those parts of the 24-h cycle when radiant energy incident on the plant exceeds a certain minimum level. However, many other aspects of the physiology and behaviour of plants and animals, not specifically limited by the prevailing environmental conditions, also show diurnal periodicity in relation to the natural cycle.

The earliest observations of this phenomenon were made with the movement of leaves. References to the observations that the leaves of some species take up a different position at night from that which they occupy during the day were recorded in the writings of both Pliny in the first century and Albertus Magnus in the thirteenth century (cited by Bünning, 1960). The first experimental confirmation of the entrainment of rhythmic functions by the periodic repetition of environmental stimuli was made by Hill (1757). He inverted the cycles of light and darkness to which plants were exposed by using artificial light at night and covering the plants during the day. In these experiments it was

observed that the diurnal rhythms of leaf movement rapidly became adjusted to the new cycles and adopted a phase relationship to the entraining cycle similar to that previously maintained in relation to the natural cycle. From this he concluded that light-dark cycles exert a controlling influence over rhythmic behaviour. The same conclusion was later drawn by DeCandolle (1832) from the results of similar experiments.

Rhythms may often be entrained with extremely weak or brief signals if these are repeated at 24-h intervals. Kleinhoote (1929) showed that the rhythmic leaf movement of Canavalia ensiformis is entrained by light-dark cycles consisting of 1 h of light alternating with 23 h of darkness (LD 1:23). The minimum exposure to light required to entrain a rhythm in 24-h cycles varies between organisms. In contrast to the leaf movement rhythm of Canavalia, that of Pharbitis nil seedlings requires between 1 and 4 h of light in each 24-h cycle (Bollig, 1974). Similarly there is a requirement for a minimum duration of darkness in each 24 h which often must be considerably longer than the minimum effective duration of light (Bünning, 1973).

Entrainment of a rhythm may also occur when an organism receives regular 24-h cycles consisting of darkness interrupted by two or more brief exposures to light. Rhythms in some insects (Minis, 1965; Pittendrigh, 1965) and in the plant Lemna (Hillman, 1970) may be entrained by two 15-minute light exposures. The phase of rhythms entrained in this manner is similar to that obtained with a complete photoperiod of which the short light breaks mark the beginning and end. There are differences between organisms as to the effectiveness of such treatments. The time of the maximum opening of the leaves of Portulaca grandiflora depends on the length of the photoperiod (Karvé and Jigajinni,

1966a). The complete photoperiod may be substituted by two light breaks of 1 or 2 h marking the beginning and end of the photoperiod. If however the plants are exposed to light breaks of only 30 min, entrainment does not occur (Karvé and Jigajinni, 1966b).

Although they have been less well investigated than light-dark cycles, it has become apparent that periodic fluctuations in temperature may also influence rhythmic behaviour. Stern and Bünning (1929) first demonstrated that periodic changes in the ambient temperature can induce rhythms in otherwise arhythmic organisms, and that a specific phase relationship is established between the entraining rhythm and the temperature cycles. Both sinusoidal and step type temperature changes can be effective. Entrainment of the petal movement rhythm of Kalanchoë blossfeldiana is achieved with a temperature fluctuation of 1°C (Oltmanns, 1960). With Pilobolus it was found that only a relatively brief exposure to an increased temperature during each 24-h cycle is required to entrain the rhythm, a 5°C increase in temperature for 1 h being effective (Ubelmesser, 1954).

The importance of environmental factors is also apparent from the entrainment of rhythms to frequencies other than 24 h. At a uniform temperature alternate exposure to light and darkness may entrain rhythms to periodicities ranging from 6 to 48 h (Bruce, 1960). In most organisms there appears to be a limit to the rapidity with which a rhythm can be forced to oscillate by means of imposed cycles of light and darkness. The leaf movement rhythm of Phaseolus multiflorus for example can be entrained to an 18-h period by LD 9:9, but when exposed to LD 6:6 does not show a 12-h period but its natural frequency (Bünning, 1957). The limit to the rapidity with which the rhythm of carbon dioxide output from leaves of Bryophyllum fedtschenkoi may be forced to oscillate depends

on the light intensity employed. With a light intensity of 1,000 lux, entrainment to LD 3:3, 6:6 and 8:8 occurs. At 500 lux, LD 6:6 but not 3:3 are effective, while at 100 lux, LD 6:6 are ineffective (Wilkins, 1962a). Temperature cycles differing from the periodicity of the natural environment have also been shown to entrain the rhythms of sporulation in Oedogonium cardiacum to cycles of 18 h but not of 12, 30 or 48 h (Bühnemann, 1955b).

The first indications that the processes which showed periodic response to the day-night cycle represented anything other than a direct response to this imposed periodicity came from the experiments of DeMairan in 1727 (cited by Bünning, 1960). He found that leaf movements of plants transferred to a darkened room continued in a rhythmic manner for a number of days. DeCandolle (1832), by placing plants in artificial light every night, showed that the leaf movement rhythm of Mimosa persists in continuous light. However, in neither of these investigations was the ambient temperature controlled and might therefore have fluctuated rhythmically. The experimental procedure of DeCandolle must have resulted also in regular 24-h changes in the light intensity.

Duhamel (1758) confirmed the continuation of leaf movement rhythms in constant darkness with experiments carried out in caves where any large temperature fluctuation could be ruled out. Sachs (1863) recorded the leaf movement rhythm of Acacia lophantha in constant darkness and found no correlation between leaf movement and fluctuations in the regularly recorded temperature.

Pfeffer (1875) suggested that the rhythmic leaf movements were in some way after-effects of the natural light-dark cycle to which the plants had previously been exposed. However, an alternative suggestion

was put forward by Darwin and Darwin (1880), that the movements of the leaves were innate and that the alternations of light and darkness merely set the phase of the rhythmic movements.

Critical evidence against the idea that rhythms were acquired by experience was obtained by Semon,(1905, 1908) using seedlings of Acacia lophantha which had been grown from germination in LD 6:6 or 24:24. These seedlings still invariably showed leaf movement which followed a 24-h pattern rather than one of 12 or 48 h after the plants were placed in continuous light. This observation has been confirmed in similar experiments for a number of organisms, both plants and animals (Kleinhoote, 1932; Ubelmesser, 1954; Bühnemann, 1955b; Hastings and Sweeney, 1958). In only one case has evidence been presented for the retention in constant conditions of a previously experienced period differing markedly from 24 h. The rhythms of oxygen production and rate of filament elongation of the alga Hydrodictyon reticulatum, entrained to LD 12:12, 10.5:7 or 6:6, persists with the corresponding periodicities of 24, 17.5 and 12 h after transfer to continuous light or darkness (Pirson et al, 1954; Schön, 1955).

The concept of rhythmicity resulting from previously experienced periodicity was further refuted by experiments in which oscillations were initiated in organisms which had been raised under constant conditions. Initiation was achieved by a single transfer to a different set of conditions. This has been reported for the leaf movement rhythm of Phaseolus seedlings (Bünning, 1932) and for rhythms in a number of other organisms (Park and Keller, 1932; Horstmann, 1935; Ball and Dyke, 1954). This phenomenon occurs with Phaseolus seedlings even when the previous generation has experienced only constant conditions (Bünning, 1932). Furthermore, the exposure of Drosophila to constant conditions for 15

consecutive generations failed to prevent rhythmic behaviour becoming apparent after a transfer to different conditions (Bünning, 1935a). Similarly a rhythm of luminescence can be induced in a previously arhythmic culture of Gonyaulax polyedra by a single change in the light intensity in which the cultures were grown. The ability to oscillate remains, even in cultures maintained in continuous light for 3 years (Sweeney and Hastings, 1962). Thus environmental factors such as light and temperature need not necessarily be oscillating in order for a biological process to function rhythmically. Neither do they exert a lasting influence on the periodicity of an organism after its transfer to constant conditions. These conclusions suggest either, that variation in some other environmental factor maintains the rhythm in supposedly constant conditions, or that the rhythm is endogenous and is generated within the organism in the manner of a self-sustained oscillation, following an initiating stimulus.

Before a rhythm can be considered to be endogenous, a number of its properties must be examined. It is generally accepted that an endogenous circadian rhythm must display five characteristics first proposed by Pittendrigh (1954).

1. The rhythm must continue in an environment held as constant as is possible.
2. The phase of the rhythm should be able to be shifted and subsequently retained under constant conditions, irrespective of its phase relationship to external cycles.
3. The rhythm should be initiated by a single stimulus.
4. The phase of the rhythm should be delayed under hypoxia.
5. The period of the rhythm should, under certain conditions, vary from 24 h.

The first condition is perhaps the most obvious, in that if rhythmicity persists in the absence of external periodicity then it must be of endogenous origin. However, this criterion is one of the most difficult to establish. Although periodic variation in light intensity, temperature, humidity and barometric pressure can now fairly easily be suppressed, it may still be argued that some other subtle geophysical or unknown factor is responsible. Attempts have been made to establish conditions from which have been eliminated those periodic variables which occur as a result of the Earth's rotation. Hamner et al (1962) attempted to create such conditions by mounting various subjects on a rotating platform near the Earth's geographic south pole and found that higher and lower plants as well as animals and insects continued to display rhythmic behaviour. These results indicate that in none of these organisms is rhythmicity dependent upon periodicity imposed by the Earth's rotation.

Numerous observations and experiments have been carried out on organisms maintained in conditions which are held, as far as is possible, constant. In many organisms, whether or not rhythmicity can be observed depends on the conditions experienced. In some cases rhythms may persist well in one set of conditions but rapidly fade out in another. In Hydrodictyon (Pirson et al, 1954; Pirson, 1957) and Phaseolus seedlings (Bünning and Lorcher, 1957) the rhythms in question persist well in both darkness and low intensity light. In Gonyaulax however rhythms are inhibited by continuous high intensity light, damp strongly in continuous darkness, but persist for at least 14 days in continuous low intensity light (Hastings and Sweeney, 1958). The rhythms in Oedogonium persist in continuous light but not in darkness (Bühnemann, 1955a), while the reverse is true in a second alga, Chlorella (Hesse, 1972).

The absence of oscillation and the rapid damping out observed under some conditions does not exclude the operation of a self sustaining oscillator. A particular set of environmental conditions may be highly unfavourable to the specific physiological function which is being measured as an overt rhythm. It is also possible, when the rhythms of populations of organisms are being monitored, that the observed fade-out occurs as a result of a loss of synchronization between individuals, or even different cells of individuals. Desynchronization of individuals in a population has been demonstrated for the flower opening rhythm of Cichorium intybus (Todt, 1962). In continuous light synchronization is lost first among the individual plants, then among different composite flowers of a single plant, and finally among the petals of individual flowers. Clearly in this case the rhythmicity of the individual petals is still apparent, but if the response of the population was being recorded the process would have appeared to damp out due to desynchronization. Nevertheless, studies with single celled organisms, particularly Gonyaulax (Sweeney, 1960) and Chlorella (Hesse, 1972) have demonstrated that after prolonged exposure to constant conditions the rhythms do indeed fade out in individual cells.

The long term persistence of rhythms in constant conditions seems to be more common in animals than in plants. Rawson (1960) has followed the activity rhythm of the bat Eptesicus fuscus for a period of 5 months. Rhythms can also persist during hibernation (Menaker, 1961) and in successive generations of mice (Aschoff, 1955).

Examples of the initiation of a rhythm after prolonged exposure to constant conditions have been cited as evidence against the concept of periodicity learnt by experience. These results also conform to one of the conditions considered as necessary for the establishment of the

endogenous nature of a rhythm, its initiation by a single stimulus. Many other investigators have confirmed this ~~principal~~ principle and demonstrated that a range of stimuli may be effective. In Gonyaulax (Hastings and Sweeney, 1959) and leaves of Bryophyllum fedtschenkoi (Wilkins, 1959) oscillation begins after a transfer from continuous light to darkness. A transfer from darkness to light will initiate a rhythm in Oedogonium (Bühnemann, 1955a), and rhythms initiated by both 'light-on' and 'light-off' stimuli have been reported for the petal movement of Kalanchoë (Engelmann, 1960) and the leaf movement of Xanthium (Hoshizaki et al, 1969). In Bryophyllum leaves a decrease in the light intensity is sufficient to initiate the oscillation (Wilkins, 1960a). Similarly, a decrease in the ambient temperature is effective in this organism (Wilkins, 1962b). The initiation of a rhythm can also occur in response to a relatively brief, temporary change in the constant conditions. The leaf movement rhythm of Phaseolus seedlings in darkness may be initiated either by an exposure to red light for 1 h or by an increase in temperature of 5°C for 1h (Bünning, 1931).

The endogenous nature of rhythms is also implied by their requirement for metabolic energy. A temporary exposure to anaerobiosis inhibits rhythmicity in Avena coleoptiles (Wilkins and Warren, 1963), Phaseolus seedlings (Bünning et al, 1965) and Bryophyllum leaves (Wilkins, 1967). Similarly, as metabolism is considerably reduced by low temperatures, circadian rhythms would be expected to be inhibited at, or a little above, freezing point. In each of these three organisms, prolonged exposure to low temperature does delay the phase of the rhythm (Ball and Dyke, 1957; Bünning and Tazawa, 1957; Wilkins, 1962b). While exposures to low temperatures or anaerobiosis in some parts of the cycle cause phase delays, at other times such treatments are without effect. In both Bryophyllum and Phaseolus the times during the cycle at which low temper-

ature and anaerobiosis are effective are quite different. However, the conclusion may still be drawn, that since metabolic energy is required for the rhythm to proceed through certain phases, the oscillation must therefore be endogenous.

A further implication of the results of a temporary exposure to low temperature or anaerobiosis is that the phase of a rhythm can be set to occur at any time of day. This may also be achieved by making use of the fact that a rhythm adopts a specific phase relationship to its initiating stimulus. The treatments most effective in this respect are those which normally entrain the rhythm to the imposed periodicity of the natural environment. Thus the transfer from light to dark sets the phase of the oscillations in Gonyaulax (Hastings and Sweeney, 1959) and Bryophyllum (Wilkins, 1960a), as does a decrease in light intensity in these organisms. The phase of the rhythm in Bryophyllum is also reset by a transfer from high to low temperature (Wilkins, 1962b).

The phase of a rhythm persisting in an organism held in constant darkness can be shifted by a brief exposure to light, typically of a few hours duration. Similarly the phase of circadian rhythms in an irradiated organism can be changed by a single exposure to darkness (Wilkins, 1960a). An exposure to elevated temperature for a few hours shifts the phase of the Phaseolus leaf movement rhythm (Moser, 1962), while a prolonged decrease in temperature causes a phase shift in the leaf movement rhythm of Coleus (Halaban, 1968b). In both cases the new phase is retained after a return to constant conditions.

Whether a phase shift occurs and its magnitude depends on the time in the circadian cycle at which a change in the environment is experienced. When, for example, an organism is exposed to light during the part of

the circadian cycle normally associated with the day time (the subjective day), little or no phase shift is induced. In ~~contrast~~ contrast, an identical exposure during the part of the circadian cycle normally associated with night (the subjective night), results in a large phase shift. In general high temperatures cause a minimum phase shift during the subjective day while producing a considerable shift during the subjective night. In contrast the minimum and maximum effectiveness of low temperatures in inducing phase shifts occur during the subjective night and day respectively.

The most convincing evidence for the endogenous nature of rhythmic phenomena is the fact that the period of the oscillation is often found to vary somewhat from 24h. In constant conditions rhythms show periods both longer and shorter than 24 h, most frequently within the range 20-28 h. This is despite the fact that any hypothetical, external, entraining cycle resulting from the periodicity of the Earth's rotation would have a precise 24-h periodicity. Period variation can be seen in the early results of DeCandolle (1832) who found a 22-23h period for the leaf movement rhythm of Mimosa, and of Semon (1905, 1908) who reported periods of leaf movement rhythms in excess of 24 h. A large number of plant and animal rhythms have now been shown to have a free-running period differing significantly from 24 h. For example, the periods of the rhythms of leaf movement of Phaseolus (Leinweber, 1956) and carbon dioxide emission from Bryophyllum leaves (Wilkins, 1962b) are 28 and 22.4 h respectively at 25-26°C.

In any one organism the period may vary with the prevailing conditions. The period is often different in constant darkness and in constant light, and may depend on the light intensity. The effect of light intensity on leaf movement varies considerably from species to

species. Thus, while the rhythms of Phaseolus (Moser, 1962) and Canavalia (Kleinhoote, 1932) persist under continuous white light of different intensities without significant variation in their period, the periods of rhythms in Coleus (Halaban, 1968a) and Pharbitis (Bollig, 1974) increase with increasing light intensity. The rhythm of luminescence in Gonyaulax (Hastings and Sweeney, 1959) decreases with increasing light intensity.

A small but significant effect of ambient temperature on the free-running period in light or darkness has also been reported a number of times. Again this effect is species dependent. No effect is found with Uca (Brown and Webb, 1948) while the period is shorter at higher than at lower temperatures in Phaseolus (Bünning, 1931) and the opposite is true for rhythms in Gonyaulax (Hastings and Sweeney, 1957; Sweeney and Hastings, 1958) and Oedogonium (Bühnemann, 1955b). Although the periods of free-running rhythms may vary by only a few hours from the 24-h natural cycle, these variations are indisputable. Assuming that the possession of rhythmicity is of adaptive value, it is not surprising that natural selection has favoured rhythms with free-running periods close to those of the natural environment. Some degree of compensation with respect to irregular and quite large variations in temperature and light intensity, not directly correlated with the 24-h periodicity of the environment, can also be expected as a prerequisite for accurate timing, and hence very likely to have arisen through natural selection.

Further evidence for the endogenous nature of the oscillation is implied in our knowledge that the period is specified in the genotype of an organism. This has been demonstrated by obtaining hybrids ~~hybrids~~ from parent strains with different periods (Bünning, 1932, 1935b). While

unequivocal demonstration of Mendelian segregation was not possible the original periods did appear in later generations. Konopka and Benzer (1971) isolated mutants of Drosophila which were arhythmic or displayed periods very different from 24 h . In this case each of these mutations appeared to involve the same functional gene on the X-chromosome.

Although rhythms which meet the five conditions discussed previously are now generally considered to arise endogenously rather than exogenously this view has not received universal acceptance. Brown and co-workers were led by the temperature independence of the rhythm they were studying to suspect an exogenous basis for the oscillation (Brown et al, 1954). Furthermore they reported persistent daily, monthly and lunar rhythms in the respiration rates of Uca pugnax, U. pugilator and Triluris viridescens which showed statistical correlation with the concurrent rate of barometric pressure change (Brown et al, 1955). Using hermetically sealed containers, which ruled out the pressure changes, this research group claimed to find a correlation between circadian rhythms of oxygen production in potatoes and the intensity of primary cosmic radiation (Brown, 1957; Brown et al, 1957). Further experiments have suggested the same relationship between cosmic radiation and rhythms in Fucus, Uca, carrots, oysters and rats (Brown, 1969). Later investigations showed that the rhythms could be correlated with variations in the atmospheric electromagnetic fields and the Earth's field. This was not unexpected, as the primary cosmic radiation would vary inversely with the strength of the Earth's field (Brown, 1968).

Further studies especially with the changing of the lunar orientation rhythms of Dugesia and Marsarium demonstrated that rhythms could be affected by imposed changes in magnetic field strength (Brown,

1962; Brown et al, 1960a, 1960b, 1960c; Brown and Park, 1965, 1967; Brown et al, 1964). Supporting evidence of positive relationships between circadian rhythms and changes in the magnetic field has been reported for earthworms (Bennet and Huguenin, 1969), gerbils (Stutz, 1972) and humans (Wever, 1967).

A number of assumptions which are difficult to test experimentally have been made by the supporters of exogenous rhythmic control. To explain the experimental flexibility of the phase of a rhythm relative to the natural cycles, it has been suggested that overt rhythms may be maintained in any phase relationship to a basic oscillator, hypothetically driven by external cycles. Since we know neither the nature of the basic oscillating mechanism nor its relationship to overt rhythms, it is at present impossible to refute the hypothesis of exogenous timing on this point alone.

The major difficulty in the exogenous timing concept is the repeated demonstration of free-running periods differing from 24 h. In an attempt to explain these observations, Brown has proposed a mechanism which he terms autophasing. This proposal invokes the observed rhythmic variation in sensitivity to phase resetting by light or temperature signals. It is suggested that the phase of an exogenously controlled rhythm in an organism in constant light will be shifted when the light sensitive part of the cycle is reached. Therefore although the light is constant, its effectiveness in inducing a phase shift is rhythmic as a consequence of the rhythm of the organisms own responsiveness. The same principle would presumably apply for variations of the period from 24 h which depend on the ambient temperature. In the absence of light a rhythmic responsiveness to darkness might be considered responsible. The occurrence of periods both longer and shorter than 24 h may

be explained in this hypothesis, as the phase shift induced when the sensitive part of the driven cycle is stimulated, could be either an advance resulting in shorter periods or a delay resulting in longer periods.

The concept of an exogenous origin of periodicity as proposed by Brown has been subjected to critical examination. Criticism has been made of the evidence on which the theory is based, that of correlation of rhythms with environmental variation. Many of these correlations were obtained by statistical analysis of data averaged over several weeks or months. Enright (1965) has reanalysed 17 sets of such data and claims that none provide convincing evidence for accurate rhythms corresponding to tidal movement (24.8-h), and the presence of accurate diurnal components (24-h) represents a tentative interpretation for only three sets out of the 17. He concludes that from the available data there is insufficient logical basis to uphold the claim for a persistent accurate geophysical component to rhythmicity. Although the influence of changes of magnetic field strength on the rhythms in some organisms has been reported as supporting the exogenous timing hypothesis, this effect is not common to all circadian rhythms. Ditz and Sargent (1974) examined the effect of low strength magnetic fields on the circadian rhythms of Neurospora crassa. No effect was found whether the fields were continuous, pulsed 20 min daily or applied in 12:12 cycles. Therefore, although variation in geophysical factors may exert a small influence on some organisms, no hypothesis or evidence has yet been advanced which convincingly establishes periodic variations in these factors as the driving mechanism for the basic circadian oscillator.

Rhythms which satisfy the conditions to be considered endogenous

occur in all the major groups of plants (Wilkins, 1969) and animals (Harker, 1958). Notable exceptions to the list of organisms possessing circadian rhythms are the prokaryotic cells. Circadian rhythms in bacteria and blue-green algae are unknown with one possible exception. This exception involves the rate of growth of Escherichia coli (Rogers and Greenbank, 1930). Ehret (1960) has advanced the theory that rhythms with periods close to 24 h are common to every eukaryotic cell. Rhythms have not however been found in all such organisms in which they have been sought. Reports have been made of rhythmicity in a particular process occurring in some members of a family of plants but not in others (Ball et al, 1957). Sometimes, of two closely related species, one may possess a persistent rhythm and the other not. A persistent rhythm of spore discharge is found in Pilobolus sphearosporus but not in P. crystallinus (Ubelmesser, 1954). The possibility remains however, in organisms where rhythms have not been detected, that either the right process may not have been monitored, or unsuitable conditions may have been chosen for the investigation.

The selective value of endogenous circadian rhythms is apparent in situations where a 'time-sense' is important to an organism. We can recognise the necessity of many organisms to perform certain functions at specific times of day, both in terms of synchronization with favourable environmental conditions, and in terms of biological synchronization. Thus, many flowers open and offer nectar and pollen to insects at the time of day when the insect vector is most active (Kleber, 1935). The possession of an endogenous circadian timer also allows an element of preparedness in that the organism does not have to wait for a specific environmental stimulus in order to commence a cycle of behaviour or biochemical processes. In this context we can see that the activity of many birds and animals begins before dawn (Bünning, 1973).

Bünning (1960) has further stated that the possession of oscillations in their own right is necessary to keep the cells alternating between extreme physiological states and that if these extreme states are not reached certain physiological processes fail to function. In support of this, Bünning (1973) cites the damage caused to higher plants (Arthur et al, 1930; Arthur and Harvill, 1937) and lower plants (Ruddat, 1960) raised in continuous light. This damage can be relieved by an occasional exposure to darkness or to cycles of high and low temperature (Hillman, 1956) which would initiate or entrain an oscillation.

The possession of a continuing circadian oscillation enables an organism to store 'temporal information' over a period of several days in which there exists no factor capable of initiating a new measuring process. This aspect of biological rhythms is important in the behaviour patterns of some animals although it is probably not of significant value to plants. An example of stored information is the accurate timing of the return of bees to a location at which they had regularly found food at a particular time several days previously (Beling, 1929). Another aspect of the use of circadian rhythms peculiar to animals is that of solar navigation, to determine the correct angle between the direction of the sun and the animal's own locomotion, by compensating for the relative movement of the sun across the sky during the day. There is clear evidence that animals use endogenous rhythms to achieve this compensation (Hoffmann, 1960).

Plants and animals have evolved a whole series of adaptive changes on an annual basis. For example, to survive unfavourable times of the year plants form seeds, tubers, or dormant buds, while vertebrates hibernate, and some insects go into diapause. In contrast, growth and

reproduction occur during those parts of the year when favourable conditions are encountered. Often these responses occur as a result of changes in the day length and Bünning (1936) has proposed that the timing mechanism involved in circadian rhythms also serves as the basis for this photoperiodism. Endogenous circadian rhythms are envisaged as providing a reference against which the length of the day can be measured with respect to the length of the night. Much evidence is available to suggest the involvement of circadian rhythms in photoperiodic responses, particularly the induction of flowering in plants, and of eclosion in insects. However unequivocal evidence for circadian rhythms can only be obtained from experiments using abnormal cycle lengths (Carr, 1952; Engelmann, 1960) or skeleton photoperiods (Hillman, 1964; Minis, 1965; Pittendrigh, 1966). Thus, the true significance of rhythms in photoperiodism under natural conditions, while almost certainly of great importance, has not yet been satisfactorily determined.

Recently the attempt to identify the basic oscillating mechanism has become one of the two most active areas of rhythm research, the second being the investigation of how circadian rhythms are controlled and modified by the external environment. A number of attempts have been made to determine the level of organization at which the oscillation operates. Further attempts have been made to decide between the possibilities of an universal basic oscillator or many oscillating systems. In the case of the latter these might differ from cell to cell, species to species or depend on the degree of complexity of the organism.

In higher animals the overt rhythms are often directed by an organ or tissue spatially separate from that in which the rhythm is manifested. Hormonal or nervous transmission may be shown to mediate

this control. In this sense the integrity of the organism is required for the expression of rhythmicity. However, the organ or tissue ultimately responsible for generating the oscillation can often be shown to oscillate in isolation from the rest of the organism (Bünning, 1973). No particular organisation of tissues seems to be required for the expression of some plant rhythms, since small pieces of mesophyll cut from leaves of Bryophyllum show a rhythm of carbon dioxide emission irrespective of the part of the leaf from which they are taken (Wilkins, 1959). Further it has been shown that rhythms persist in undifferentiated callus cultures of carrot (Enderle, 1951) and Bryophyllum (Wilkins and Holowinsky, 1965). The extent to which an organism can be disrupted without the loss of rhythmicity does depend however on the preservation of the function assayed for rhythmicity.

Populations of unicellular organisms have been demonstrated to be rhythmic (e.g. Pohl, 1948; Ehret, 1959a). Sweeney (1960) has shown the occurence of a persistent rhythm in photosynthetic capacity in single cells of Gonyaulax. This suggests that even in multicellular organisms the basic oscillation may be generated in individual cells.

The possible ~~occurence~~ occurrence of several oscillators in the cell, each responsible for a separate, overt rhythm, has been examined using organisms which exhibit more than one rhythm. If these rhythms are the products of a single oscillator they should retain identical periods and maintain a definite phase relationship to each other. If each is controlled by its own oscillating mechanism, some degree of dissociation might be observed. Such a dissociation has been found in man under artificial cycle lengths of 21 and 27 h. The rhythm of body temperature is immediately entrained to these cycle lengths while the K^+ excretion rhythm initially maintains a 24-h period (Loban, 1965). Further, in

some humans under constant conditions, body temperature and activity rhythms show different periods (Aschoff et al, 1967). However, the organs and tissues controlling these functions in man are relatively independent and may also differ in their response to external conditions. Thus, studies of rhythmic desynchronisation in higher animals do not permit a conclusion to be drawn on the number of basic oscillators involved at the cellular level. McMurray and Hastings (1972) have shown with Gonyaulax that the phase relationship among the circadian rhythms of photosynthetic capacity, glow, cell division, and luminescence capacity remain unchanged during several weeks under constant conditions. Further, an exposure to a phase shifting treatment alters the phase of all the rhythms to an equal extent. While this absence of desynchronisation suggests the ~~occurrence~~ occurrence of only one oscillator, it is still impossible from these results to discount the presence of several oscillators possessing precisely the same period and response to external stimuli.

Some fifteen rhythmic phenomena, including the rhythmic sensitivity to flower induction and the activity of several key enzymes, have been observed in Chenopodium rubrum, and are summarized by Wagner et al, (1974). Periods ranging from 12 to 30 h often with regular, more frequent subpeaks of actitity have been recorded. Thus, a number of rhythmic processes appear to operate simultaneously in cells but with quite different periods. Chia-Looi and Cumming (1972) have also studied circadian rhythms in Chenopodium and report different periods for several rhythms recorded in similar conditions. In the latter case, however, some doubt must be cast on the accuracy of the period estimation, from data displaying large arhythmic variation. On the basis of the results obtained with Chenopodium it appears that more than one oscillator may be involved. However, Wagner et al (1974) attribute the

occurrence of a number of periods to a common, high frequency oscillation subject to different degrees of frequency demultiplication. Our present lack of knowledge of the nature of the oscillator prevents us from confirming or denying this possibility.

In Drosophila, while there is no evidence for desynchronisation of different overt rhythms (Konopka and Benzer, 1971), the response of the eclosion rhythm to phase shifting treatments has lead Pittendrigh et al (1958) to propose that two distinct oscillating systems underly the overt rhythm. Following a light treatment a number of transients occur before a different stable phase is established. Following a short temperature pulse, a temporary derangement of the rhythm occurs and transients which follow lead to the rhythm reverting almost to the initial phase. This is explained by Pittendrigh et al (1958) as representing an ultimate oscillator, termed A, which is sensitive to light but not to temperature, and a second oscillator B which is coupled to and driven by A, and is light insensitive but temperature sensitive. The overt rhythm follows oscillator B. After a light signal the phase of A is immediately reset by the signal but it takes several cycles before B is reentrained by A and transients occur. After a temperature pulse, the B oscillator is shifted, but ultimately the temperature insensitive pacemaker A regains control of B, and transients occur as B reverts to the phase of A. Further critical evidence in favour of this hypothesis was obtained from experiments with two light pulses (Pittendrigh and Minis, 1964). Following the first light pulse, the overt rhythm passes through transients with periods longer or shorter than the free-running period. The steady state phase shift induced by a second light pulse is precisely that expected if the oscillator was immediately reset by the first pulse, rather than dependent on the phase of the transient cycles at which the second pulse is applied.

Evidence in support of the coupled oscillator theory may also be inferred from the results of investigations with higher plants. Halaban (1968b) has been able to explain, by the coupled oscillator hypothesis, the results on the leaf movement rhythm of exposing Coleus seedlings to two light pulses, or a light pulse followed by a temperature pulse. With Coleus, a temperature decrease of 7°C for 8 h caused only transient phase shifts and was presumed to influence only the B oscillator. A temperature decrease of 11°C for 10 h appeared also to shift the phase of the A oscillator. The nature of neither the A nor B oscillators postulated in this hypothesis is known nor is the relationship between them.

Thus no unequivocal evidence is available which reveals the complexity of the oscillating mechanism or the possible diversity of oscillators. A number of hypotheses have been formulated for the mechanism of a basic oscillator. In each case the common features of circadian rhythms and their almost certain location at the cellular level, has prompted the proposal for a single universal oscillating mechanism. Because of its central role in cell metabolism, the nucleus has been suggested as the site of the basic oscillator, and changes in the rate or pattern of RNA or protein synthesis as the primary mechanism. This is the view put forward in the 'chronon concept' of Ehret and Trucco (1967). This concept holds that circadian rhythms result from cycling of a sequential nature that regulates the transcription of RNA from DNA. The chronon is envisaged as a large polycistronic complex of DNA whose transcription rate is limited by some function of eukaryotic organisms that is relatively temperature independent. It is supposed that each eukaryotic cell contains hundreds of chronons on each of its nuclear chromosomes and many sets of extra nuclear chronons in its cell organelles. It is envisaged that RNA transcription proceeds unidirect-

ionally from an initiator cistron to a terminator cistron. The eventual products of translation of the message of the terminator cistron cause an initiator substance to accumulate. When this arrives at the initiator cistron the system proceeds to its next ciradian cycle.

Evidence of a nuclear origin for the mechanism of circadian rhythms comes from the effectiveness of those wavelengths which are absorbed by nucleic acids. These wavelengths reset the phase of the mating rhythms of Paramecium (Ehret, 1959b) and the luminescence rhythm of Gonyaulax (Sweeney, 1963). UV radiation of similar wavelength does not however shift the phase of the carbon dioxide emission rhythm of Bryophyllum (Wilkins, 1973). The involvement of transcription in circadian rhythms is not supported by the observation of a circadian rhythm of oxygen uptake in dry onion seeds (Bryant, 1972), since DNA replication, transcription, and perhaps translation do not occur in this quiescent state (Weeks and Marcus, 1971).

The possible role of the nucleus and its associated processes in the control of circadian rhythms has been tested by enucleation of the giant single-celled alga Acetabularia. It has been found that the rhythm in photosynthetic capacity continues in normal and enucleate cells in a similar manner (Sweeney and Haxo, 1961), and further that the phase of the rhythm is shifted by light in both the presence and absence of the nucleus. This implies that the basic oscillating system is not located in the nucleus. However, transplanting the nuclei of cells which have been grown in cycles of light and darkness differing by 12 h and transferring the cells to constant conditions reveals that the cycles experienced by the nucleus predominate in phase determination (Schweiger and Schweiger, 1965). The continuation of the rhythm in the absence of the nucleus does not rule out the involvement of nucleic acids in the oscillating mechanism. Chloroplasts isolated from

Acetabularia contain considerable amounts of DNA (Gibor and Izawa, 1963) and direct the formation of RNA (Schweiger and Berger, 1964). The synthesis of RNA in enucleated cells of Acetabularia has been clearly demonstrated (Schweiger and Bremer, 1961), and permits an explanation, in terms of the chronon concept, for the continuation of rhythmicity in anucleate cells.

If the oscillator does indeed proceed by sequential transcription we might expect to find rhythmicity in nucleic acid metabolism or in protein synthesis, the initial result of translation. Rhythmic incorporation of labelled amino acids into protein has been reported in Euglena under conditions in which the rhythm of phototaxis persists (Feldman, 1968). Rückebeil (1961) showed that the incorporation of ^{32}P into RNA varies rhythmically in some organisms such as Phaseolus but that this is not the general rule. No rhythmic differences in ^{32}P incorporation into RNA in populations of Gonyaulax could be detected by Hastings (1960). The apparent absence of rhythmic RNA synthesis does not completely rule out a central role for the transcription process, since only a small fraction of RNA might be involved in rhythmicity. Any change in a small RNA fraction might be masked by changes in the rate of synthesis of the larger nonrhythmic fraction.

Rhythmic activity of the enyzme luciferase appears to be partly responsible for the luminescence rhythm of Gonyaulax (Hastings and Keynan, 1965). Variations in the extractable activity of this enzyme are thought to result from changes in the amount of this enzyme present in the cells. In contrast, the rhythm of photosynthesis in Acetabularia cannot be attributed to changes in the activity or level of ribulose ~~diphospho~~diphosphate carboxylase (EC 4.1.1.39), the enzyme responsible for carbon dioxide fixation, nor to the activities of eight other enzymes closely

associated with the photosynthetic process. Thus a simple, direct link between enzyme synthesis and the overt rhythm can be ruled out. However, control could be exerted by the rhythmic synthesis of enzymes which catalyse reactions biochemically quite different from, but ultimately coupled to, those directly involved in the overt rhythm.

If the chronon concept is to be considered as valid, inhibitors of protein and RNA synthesis should be shown to influence the oscillation. One of the striking features of circadian rhythms is their insensitivity to inhibitors of these processess. Actinomycin D which is thought to block DNA-dependent RNA synthesis (Reich and Goldberg, 1964) inhibits the glow rhythm in Gonyaulax but not the photosynthetic rhythm (Karakashian and Hastings, 1962). The abolition of an overt rhythm by an inhibitor, however, is difficult to interpret and may not represent the inhibition of the basic oscillator. Inhibition of the overt rhythmic process or a breakdown of the mechanism linking the oscillator and the overt rhythm might occur while the oscillator continues to function normally. Convincing evidence from experiments with inhibitors would require either a change in the period of a rhythm in the presence of the inhibitor or a phase shift induced by a brief, reversible exposure to the inhibitor. Karakashian and Hastings (1963) applied pulse type treatments with inhibitors of nucleic acid and protein synthesis at various phases of the circadian cycle in Gonyaulax but could not find any significant phase shifts.

Strumwasser (1965) has shown that the rythmic firing of a single neuron of Aplysia can be induced to start earlier by the addition of actinomycin D. This compound is also effective in lengthening the period of the rhythmic exudation of sap from decapitated tobacco plants when present in the nutrient solution surrounding the roots (MacDowell, 1964).

Cycloheximide, an inhibitor of protein synthesis, has been observed to lengthen the period of rhythms in Euglena (Feldman, 1967). In contrast, the phase and period of the photosynthetic capacity rhythm of Acetabularia are unaffected by high concentrations of actinomycin D, puromycin and chloramphenicol which markedly reduce RNA and protein synthesis (Sweeney et al, 1967). Although these latter results at first sight suggest that rhythmicity is independent of RNA and protein synthesis the authors found that a considerable fraction of RNA synthesis appeared to be unaffected by actinomycin D. As only a small fraction of the total nucleic acid and protein synthesis might be involved in the circadian oscillation, this might remain unaffected by the inhibitor. Thus, while there is clear evidence for an influence of the nucleus and of RNA and protein synthesis on the operation of circadian rhythms in some organisms, it remains unclear whether the nucleus or processes involving the nucleic acids are the principal or only source of rhythmicity.

The structure and function of membranes, common to all eukaryotic cells has led to the formulation of several models which attempt to explain the generation of circadian rhythms as a membrane function. Njus et al (1974) have proposed a feedback oscillator based on ion gradients and the membrane bound ion transporting elements envisaged in the fluid mosaic model of cellular membranes (Singer and Nicolson, 1972). It is postulated that the proteins which make up the ion transporting channels respond to changes in concentration gradients of specific ions by grouping to form such channels when the gradient is small, and dispersing to a non-transporting mode when the gradient is large. A reduction in the magnitude of the gradient by passive transport is assumed, which then completes the feedback pathway and oscillation recommences. The activity changes in the membrane protein, brought about by the circadian ion fluxes, could involve either synthesis and degradation or activation and

inhibition. Since it is known that protein synthesis is not always required for the operation of the circadian clock, Njus et al (1974) favour activation and inhibition of transport proteins as being responsible.

To account for the observed effects of light on rhythms, Njus et al propose that light acts by perturbing ion gradients across membranes. In many organisms the photoreceptors would be closely coupled to the oscillator and would operate directly as photosensitive ion gates. In other organisms hormones would mediate between specialized photoreceptor cells and those cells responsible for overt rhythmicity. Consequently the coupling hormone would act by inducing a permeability change in its target membrane. A light pulse, decreasing the gradient, would have one of two effects. Applied at a certain time when the membrane is in the active mode, it would cause a phase delay because the ion accumulation would need to be repeated before the maximum concentration was reached. If applied while the membrane was in the passive mode however, a light pulse would be expected to advance the phase of the rhythm by increasing the rate of disappearance of the ion gradient.

Both the observed effects of temperature on the rhythm, and the high degree of temperature compensation, may result from changes in the membrane lipids. According to the fluid mosaic concept of membrane structure, proteins are intercalated into the lipid bilayer, and can move in the plane of the membrane through the fluid lipid matrix. Therefore the kinetics of the grouping of proteins to form ion transporting channels in response to changes in ion gradients would depend on the fluidity of the membrane lipids. This fluidity may be controlled by lipid adaptation, and be relatively independent of temperature (Barańska and Wlodawer, 1969). Lipid adaptation also compensates for temperature, the rate of passive ion diffusion through the lipid bilayer (Haest et al,

1969), which constitutes one phase of the model oscillator. The resetting of the phase of rhythms by temperature pulses or steps, of only a few degrees, may result from the effect of temperature on ion gradients through an incomplete temperature compensation of membrane lipid fluidity. In the case of more extreme temperatures these may fall outside the range over which compensation is possible.

The evidence in favour of the membrane-ion gradient model of circadian oscillation is largely circumstantial, since direct measurements of the changes in the transporting properties of the membranes during the circadian cycle have not been made. Some rhythmic processes can be readily attributed to the movement of ions. Satter and Galston (1971a) showed that Albizzia leaflets close when potassium ions move into the dorsal and out of the ventral pulvinule motor cells, and open when the flux is reversed. This is true whether leaflet movement is controlled by an endogenous rhythm or by phytochrome. The movement of potassium ions across the pulvinal membranes during the daily cycle has also been reported in Trifolium (Scott and Gulline, 1975) and Samanea (Satter et al, 1974). Changes in the osmotic potential of the upper epidermis of Kalanchoë petals have been found which correspond to the rhythm of opening and closing of the flowers (Schrempf, 1975). These changes are associated with the rhythmic movement of potassium and sodium ions between the upper epidermis and the rest of the petal, although no change in the calcium ion concentration was found.

Sweeney (1974) has reported a circadian rhythm in the intracellular level of potassium in Gonyaulax polyedra. In this organism, exposure to a low concentration of ethanol causes phase shifts, the magnitude and direction of which are dependent on when in the cycle cells are treated. The phase response curve obtained with ethanol treatment resembles that for light. Ethanol may act by changing the ionic permeability of bio-

logical membranes (Gutknecht and Tosteson, 1970) and, in Gonyaulax, ethanol treatments which cause phase shifts were shown to result in up to a 50% decrease in the intracellular level of potassium (Sweeney, 1974). Ethanol also induces phase shifts in the leaf movement rhythm of Phaseolus seedlings (Bünning and Baltes, 1962), and lengthens the free-running period of this rhythm (Keller, 1960).

Valinomycin, a chemical which is thought to influence membrane permeability (Willert, 1972), induces phase shifts in the Phaseolus leaf movement rhythm when applied through the transpiration stream (Bünning and Moser, 1972). When applied in ethanol, valinomycin prevents the effect of ethanol alone on the phase of the circadian rhythm and the potassium content of Gonyaulax (Sweeney, 1974). However, valinomycin given either as a pulse or continuously had no effect on the petal movement rhythm of Kalanchoë (Schrempf, 1975).

Heavy water (D_2O) is another chemical which may influence membrane permeability or the ion balance across cellular membranes. This induces phase shifts and lengthens the period of the rhythm in Phaseolus (Bünning and Baltes, 1963), and lengthens the period of the rhythms in Euglena (Bruce and Pittendrigh, 1960), Excirolana (Enright, 1971) and deer mice (Sutter and Rawson, 1968). A cyclic change in the physical properties of cellular membranes has also been suggested by Wagner and Cumming (1970) to account for the rhythmic leakage into the incubating medium of betacyanin from seedlings of Chenopodium.

If changes in ion gradients across membranes are central to the oscillating mechanisms, an exogenous supply of ions might be expected to modify these gradients and hence alter the course of the rhythm. The rhythmic firing of the optic nerve of the excised eye of Aplysia can be

phase shifted by higher than normal concentrations of potassium ions (Eskin, 1972). Increasing the external concentration of potassium or sodium ions had no effect on the rhythm of stimulated luminescence in Gonyaulax (Sweeney, 1974). Temporary or prolonged incubation on a solution containing potassium ions did not significantly influence either the phase or the period of the Kalanchoë petal movement rhythm (Schrempf, 1975). This circadian rhythm did however show a longer period under the continuous influence of lithium ions, but a pulse of up to 12 h had no effect on the phase of the rhythm. Lithium ions were also demonstrated to slow down the circadian activity rhythm of a mammal Meriones crassus (Engelmann, 1973). Lithium ions may themselves influence membrane permeability (Bose and Lowenstein, 1971) but the significance of these results in terms of the mechanism of oscillation is not clear, since it is reported that lithium may also effect nucleic acid synthesis (Volm et al, 1970).

Wagner and Cumming (1970) and Wagner et al (1974) also conclude that membranes are the primary site of regulation of endogenous rhythms and are responsible for the perception and amplification of internal and external stimuli. However, unlike the model of Njus et al (1974) which supposes a feedback oscillator involving ion gradients and membrane function, Wagner and co-workers consider that endogenous rhythmicity originates from high frequency oscillations. These oscillations, in the enzymes associated with energy metabolism, have periods ranging from less than one minute to several minutes. Such high frequency oscillations have been demonstrated for the enzyme activities involved in yeast glycolosis (Betz and Chance, 1965a, 1965b; Pye, 1969), for several other enzyme systems (Hess and Boiteux, 1971) and for hydrogen and potassium ion fluxes from isolated mitochondria (Chance and Yoshike, 1966). The short periods of these oscillations are difficult to reconcile

with the 24-h periods of circadian rhythms. If high frequency oscillations do in fact underly circadian rhythms, some frequency demultiplication process must clearly operate. One approach to this problem considers the oscillator to be a biochemical network of self sustaining oscillators coupled so as to give negative feedback inhibition. Individual oscillations thus become synchronized at a much lower than normal frequency (Pavlidis, 1969; Pavlidis and Kaufmann, 1969; Pye, 1969). Spatial separation might also reduce the frequency in a system of coupled, high frequency oscillators. The model proposed by Wagner suggests that rhythms arise by the coupling of high frequency oscillations in the pathways of energy production and utilization in separate compartments within the cell. These coupled oscillators are linked to the induction of structural changes in the membranes of the intracellular compartments, and hence regulate the pool sizes and availability of intermediates and co-factors for the separate pathways. Endogenous rhythms with circadian periods might then be generated in the overt reaction of these distinct pathways. It is assumed that circadian rhythms in growth and behaviour results from those in energy metabolism (Cumming and Wagner, 1968; Frosch and Wagner, 1973a, 1973b; Frosch et al, 1973; Wagner et al, 1974).

The occurrence of non-circadian rhythms, and of circadian rhythms which also show underlying oscillations of higher frequency, as revealed by sub-peaks in the activity of certain enzymes of energy metabolism, may be considered as evidence in favour of the involvement of high frequency oscillations (Wagner et al, 1974). The involvement of compartmentation in rhythmicity is supported by the fact that there are rhythmic changes in the ultrastructure of chloroplasts and mitochondria in daily light-dark cycles (Heber, 1969; Könitz, 1965; Schor et al, 1970). Within the same cell, mitochondria swell and chloroplasts con-

tract upon irradiation while the reverse occurs in darkness (Murakami and Packer, 1970; Packer et al, 1970). Furthermore there is evidence that endogenous rhythms in chloroplast swelling and contraction are a structural corollary of oscillations in enzyme activity (Busch, 1953; Driessche, 1966). However, while these changes suggest that rhythmic enzyme activities can occur in co-operation in different cell organelles and support the involvement of compartmentation, they do not confirm the hypothesis of Wagner et al (1974), and might equally result from membrane control of ion gradients or represent merely a secondary response to some quite distinct oscillating mechanism.

Investigation of the environmental control of circadian rhythms has been concentrated primarily on the effects of radiant energy, since this appears to be the principal synchronizing agent under natural conditions. A number of attempts have been made to elucidate the pigments responsible for the initial photoreception. The identification of photoreceptor pigments is usually achieved by determining action spectra for a defined response of a circadian rhythm to light. In an ideal system the action spectrum for a reaction is directly related to the absorption spectrum of the participating photoreceptor although inactive pigments often interfere with absorption. In addition to the few detailed action spectra which have been obtained for photocontrol of circadian rhythms, there are a number of reports of the effects of broad spectral bands. Radiant energy can be involved in the initiation of a rhythm by transfer from light to dark or vice versa, in the entrainment of the rhythm to light-dark cycles, in phase shifting the rhythm with a single exposure, and in influencing the free-running period of the rhythm. In many investigations only one of these responses has been studied, although in a few organisms two or more responses have been examined for spectral dependence.

In many animals resetting of the circadian oscillation by light signals is accomplished indirectly via hormones or the nervous system. In higher animals the perception of light is generally by the eyes so that in the absence of eyes or with the eyes covered a rhythm will persist with a free-running period differing from the 24-h period of the natural light-dark cycle (Bünning, 1973). Synchronization without eyes might occur in some mammals however, since light can penetrate into the brain (Brunt et al, 1964). Photoperiodic responses in mammals which presumably depend on the interaction of light-dark cycles and a circadian rhythm are indeed often independent of the presence of eyes (Bünning, 1973). In insects as well as vertebrates, light effects other than via the eyes are known. Light-dark cycles are effective in entraining the rhythm of an eyeless mutant of Drosophila melanogaster (Engelmann and Honegger, 1966).

Frank and Zimmerman (1969) have determined action spectra for light induced phase shifts of the circadian rhythm of adult emergence in Drosophila pseudoobscura. The action spectra for both advancing and delaying the phase of the rhythm are similar, with maximum activity between 420 and 480 nm and a sharp cut-off above 500 nm. The initiation of the circadian rhythm of egg hatching in the moth Pectinophora gossypiella by a brief light pulse has an action spectrum similar to that for phase shifting the Drosophila rhythm (Bruce and Minis, 1969). These regions of the spectrum are also responsible for control of the photoperiodic diapause of Pieris brassicae which appears to involve an endogenous oscillator. The extension of short days or the interuption of a long night inhibits diapause, and only wavelengths below 550 nm are effective (Bünning and Jorrens, 1960).

In the fungi also, photoresponses are usually dependent on the

blue region of the spectrum. The expression of a circadian rhythm of conidiation by a strain of Neurospora crassa is inhibited by growth in continuous white light. The action spectrum for this effect has a large peak with minor sub-peaks in the blue region of the visible spectrum and a broad shoulder in the near UV (Sargent and Briggs, 1967). Muñoz and Butler (1975) conclude that a flavin is the photoreceptor involved in this response and that the absorption of light by this pigment is also responsible for the less precise action spectra found for Drosophila (Frank and Zimmerman, 1969) and Pectinophora (Bruce and Minis, 1969). The influence of different wavelengths on the sporulation and mitosis rhythms of the alga Oedogonium have been investigated by Bühnemann (1955c) with the conclusion that in this organism also, wavelengths below 550 nm are those principally involved in photocontrol.

In unicellular organisms light clearly exerts its effect directly on the cells in which the oscillation is occurring. The action spectrum for phase shifting the luminescence rhythm in Gonyaulax has a major peak in the blue region of the spectrum at 475 nm and a minor peak in the red region at 650 nm (Hastings and Sweeney, 1960). The peaks of this action spectrum do not coincide exactly with the absorption spectrum of any one of the pigments in Gonyaulax but correspond roughly to the total absorption of the cell which principally reflects the pigments involved in photosynthesis (Sweeney, 1969). The rhythm of mating capacity of Paramecium bursaria is shifted by light and the action spectrum for this response shows greatest activity in the red (600 - 700 nm) region of the spectrum with peaks also occurring at 440 nm and in the near UV (< 380 nm) (Ehret, 1960). However the author considers that the action spectrum is too poorly defined to implicate any particular group of pigments.

Both Gonyaulax (Sweeney, 1963) and Paramecium (Ehret, 1959b)

show stable phase shifts in response to UV radiation, principally at 254 nm. In contrast to the relatively long exposure to visible light required to shift the phase, an exposure to UV of only a few minutes is effective. The sensitivity to resetting by UV varies cyclically, as does the response to visible light. However, whereas visible light causes both phase advances and delays, depending on the circadian time of application, UV radiation induces only phase delays in Paramecium and only phase advances in Gonyaulax. In Paramecium, but not Gonyaulax, visible light appears to reverse the phase shift induced by exposure to UV (Ehret, 1959b). It seems certain that the effects of UV are essentially different from those of visible light. The involvement of nucleic acid metabolism is suggested since nucleic acids absorb radiant energy in the UV region of the spectrum and some UV effects such as chromosome breakage are photoreversible by visible radiation (Jagger, 1958).

In higher plants the red end of the visible spectrum is commonly found to be the most active in mediating the photoresponses of circadian rhythms. In some higher plants, however, both red and blue regions of the spectrum are effective, and in a minority of plants only blue light is active. Greater complexity in some higher plants can be seen from the fact that different aspects of the photocontrol of circadian rhythms appear to involve different pigments as primary photoreceptors.

Possibly the most conclusive demonstration of the nature of the photoreceptor comes from studies of entrainment of the carbon dioxide output rhythm of Lemna perpusilla (Hillman, 1971), although an action spectrum has not been determined for this response. A transfer from light to darkness initiates a rhythm in the rate of carbon dioxide output by axenic cultures of Lemna which rapidly damps out after one or

two cycles. If cultures, otherwise maintained in darkness, receive 15 - min pulses of red or far-red radiation at 8-h intervals the carbon dioxide output is arhythmic. An entrained rhythm becomes apparent however if cultures are exposed to only one pulse of red or far-red radiation, or to two pulses 8 h apart, in each 24 - h cycle. Far-red reversal of the action of red radiation, characteristic of the involvement of phytochrome, can be observed. The carbon dioxide output, normally arhythmic when cultures receive pulses of far-red radiation at 8-h intervals, becomes rhythmic if one of the pulses is immediately followed by an exposure to red radiation and is entrained in a definite phase relationship to that pulse. If the red pulse preceeds the far-red treatment however, carbon dioxide output remains arythmic, showing that far-red radiation reverses the effect of red. Blue light is active and acts like red in schedules consisting of one pulse of blue and two pulses of far-red arranged so that the three pulses are given at 8-h intervals. On the other hand blue light acts like far-red when one pulse of blue is accompanied by two pulses of red light at 8-h intervals in each 24-h cycle. It was concluded from these studies that a regularly imposed change in the level of the far-red absorbing form of phytochrome (P_{fr}) can entrain the carbon dioxide output rhythm, the principal synchronizer being the sharpest transition from a low to a high level of P_{fr} that occurs with a periodicity of 24 h. Hillman (1971) attributes the effects of blue as well as those of red and far-red radiation to phytochrome.

The rhythm of carbon dioxide output in leaves of Bryophyllum fedtschenkoi is inhibited by high flux densities of radiation containing wavelengths longer than 565 nm (Wilkins, 1960a). The phase of this rhythm is shifted by red but not by blue light (Wilkins, 1960a). More recently an action spectrum has been determined for the inhibi-

of phase shifts in the Bryophyllum rhythm by visible radiation (Wilkins, 1973). This shows only one activity peak, located between 600 and 700 nm. The importance of the red region of the spectrum in shifting the phase of the circadian rhythm in Bryophyllum was confirmed by Jones (1973) in experiments with the rhythm in carbon dioxide compensation. This persists at high light intensities and can be phase-shifted by a 5-h exposure to darkness or to light in several spectral bands. While all spectral bands tested induced a phase shift, red light had markedly less effect than blue or green light. The smaller effect of red light in this case indicates it to be the most like continuous white light and hence most active in photocontrol. The phase of the rhythm in Bryophyllum leaves, unlike that of rhythms in Gonyaulax (Sweeney, 1963) or Paramecium (Ehret, 1959b), is unaffected by UV radiation at 254 nm (Wilkins, 1973).

The rhythmic petal movement of Kalanchoë blossfeldiana flowers fades out after several days in continuous light or darkness, but can be reinitiated by transferring the flowers from one continuous condition to the other (Engelmann, 1960). On the basis of action spectra for the induction of this rhythm, which show peaks in both the red and blue regions of the spectrum, Karvé et al (1961) suggested that light absorbed by chlorophyll mediates this response. The general tendency of the flowers to close their corollas when continuously irradiated result in marked effects on the amplitude but not the phase or periods of the rhythm. This effect is induced principally by red light and can be reduced by a simultaneous exposure to far-red radiation, suggesting phytochrome to be the photoreceptor. An action spectrum for the induction of phase shifts has been obtained which is quite different from that for the initiation of the Kalanchoë rhythm (Schrempf, 1975). A peak of activity is found in the 600-700 nm region and closely resembles that found for Bryophyllum (Wilkins, 1973). A second

peak occurs in the near UV (300-380 nm); this region of the spectrum being appreciably more active than the red region. Schrempf (1975) suggested that phytochrome and possibly one other pigment are responsible for phase shifting in Kalanchoë although no red/far-red reversibility could be detected in a variety of schedules where red and far-red radiation were given sequentially or together.

The effect of different spectral bands on the rhythm of leaf movement in Phaseolus multiflorus seedlings has been studied in considerable detail particularly by Lörcher (1958). Photocontrol of this rhythm appears to be rather complex. The period of the rhythm is lengthened by radiant energy from fluorescent lamps which is rich in red but deficient in far-red radiation, and is shortened by radiant energy from tungsten lamps which is rich in both red and far-red radiation (Lörcher, 1958). Using broad band transmission filters Lörcher (1958) confirmed the lengthening effect of red and the shortening effect of far-red radiation on the period. The leaf movement rhythm of Phaseolus seedlings which had, prior to the experiment, been maintained in a greenhouse under the natural light regime, was entrained to a 25-h period by cycles consisting of 10 h red or far-red radiation and 12 h of darkness. When exposed to 22-h cycles of 10 h of blue or green light alternating with 12 h of darkness the rhythm displayed a period of 26 h. As this is also the period of the free-running rhythm in continuous darkness it is clear that blue and green light are ineffective in mediating this photoresponse. If plants were grown in darkness before the experiments, a rhythm was induced by a single 12-h exposure to red or blue, but not far-red radiation. In similar plants rhythmicity can be observed in light-dark cycles with red, blue or far-red radiation but only persists in subsequent darkness after cycles including red or blue light.

Some evidence has been obtained for the interaction of red and far-red radiation in control of the Phaseolus leaf movement rhythm. Plants transferred from continuous darkness to red light show a circadian rhythm. However, no rhythm can be detected if they are simultaneously exposed to far-red radiation. The induction of a rhythm by a single 12-h exposure to red light can be reversed by a subsequent 12-h exposure to far-red radiation. A further 12 h of red light reinitiates the rhythm, an effect which can again be reversed by 12 h of far-red radiation. The initiation of a rhythm in response to a 12 or 24-h exposure to red light occurs however, irrespective of whether the plants receive simultaneous exposure to far-red radiation. 6 h but not 1 h of red light are sufficient to initiate a rhythm, and 1 h of far-red radiation reverses the effect of 6 h of red. A further 1-h exposure to red light is sufficient to reinitiate the rhythm. The red/far-red interaction observed in several of these responses led to the suggestion that phytochrome is involved in control of the phase and period of the rhythm (Lörcher, 1958). A more complicated interaction became apparent however, with the observations of Bünning and Moser (1966) that although both red and far-red radiation induce phase shifts, the phase response curves for the two spectral bands are quite different. Exposure to red light results in a phase response curve identical to that obtained with white light, whereas far-red radiation induces phase advances but no phase delays. Furthermore, while the red light effects can be induced by irradiating only the pulvinus, far-red radiation is virtually without effect when applied to the pulvinus and acts only when perceived by the leaf blade. Thus it seems that red and far-red radiation may have very different effects. While phytochrome may be involved in the photocontrol of the Phaseolus rhythm, caution should be exercised in interpreting the red/far-red reversibility effects as confirming this.

An investigation of the leaf movement rhythm of Coleus blumeii X C. frederici led Halaban (1969) to propose that more than one unidentified pigment is responsible for photocontrol. In continuous blue light the period is significantly lengthened, while under continuous red light the period is shortened, compared with that in darkness or in constant green or far-red radiation. An 8-h exposure to red light is effective in advancing the phase of the rhythm in Coleus plants in continuous green light, at times in the cycle when an exposure to white light also induces a phase advance. Red light is ineffective however, when applied at circadian times at which white light induces phase delays. In contrast, an exposure to blue light induces phase delays but not phase advances. Far-red radiation produces neither phase advances nor delays.

Two other, less detailed studies have been reported which show the effects of different regions of the spectrum on leaf movement rhythms. Karvé and Jigajinni (1966b) showed that cycles of either red or blue light and darkness entrain the rhythm of Portulaca grandiflora. A longer exposure to red than to blue light is necessary in order to achieve entrainment with skeleton photoperiods in which red or blue light pulses mark the beginning and end of the complete photoperiod. Holdsworth (1960) found that with Bauhinia monandra in LD 12:12 only red light has an effect similar to that of white light when used to shorten the dark period. In view of the results obtained with Coleus and Phaseolus, it seems possible that these latter results do not represent a complete analysis of photocontrol of rhythms in the respective organisms.

There is considerable evidence for the involvement of both circadian rhythms and phytochrome in the photoperiodic induction of flowering in higher plants. This has led a number of investigators

to enquire whether radiant energy absorbed by phytochrome controls the entrainment of the circadian rhythm of flower induction or whether it merely interacts with the rhythm to promote or inhibit flowering (Takimoto and Hamner, 1965a, 1965b; Salisbury, 1965; Papenfuss and Salisbury, 1967; Denney and Salisbury, 1970; King, 1974a, 1974b). The results of many of these investigations are open to a variety of interpretations, including the possibility of a non-rhythmic 'hour-glass' component of photoperiodic timing (King, 1974a). While Papenfuss and Salisbury (1967) concluded that phytochrome may influence only the phase of the clock and not other aspects of flowering, a quite different conclusion has been reached by Hamner and Hoshizaki (1974). These authors state that phytochrome is not involved in the entrainment or rephasing of basic circadian rhythms. The latter conclusion is rather surprising in view of the unequivocal demonstration of phytochrome control of a circadian rhythm in Lemna (Hillman, 1971). On the basis of evidence presently available, a dual role of radiant energy in photoperiodism appears likely, although phytochrome may be involved as the photoreceptor in both processes. Irradiation treatments which clearly promote or inhibit flowering may be of insufficient duration to phase-shift the circadian rhythm of flowering capacity (Takimoto and Hamner, 1965a, 1965b; King, 1974a). A similar dual action of radiant energy is seen in the germination of Sphaerocarpus donelli spores (Steiner, 1969). Phytochrome is held to be responsible for inducing both germination and the rhythm in sensitivity to germination promotion. Two distinct responses are implicated however, by the fact that the rhythm can be induced with intensities of red light which are insufficient to induce germination.

Thus, three conclusions can be drawn concerning the photocontrol of circadian rhythms. Despite the general similarity of rhythms in all organisms, different pigments are clearly involved in photocontrol in

different organisms. More than one pigment may be involved in one organism, mediating different aspects of the photocontrol of a rhythm, for example the initiation of the rhythm and phase or period control. Finally, it appears that more than one photoreceptor may operate in the control of one aspect of a circadian rhythm, especially phase shifting, the particular pigment involved depending on the circadian time at which the organism is irradiated. In very few organisms have each of these aspects of the photocontrol of rhythms been studied. In view of this it was decided to investigate in detail the photocontrol of one circadian rhythm, that of the rate of carbon dioxide output from leaves of Bryophyllum fedtschenkoi.

This rhythm has been studied thoroughly by monitoring, with an infra-red gas analyser, the release of carbon dioxide into a stream of carbon-dioxide-free air (Wilkins, 1959, 1960a, 1960b, 1962a, 1962b, 1965, 1967, 1973; Warren and Wilkins, 1961). Rhythmic carbon dioxide output can also be observed in leaves in a stream of normal air but fades out more rapidly than in leaves in carbon-dioxide-free air (Wilkins, 1959). On the basis of the criteria first proposed by Pittendrigh (1954) it has been established that the origin of this rhythm is endogenous. The rhythm continues in constant darkness at a constant temperature and no correlation can be found between the rhythmicity of carbon dioxide emission and changes in uncontrolled environmental variables such as barometric pressure (Wilkins, 1959). The phase of the rhythm in darkened leaves can be shifted by a few hours exposure to light. Similarly, the rhythm persisting in irradiated leaves can be shifted by a few hours darkness. The new phase established by such treatments is maintained when the leaves are returned to the original constant conditions (Wilkins, 1960a). Although no rhythm is apparent in leaves exposed to high light intensities, a single change from light to dark will initiate a rhythm. The phase of a

rhythm thus initiated is dependent on the time of transfer but independent of the sidereal time (Wilkins, 1960a). Temporarily replacing the carbon-dioxide-free air with nitrogen at certain times in the circadian cycle delays the phase of the rhythm (Wilkins, 1967). Finally, the most important confirmation of the endogenous nature of the rhythm is the fact that the period of the rhythm in leaves in constant darkness depends on the ambient temperature, showing a small but significant decrease with increasing temperature (Wilkins, 1962b).

Rhythms of carbon dioxide exchange have been observed in a number of other species of succulent plants. Schmitz (1951) demonstrated a rhythm of carbon dioxide output in excised leaves of Kalanchoë blossfeldiana in darkness and in an atmosphere initially free of carbon dioxide. Gregory et al (1954) did not detect a rhythm in the rate of carbon dioxide output of whole plants of K. blossfeldiana in darkness and in a stream of normal air. The ~~occurence~~ occurrence of a rhythm in this plant was however confirmed by Nuernbergk (1961) with continuously irradiated plants in normal air, and more recently by Queiroz (1970) in continuous light or darkness. Nuernbergk (1961) also reported circadian rhythms of carbon dioxide exchange in Bryophyllum tubiflorum, B. daigremontianum and Aloë arborescens. In a survey of the ~~occurence~~ occurrence of such rhythms in 16 species of succulent plants Wilkins (1959) confirmed their presence in B. daigremontianum and B. tubiflorum and in mature leaves of K. blossfeldiana and demonstrated rhythms in one further species of Bryophyllum, B.calycinum, in addition to that in B. fedtschenkoi. A poorly defined rhythm with an erratic period was recorded in Sedum praeltum. However not all succulent species tested displayed rhythmicity. The carbon dioxide output from B. crenatum was similar to that of rhythmic species during the first 20 h of darkness. Only one peak of carbon dioxide emission occurred after which the rate of output gradually declined. Results very similar to those for B. crenatum

were found for the remaining species tested including species of Sedum, Crassula, Cotyledon, Sempervivum, Mammillaria and Aloe.

The carbon dioxide exchange of succulent plants has also been studied using the established technique for measuring the carbon dioxide compensation point of plants (Jones, 1973; Jones and Mansfield, 1970, 1972). This technique makes use of a sealed system in which air is continuously circulated over the plant material and through an infra-red gas analyser. In contrast to this 'closed circuit', the studies by Wilkins were carried out in an 'open circuit' in which the continuous gas stream passes over the plant material and through the gas analyser only once. The 'closed circuit' technique has revealed circadian rhythms in the carbon dioxide compensation points of species of Bryophyllum, Sedum and Ananas. In 'open circuit' the rhythm of carbon dioxide output in B. fedtschenkoi can be observed in darkness and in low light intensities but not in high light intensities (Wilkins, 1960a). In 'closed circuit' gas analysis the rhythm can be observed in high light intensities but not in darkness (Jones and Mansfield, 1972). The results obtained with both techniques show that a reduction in light intensity will initiate a rhythm. A rhythm in leaves in 'open circuit' can be initiated by a transfer from light to dark while in leaves in 'closed circuit' a transfer from dark to light initiates the rhythm.

It has been known for many years that the carbon metabolism of certain succulent plants displays diurnal variation. Among the first reports of this were the observations that the acidity of B. calycinum leaves increased during the night and decreased during the day (Kraus, 1886a, 1886b). Kraus also demonstrated that this fluctation in acidity was associated with changes in the starch content, a decrease in acidity being accompanied by an increase in starch content. It had been found

much earlier that the gas intake and output of succulent plants varied during the day-night cycle (DeSaussure, 1804).

Although first observed in the Crassulaceae, the accumulation of large amounts of organic acids during the night and their disappearance during the day has been reported in many families of succulent plants. For example Milburn et al (1968) reported its occurrence under natural conditions in species of the Asclepiadaceae, Bromeliaceae, Cactaceae, Compositae, Liliaceae, Vitaceae and Orchidaceae. The diurnal variations of gas exchange and acidity are due to the operation of a metabolic pathway which has become known as Crassulacean Acid Metabolism (CAM). In the leaves of CAM plants a large fixation of atmospheric carbon dioxide is catalysed at night by the enzyme phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31) (Walker, 1957, 1962; Walker and Brown, 1957). The oxaloacetate produced is reduced to malate by malate dehydrogenase (EC 1.1.1.37) resulting in malate accumulation at night in these leaves. In the daytime carbon dioxide is evolved intracellularly from malate by malic enzyme (EC 1.1.1.40) with the production of pyruvate (Walker, 1960, 1962). Pyruvate may then undergo complete oxidation to carbon dioxide and water in the tricarboxylic acid cycle (Brandon, 1963). The carbon dioxide produced during deacidification is available for photosynthetic carbon dioxide fixation by the enzyme ribulose diphosphate carboxylase. It is assumed that most of the malate synthesised when carbon dioxide is fixed during the dark is transferred to a storage pool, probably the central vacuole.

Rhythmicity in this system may be the result of the periodic operation of one or more steps of the CAM pathway. This could be due to cyclic changes in the amount or specificity of one or more of the enzymes present. Alternatively, the activity of enzymes in the pathway may undergo

periodic promotion or inhibition. Furthermore, cyclic variation of substrates or key co-enzymes, possibly ~~occurring~~ occurring as a result of rhythmicity in a different metabolic pathway, could lead to oscillations in the overt carbon dioxide exchange. These possibilities have been examined for the rhythm of carbon dioxide output in B. fedtschenkoi leaves (Warren, 1964; Warren and Wilkins, 1961). Using $^{14}CO_2$ it was found that the amount of carbon dioxide fixed by the leaves varied rhythmically and that there was a coincidence between the occurrence of peaks of the fixation rhythm and troughs of the output rhythm.

The possibility that the control of fixation by PEP carboxylase results from the periodic availability of the substrate for this reaction, phosphoenolpyruvate was investigated by Warren (1964). Infiltration of the leaves with this substance at times when no carbon dioxide fixation occurs causes no increase in fixation. This suggests that the availability of substrate is not responsible for the observed rhythmicity. The periodic appearance of an inhibitor of PEP carboxylase in the leaves was also examined in this study. At least two inhibitory substances were found to be present in the leaves but were present at all phases of the cycle. Possible periodic variation in the availability of co-enzymes has not been investigated.

Queiros (1965, 1966, 1967, 1968a, 1968b, 1969, 1970, 1972a, 1972b) and Queiros et al (1971, 1972) have investigated the control of CAM in leaves of K. blossfeldiana. During light-dark cycles with a short photoperiod, large variations in the activities of PEP carboxylase and malic enzyme occur in crude extracts of leaves, together with variations in the activities of aspartate aminotransferase (EC 2.6.1.1.) and alanine aminotransferase (EC 2.6.1.2.), enzymes closely associated with CAM. These variations are associated with the changes in the level of malate

brought about by dark carbon dioxide fixation. The activity of PEP carboxylase decreases during the night as malate accumulates, and increases towards the end of the day as malate is metabolised. In contrast, the activity of malic enzyme increases through the night as malate accumulates, and shows a decrease during the day which parallels the declining malate content of the leaves.

Malate is a strong inhibitor of the enzyme PEP carboxylase (Kluge and Osmond, 1972). Queirox (1966) has further demonstrated that the extractable activity of malic enzyme at the beginning of the day is dependent on the level of malate present, suggesting a promotive effect of malate on malic enzyme activity. In view of these observations Queiroz (1970, 1972a) has explained the diurnal pattern of CAM metabolism in terms of promotion and inhibition of enzyme activities by malic acid. He suggests that fixation occurs at the beginning of the night and that malate accumulates until PEP carboxylase becomes inhibited and further fixation ceases. The activity of malate dehydrogenase appears never to be limiting, with the result that oxaloacetate is rapidly converted to malate after the primary fixation reaction (Queiroz, 1970). The high level of malate then favours the action of malic enzyme, decarboxylation becoming apparent at the expense of a fraction of the accumulated malate. The activity of PEP carboxylase increases in the absence of the inhibitory malate and the cycle recommences, at the start of the next dark period. However, caution must be exercised in interpreting the results of experiments where enzyme activities have been measured in crude extracts, since it has been estimated that as little as 1% of the malate accumulated in the vacuole would, if present in the cytoplasm, completely inhibit PEP carboxylase activity (Kluge and Osmond, 1972). Thus fixation must be ~~occurring~~ occurring even when the total malate content of the cell is sufficient, when measured in crude extracts, to completely inhibit PEP carboxylase.

The concept of control by feedback inhibition has been refined by considering the fixation phase of the diurnal process as consisting of both the fixation of carbon dioxide and the active transport of malate to the vacuole (Kluge and Heininger, 1973; Kluge and Lüttge, 1974). The inhibition of PEP carboxylase would then depend on the level of the cytoplasmic pool of malate. This would, according to these authors, depend on the rate of influx or efflux of malate to or from the vacuole. Kinetic studies of the release of ^{14}C-malate into buffer solutions from tissue slices of Bryophyllum leaves labelled by the fixation of ^{14}C-carbon dioxide indicate that this consists of three phases, involving malate efflux from free space, the cytoplasm, and the vacuoles. From the data obtained it was estimated that the cytoplasmic pool of ^{14}C-malate is higher in acidified tissue than in deacidified tissue. Furthermore the efflux of ^{14}C-malate into the external solution from the vacuoles is also higher in acidified than in deacidified tissues and increases when the malate solution enclosed in the vacuole is made more concentrated by increasing the osmotic potential of the buffer (Kluge and Heininger, 1973; Lüttge and Ball, 1974a). Increasing the osmotic potential of the bathing medium also affects the distribution of radiocarbon among the metabolites when the tissue is allowed to fix carbon dioxide in the light. This suggests that the rate of malate flux from the vacuoles to the cytoplasm may be involved in control of CAM. In deacidified tissue the incorporation of ^{14}C into malate is inhibited at high external osmotic potentials whereas ^{14}C found in carbohydrates produced by photosynthesis remains at the same level. This effect is explained by inhibition of PEP carboxylase by a growing cytoplasmic malate pool. This would result from the retarded malate flux from the cytoplasm into the vacuole and also the increased malate efflux from the vacuole which would occur under these conditions. It is thus envisaged that during the night fixation of carbon dioxide occurs and the malate is transported to the vacuole.

As the vacuolar pool of malate increases the transport of malate to the vacuole decreases. The rate of efflux of malate from the vacuole then increases and the consequent increase in the size of the cytoplasmic pool of malate results in the inhibition of carbon dioxide fixation. The deacidification phase of the diurnal cycle would then involve passive leakage of malate from the vacuole.

Further evidence on the importance of the transport of malate in CAM control comes from experiments with the chemical α-isopropyl-α-[(N-methyl-N-homoneratyl-γ-aminopropyl] -3,4-dimethoxyphenylacetonitrile (verapamil). This chemical influences the transporting properties of membranes and causes an increase in the efflux of malate from slices of Bryophyllum leaves (Willert and Kluge, 1973). The présence of verapamil in the bathing medium reduces the ability of the tissue to accumulate malate in the dark. Thus it appears that the ~~occurence~~ occurrence of CAM is dependent on the integrity of membrane systems and the accumulation of malate in the vacuoles (Willert, 1972).

These theories were developed largely from experiments performed on tissue in light-dark cycles. It seems likely that additional or more complex reactions are involved in the control of CAM which introduce an endogenous temporal element into the sequence of reactions. The involvement of additional reactions is suggested from the observations of malate synthesis during the light period. This may remain inhibited even when the malate level has fallen below that which permitted carbon dioxide fixation in the dark (Kluge and Osmond, 1972). Queiroz (1972a) has reported that although the maximum activity of malic enzyme measured in crude leaf extracts of K. blossfeldiana is attained near the end of the dark period, none of the accumulated malate is used before the beginning of the light period. An explanation for the first observation

is that PEP carboxylase may continue to be ~~supressed~~ suppressed in the absence of malate by the products of malate degradation. These include pyruvate, the inhibitory nature of which has been demonstrated (Kluge and Osmond, 1972). A second hypothesis may be advanced which could explain the above observations and also introduce a temporal factor into the metabolic pathway. This is that the ~~occurence~~ occurrence of active and passive transport to and from the vacuole depends not only on the amount of malate but more directly on the transport properties of the membranes involved. Whether this could be brought about by the malate or associated proton fluxes themselves in a feedback manner analogous to the ion pump models suggested by Njus et al (1974) is not clear. Lüttge and Ball (1974a, 1974b) have suggested that a system analagous to the K^+ fluxes mediated by active and passive transport processes in Albizzia julibrissin (Satter and Galston, 1971a, 1971b; Applewhite et al, 1973) may operate. Lüttge and Ball (1974b) report that in Bryophyllum the flux seems specific for malate and protons and is independent of the potassium or chloride ion concentration. If such a system operates carbon dioxide fixation would occur only in conjunction with active transport of malate to the vacuole. At other times PEP carboxylase would remain inhibited by the accumulation of relatively small amounts of malate in the cytoplasmic pool.

Rhythms of carbon dioxide exchange have been observed in a number of non-succulent species. In many of these cases rhythmicity appears to result from, or is observed as, rhythmic fixation of carbon dioxide in photosynthesis. In other cases rhythms persist in conditions where photosynthetic carbon dioxide fixation cannot be responsible. The occurrence of a rhythm in the rate of carbon dioxide output of Avena sativa coleoptiles, when they are transferred from red light to darkness, has been described by Ball et al (1957). Hillman (1970) has reported a

rhythm in the carbon dioxide output of Lemna perpusilla which persists in darkness. The primary leaves of Phaseolus show one or two peaks of a carbon dioxide output rhythm in continuous darkness after transfer from light-dark cycles (Armin, Prinz zur Lippe, 1956). There is no evidence for or against the involvement of carbon dioxide fixation in these rhythms.

Chia-Looi and Cumming (1972) reported that the dry weight of Chenopodium rubrum seedlings varies rhythmically during a 72-h dark period. The only ways in which the dry weight of the tissue could increase in darkness is by either periodic uptake and release of mineral salts from the growth medium, or rhythmic net synthesis of dry matter by the fixation of atmospheric carbon dioxide. When seedlings were treated with higher levels of carbon dioxide (0.1%) in darkness, the dry weight content was greater than in seedlings supplied with normal air (0.03% CO_2). The authors consider that a rhythmic mechanism similar to that of CAM may operate in C. rubrum. Thus the rhythmic fluctation in the dry weight of the seedlings may be explicable on the basis of rhythmic changes in the rate of dark carbon dioxide fixation. The rhythm of dark respiration measured as carbon dioxide output from Chenopodium seedlings cannot however be explained by rhythmic carbon dioxide fixation. The rhythms of carbon dioxide output and dry weight differ in both phase and period. Using the same technique which revealed rhythms in the carbon dioxide compensation points of a number of succulents, Jones and Mansfield (1970) also found a rhythm in Coffea arabica. Subsequent studies showed that such rhythms are not commonly found in non-succulents and their presence even in Coffea is dependent on the age of the leaves and may be confined to young leaves (Jones and Mansfield, 1972).

The rhythm in the rate of carbon dioxide output in B. fedtschenkoi

leaves was selected for the investigation into the photocontrol of circadian rhythms for a number of reasons. Rhythms of carbon dioxide exchange are particularly suitable for investigation since the technique of infra-red gas analysis permits the carbon dioxide level in a gas stream to be monitored automatically and frequently. As the oscillation of carbon dioxide emission is believed to be generated and controlled at the cellular level, spatial separation of the photoreception and oscillator mechanisms is not expected. Although the precise mechanism of oscillation is unknown, the underlying biochemical pathways have been the subject of considerable and detailed research. This permits research into the photocontrol of the rhythm to be related to possible oscillatory mechanisms. B. fedtschenkoi is ~~a~~ an evergreen perennial plant and can easily be propagated vegetatively. Ample supplies of suitable experimental plant material can therefore be readily obtained.

Thus the object of this investigation was to study in detail, aspects of photocontrol of the circadian rhythm of carbon dioxide output in leaves of B. fedtschenkoi and in particular, to identify the pigments involved in photoreception in each of these aspects.

MATERIALS AND METHODS

1. Plant Material

The plant material was Bryophyllum (Kalanchoë) fedtschenkoi Hamet et Per. The stock of plants had been derived vegetatively as cuttings from a single original plant obtained from the Royal Botanic Gardens Kew and was the same as that used in investigations by Wilkins (1959, 1960a, 1960b, 1962a, 1962b, 1965, 1967, 1973) and Warren and Wilkins (1961). A continuous supply of experimental material was obtained by regularly taking cuttings, consisting of shoots with five pairs of well developed leaves, from stock plants. Experimental plants were grown in plastic pots either singly (pots 105 mm diameter, 100 mm depth) or with two plants per pot (130 mm diameter, 120 depth). A standard compost mixture of topsoil: 'Rumex' Sphagnum moss peat: course sand, 1:1:1, was used. Plants were maintained, until required, in a heated greenhouse with a minimum temperature of approximately 18°C.

Plants were transferred to a controlled environment room at least 7 d, and typically 3-4 weeks, before use in experiments. An 8-h photoperiod was provided from 08.00 h to 16.00 h each day by a bank of 65/80 W fluorescent lamps (Atlas 'Daylight Supafive': Stella 'Warm White', 1:1). These were mounted 1m above bench level. The radiant flux density at bench level was 47.3 J $m^{-2}s^{-1}$. The temperature was 25 $\pm$ 0.5°C during the photoperiod and 15 $\pm$ 0.5°C during the dark period. Humidity in the growth room was not controlled. The plants were watered daily.

A small number of experiments were carried out using plants which had been maintained in an unheated greenhouse with 16 h supplementary irradiation given each day from 06.00-22.00 h. Irradiation was provided by 65/80 W florescent lamps (Phillips 'Daylight') 60mm apart and 640mm

above bench level.

Experiments were carried out on large, succulent, mature leaves. The top five pairs of leaves were not selected since rhythms persisted with a lower amplitude in those than in mature leaves. It has recently been reported that young leaves may not show typical CAM (Jones, 1975). Leaves showing any signs of senescence were also rejected. The production of suitable leaves was enhanced by excising the main shoot of plants above the fourth or fifth visible leaf pair and subsequently removing axillary shoots as they were produced.

2. Carbon dioxide monitoring apparatus.

A flow diagram of the equipment used to monitor the rate of carbon dioxide output from Bryophyllum leaves is shown in Figure 1. Air from compressed air cylinders (British Oxygen Co. Ltd.) was passed through several vessels each containing 750 cm^3 of a 10% aqueous solution of potassium hydroxide to remove carbon dioxide present in the air. The potassium hydroxide in these vessels was renewed at 2-3 week intervals.

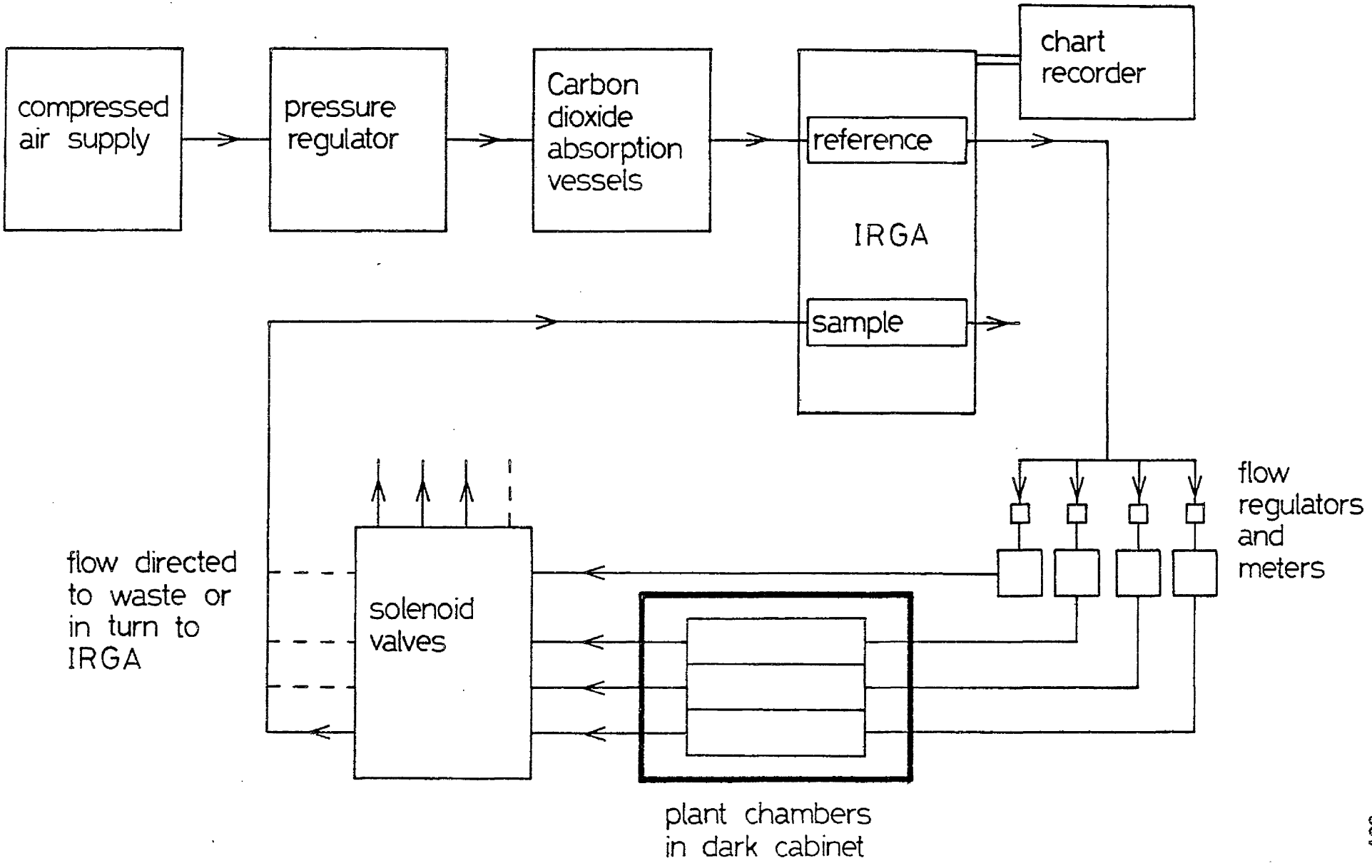
The gas stream then passed, via a device which maintained a constant pressure in the system, to the reference tube of an infra-red gas analyser (IRGA). After the stream had passed through the comparison tube of the IRGA it was divided into four streams, each of which then passed through a capillary flow meter. Flow meters were constructed from manometer tubes containing Brodie's solution and fixed against a measuring scale. The air stream passed from one arm of the manometer to the other through a capillary tube. This was fixed to the open ends of the manometer by short lengths of rubber tubing. The resistance to flow induced by the capillary caused a displacement of the solution in the manometer which was proportional to the gas flow rate. The rate of flow

FIGURE 1

Flow diagram of the apparatus for monitoring the carbon dioxide output of Bryophyllum leaves.

Arrowed lines indicate the flow of gas in the system. Broken lines show alternative flow paths operated by the solenoid valves.

FIGURE 1


compressed air supply
pressure regulator
Carbon dioxide absorption vessels
reference
IRGA
sample
chart recorder
flow regulators and meters
flow directed to waste or in turn to IRGA
solenoid valves
plant chambers in dark cabinet

was controlled both at the cylinder head and at the polythene tubing inlet to the flow meters. At the latter site either fine gas flow regulator valves or 30mm screw clips were used.

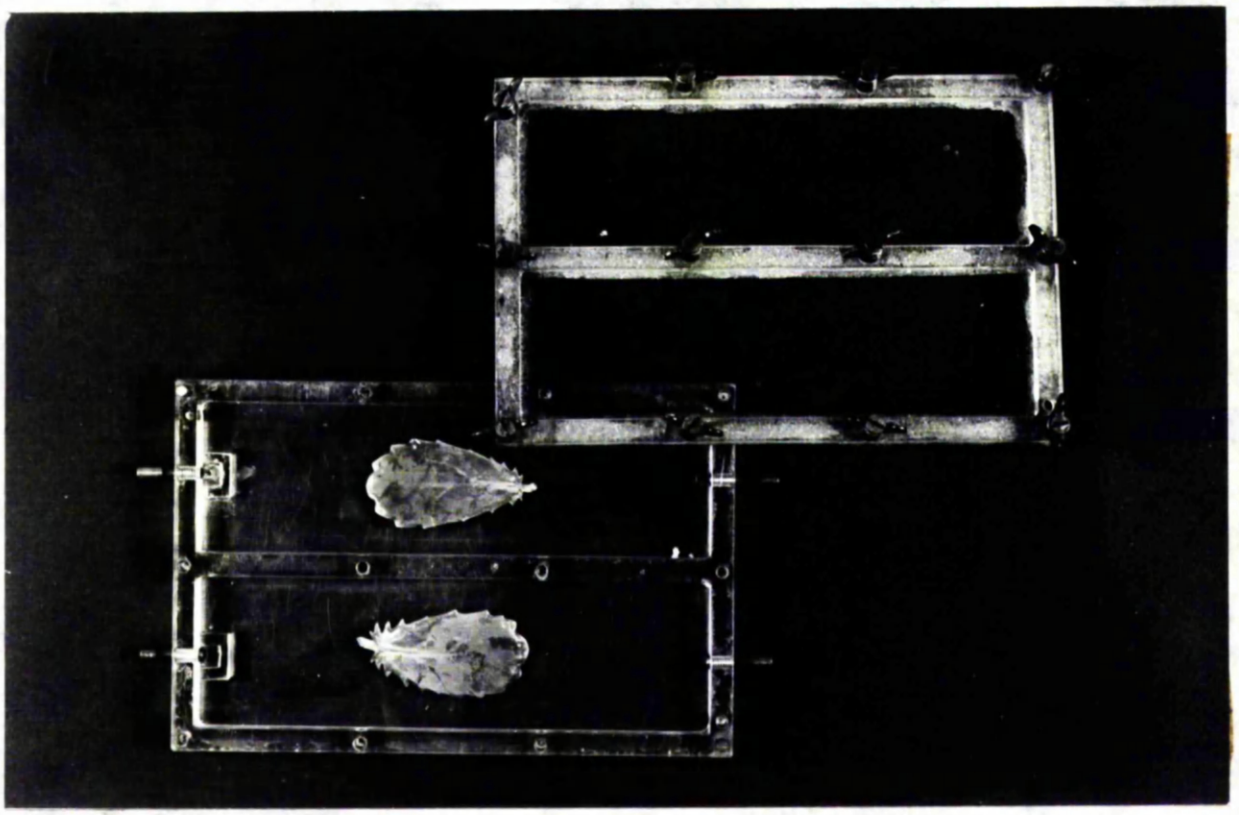
Three of the streams passed to plant chambers and then to the sample tube of the IRGA via solenoid valves. The fourth stream by-passed the plant chambers and flowed directly from the flow meter to the solenoid valves. The fourth stream thus enabled an hourly check of the zero of the IRGA to be made. The solenoid valves were operated by a cam timer in such a way that the gas flow from each chamber was directed in turn through the sample tube of the analyser for 15 min. and to waste for the remaining 45 min. in each hour. The output signal of the IRGA, representing the difference in the carbon dioxide content of the air in the reference and sample tuben of the analyser, was recorded on a chart recorder.

Chambers in which leaves were irradiated (Figure 2) were constructed from clear perspex and consisted of a hollow base divided into two. 3mm internal diameter brass or perspex tubing conducted the stream of air into and out of the chambers. The removable top of the chambers was a 2mm thick sheet of perspex. This was held in place by a perspex frame screwed tightly to the base of the chamber. Soft rubber formed an air-tight seal between the two plant chambers and a water-tight seal between the base and top. The chambers were found to be completely air and water-tight when tested at pressures far in excess of those encountered during normal use. Plant chambers designed to maintain leaves in darkness were constructed from 150 mm x 48 (or 54) mm diameter glass tubes covered with 3 layers of black insulating tape. These tubes were stoppered at each end with rubber bungs through which 6mm (internal diameter) glass tubing carried the gas flow. To avoid leakage of light the glass and

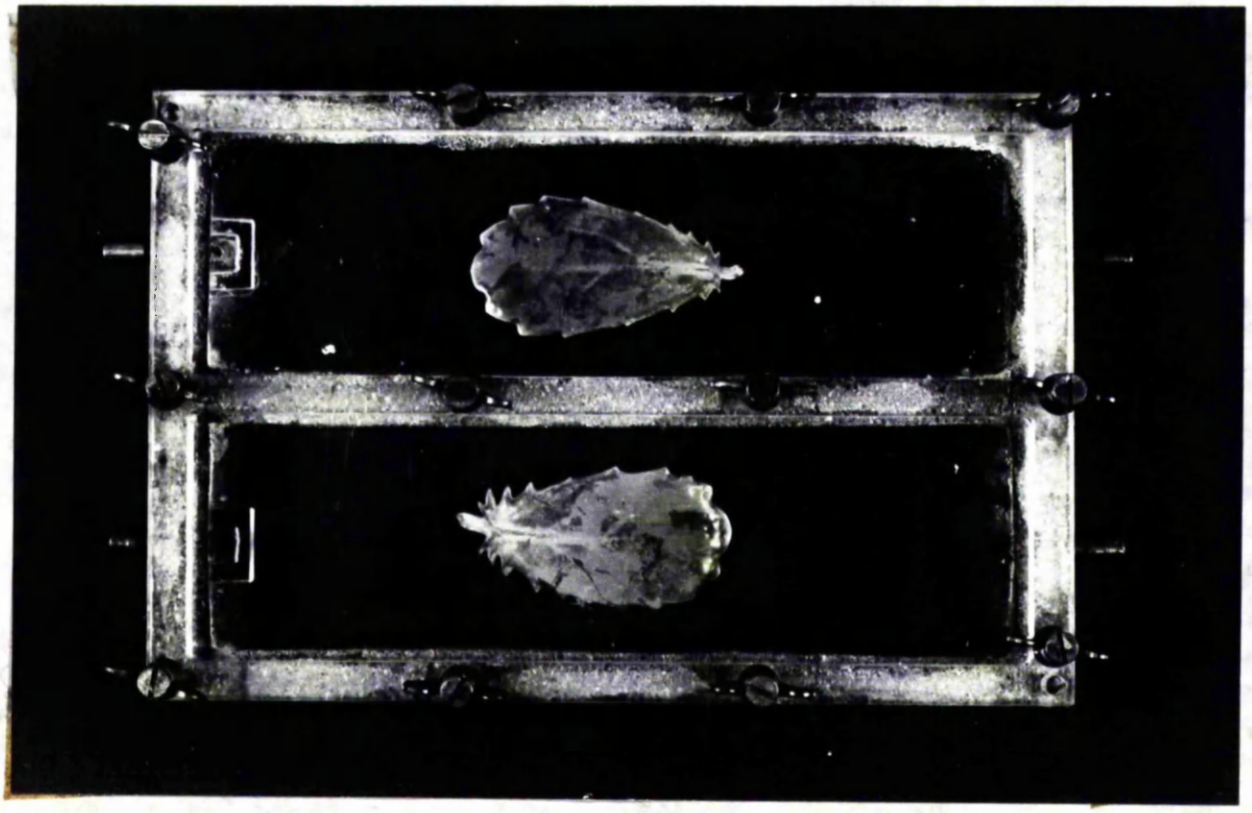
FIGURE 2

Perspex plant chambers containing Bryophyllum leaves. Photographs show chambers with the top detached (A) and attached (B) to the base

A



B



plastic tubing for a distance of 350mm either side of these chambers was also covered with black tape.

Plant chambers were maintained in water baths at a depth of 150mm. Used in conjunction with ~~refrigeration~~ refrigeration units, these water baths maintained experimental ambient temperatures $\pm$ 0.2°C. The water baths containing the plant chambers were enclosed in dark cabinets constructed from 'Handy Angle' frames and covered with two layers of black polythene (1,000 guage; Transatlantic Plastics Ltd.).

Three complete sets of equipment were used. In the first, a Grubb-Parsons IRGA (model SB1) was employed which received a power supply from a Grubb-Parsons type A,A.C voltage stabilizer. A modified Phillips valve voltmeter was used in conjunction with the SB1 IRGA and provided the high tension current to the detecting condenser of the IRGA and amplified the output signal. This signal was displayed on an Elliot type 230 chart recorder. The plant chambers were maintained at constant temperatures with a Grants water bath (Model SB4) and a Grants cooling unit (model CC15). The gas flows were directed through the IRGA in turn, by means of solenoid valves (Figure 3). These consisted of oppositely mounted solenoids, activated by a 1-h cam time switch (Elrenco Ltd. ECT/4). The solenoids tilted a three armed glass tube in such a way that, depending on which solenoid was activated, a small amount of mercury blocked one of the outer arms. The gas stream entering the centre arm was thus directed through the open tube either to waste or to the gas analyser. Every 15 min one of the valves was tilted to the 'to IRGA' position while the previously operated valve was simultaneously activated and returned to the 'to waste' position. The solenoids were operated by a 12 volt supply from a transformer.

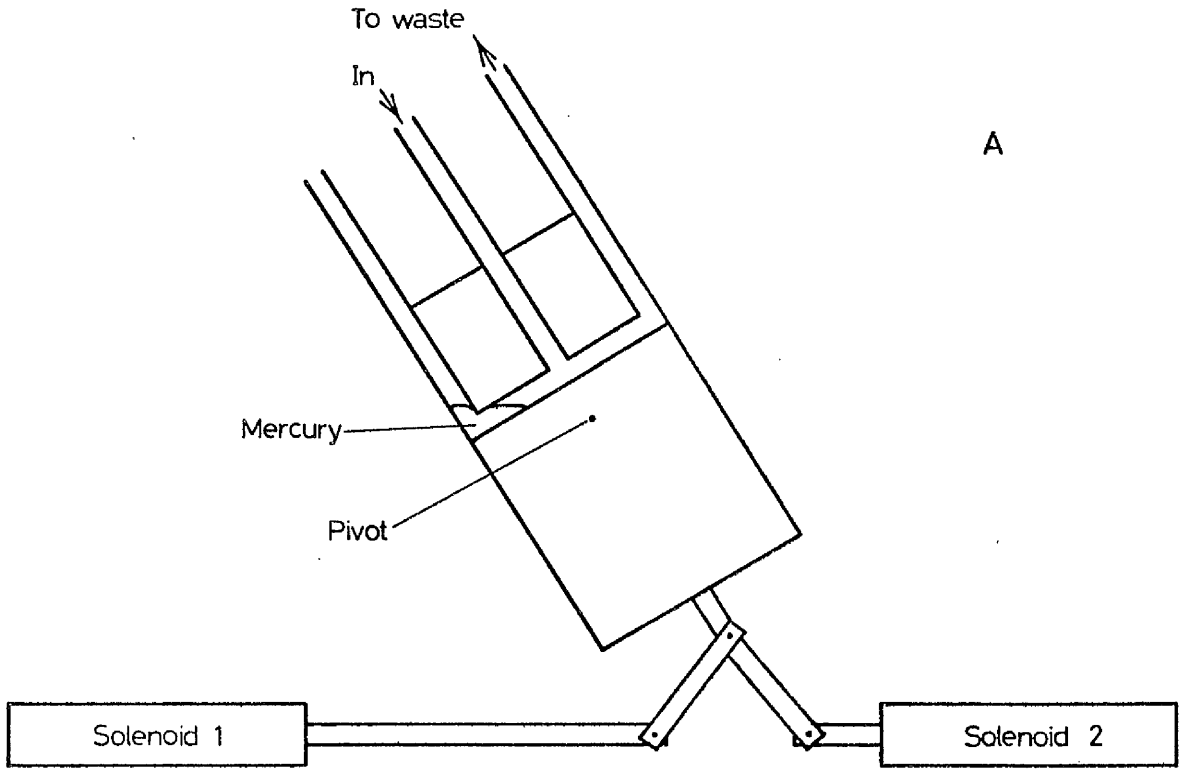
The second apparatus was basically similar to the first. A Grubb-

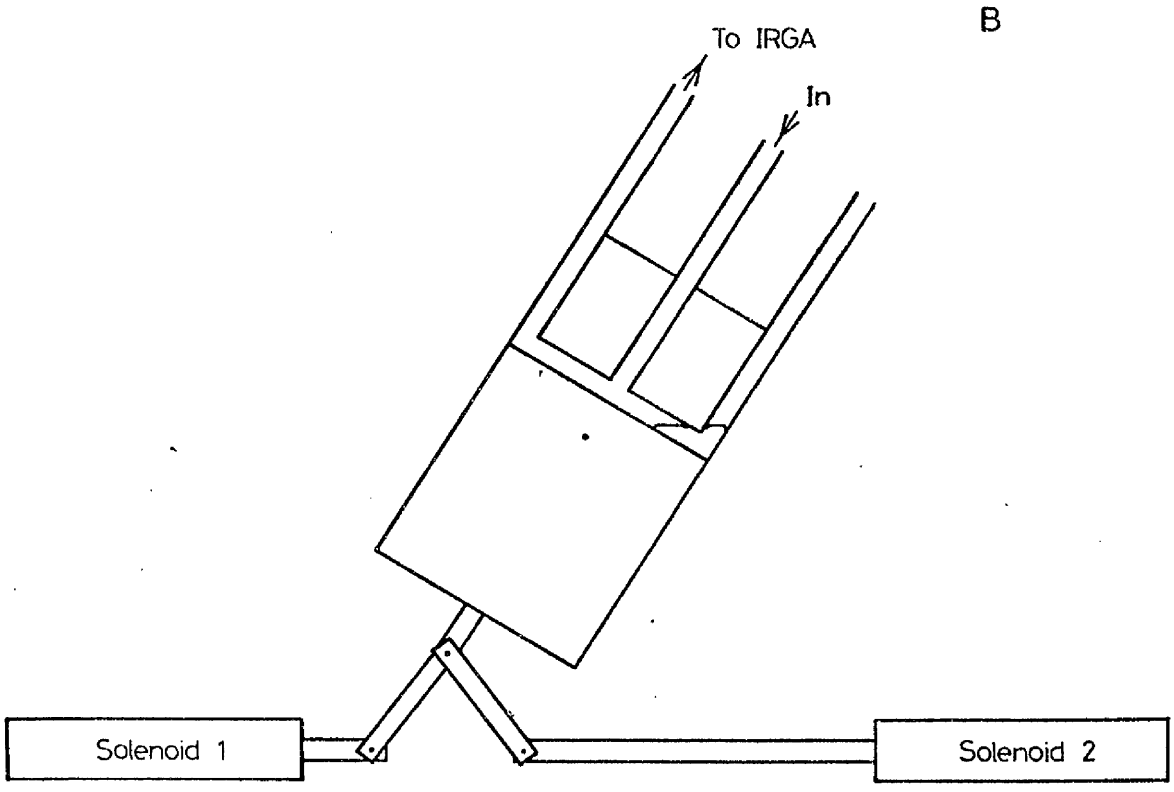
FIGURE 3

Solenoid valves used in apparatus 1.

(A) Flow directed to waste when solenoid 1 is activated.

(B) Flow directed to IRGA when solenoid 2 is activated.


To waste
In
A
Mercury
Pivot
Solenoid 1
Solenoid 2


B
To IRGA
In
Solenoid 1
Solenoid 2

Parsons SB2 IRGA was used which did not require a separate stabilizer or voltmeter. The output signal from the IRGA was recorded on a Servoscribe 1s potentiometric flatbed recorder. The gas flows were directed to waste or to the IRGA by Black model 1510, 3 way, normally open solenoid valves (Black Automatic Controls Ltd.) The third apparatus also utilized a Grubb-Parsons SB2 IRGA but differed from the first two apparati in that a Grants SB35 water bath and Schrader 457-SA, 3 way normally open solenoids valves were used. The solenoid valves used in apparati 2 and 3 were operated at mains voltage.

In apparatus 1 the internal diameter of polythene tubing used to carry the gas stream was 5mm and the flow meters were constructed from 3 mm internal diameter manometer tubes and 0.5 mm capillary tubes. In the second and third sets of equipment, 6 mm internal diameter polythene tubing, 5 mm manometer tubes and 0.5 mm capillary tubes were used. All three IRGA's were fitted with optical suppression filters which eliminated interference from infra-red radiation absorbed by both water vapour and carbon dioxide in the gas stream.

The capillary flow meters were calibrated by recording the time taken at several settings for air flowing through the meter to displace water from an inverted vessel of known volume. The calibration data so obtained were confirmed with a soap bubble flow meter. The ~~IRGA's~~ IRGAs were calibrated with air of a known carbon dioxide content. The carbon dioxide content of a cylinder of air was determined by passing a known volume of the air through a 0.073 N aqueous solution of barium hydroxide in a series of tubes. A sufficient number of tubes were used to ensure that no barium carbonate precipitation occurred in the last tubes of the series. The solution in the tubes was titrated against standard 0.1 N hydrochloric acid. The amount of carbon dioxide absorbed by the

barium hydroxide and hence the concentration of carbon dioxide in the test air sample could then be calculated. Air of known carbon dioxide content was mixed in varying proportions with carbon-dioxide-free air and passed through the sample tube of the IRGA while carbon-dioxide-free air passed through the reference tube. In this way a calibration curve for IRGA response against carbon dioxide content of air was determined under conditions similar to those used in subsequent experiments. The calibration curves obtained in this way corresponded closely to those provided by the manufacturers. Each IRGA was fitted with a calibration device which gave a reading when a wire was placed in the path of the infra-red radiation passing through the sample tube. This calibration value was recorded at the sensitivity of the IRGA and chart recorder used for calibration. The relationship between chart deflection and the carbon dioxide content of air samples at any other sensitivity settings could thus be determined by comparing a new calibration wire reading with the original one. In addition, in apparatus 1 the calibration wire built into the IRGA was operated automatically once every hour by the 1 h cam timer, as a check on the sensitivity of the IRGA. The calibration of both flow meters and IRGA's was checked periodically. Examples of the calibration data obtained are shown in Figure 4 and 5. The equations of the lines, obtained by regression analysis, were used for transforming the data.

3. Irradiation

A number of radiant energy sources were used in irradiation studies. These were mounted above the water baths in the dark cabinets. White light was obtained from either a 60 W tungsten lamp or a 20 W, 580 nm, Atlas 'White' fluorescent lamp. In the case of the tungsten lamp, the radiant flux density was adjusted to that used experimentally by means of a rheostat (Bereostat L50, 800 ohms) connected in series. Monochromatic radiation was obtained from Bausch and Lomb High Intensity Grating

FIGURE 4

Calibration data for apparatus 1 flow meters.

Each point is the mean value of at least 3 individual readings.

○ - manometer 1, $y = 0.0582x - 0.1433$

● - manometer 2, $y = 0.0770x - 0.1317$

■ - manometer 3, $y = 0.1117x - 0.2140$

▲ - manometer 4, $y = 0.1100x - 0.1623$
(line not shown)

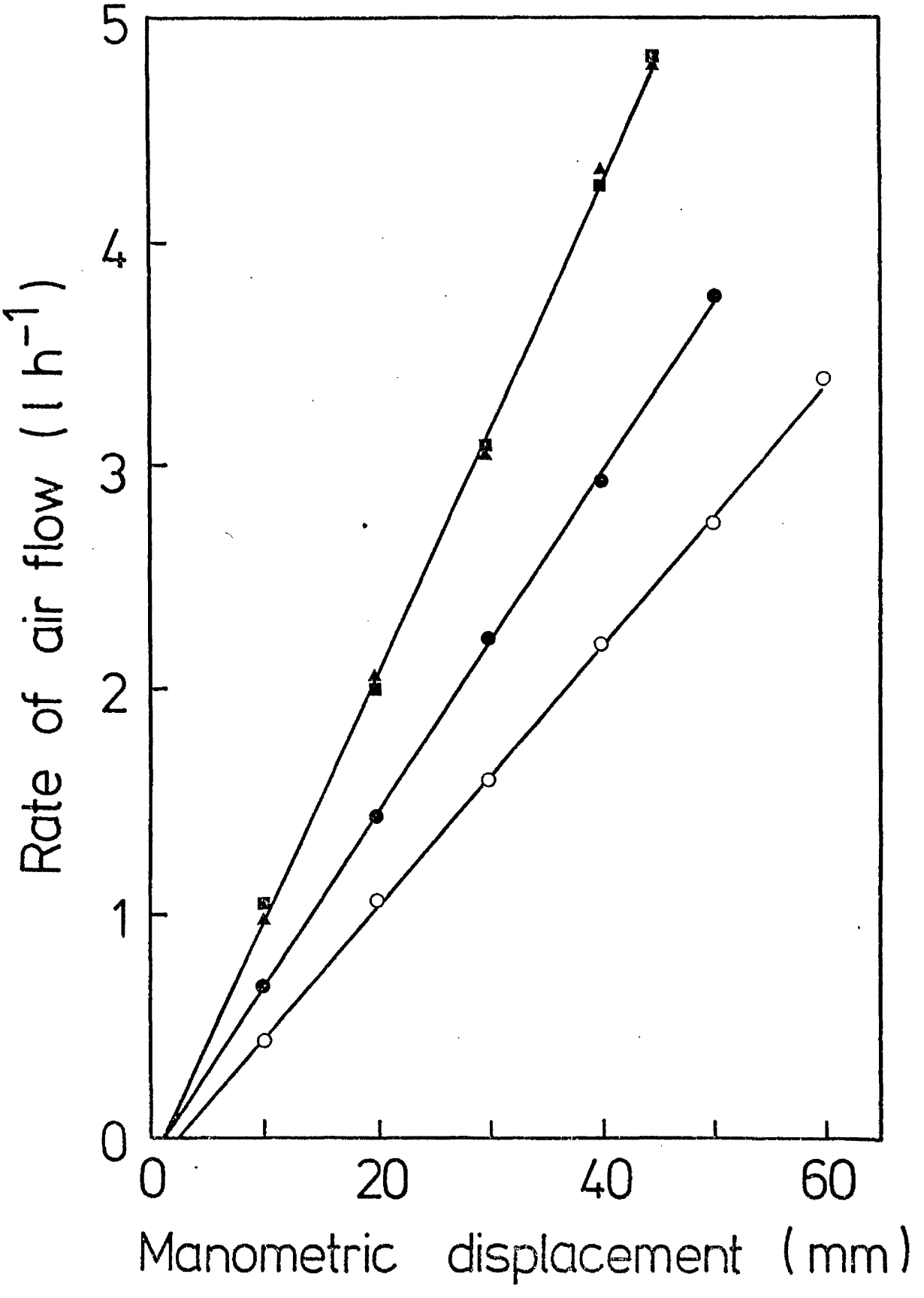
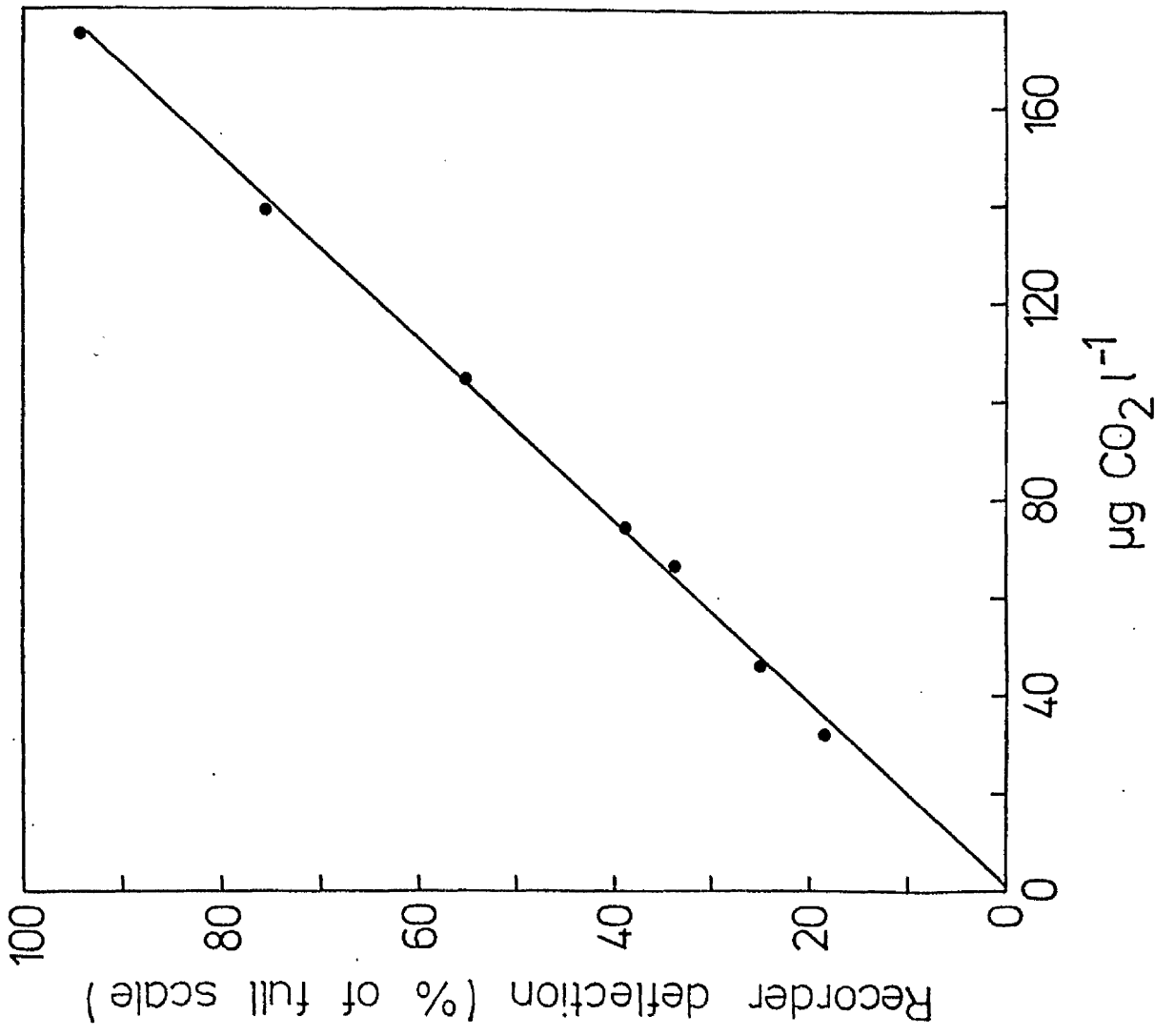

Rate of air flow (l h⁻¹)
0
1
2
3
4
5
0
20
40
60
Manometric displacement (mm)

FIGURE 5

Calibration data for apparatus 1 IRGA

$y = 0.54x - 0.79$


Recorder deflection (% of full scale)
100
80
60
40
20
0
0
40
80
120
160
µg CO_2 l^{-1}

Monochromators with tungsten light sources containing 45 W Sylvania tungsten halogen lamps and monochromators with a grating of 1350 grooves mm^{-1}. The entrance and exit slits of the monochromator were 5.36 and 3 mm respectively. The radiant flux density of monochromatic radiation was adjusted either with a rheostat connected in series or by the insertion of Kodak Wratten neutral density filters into the beam. The monochromators provided spectral bands 25 nm wide as measured with a hand spectrometer. To eliminate possible contamination of the red end of the spectrum with overlapping blue light of other order spectra from the grating two layers of Cinemoid Orange No.5 filter (Rank Strand Electric Ltd.) were inserted in the beam when the monochromators were set at wavelengths longer than 560 nm.

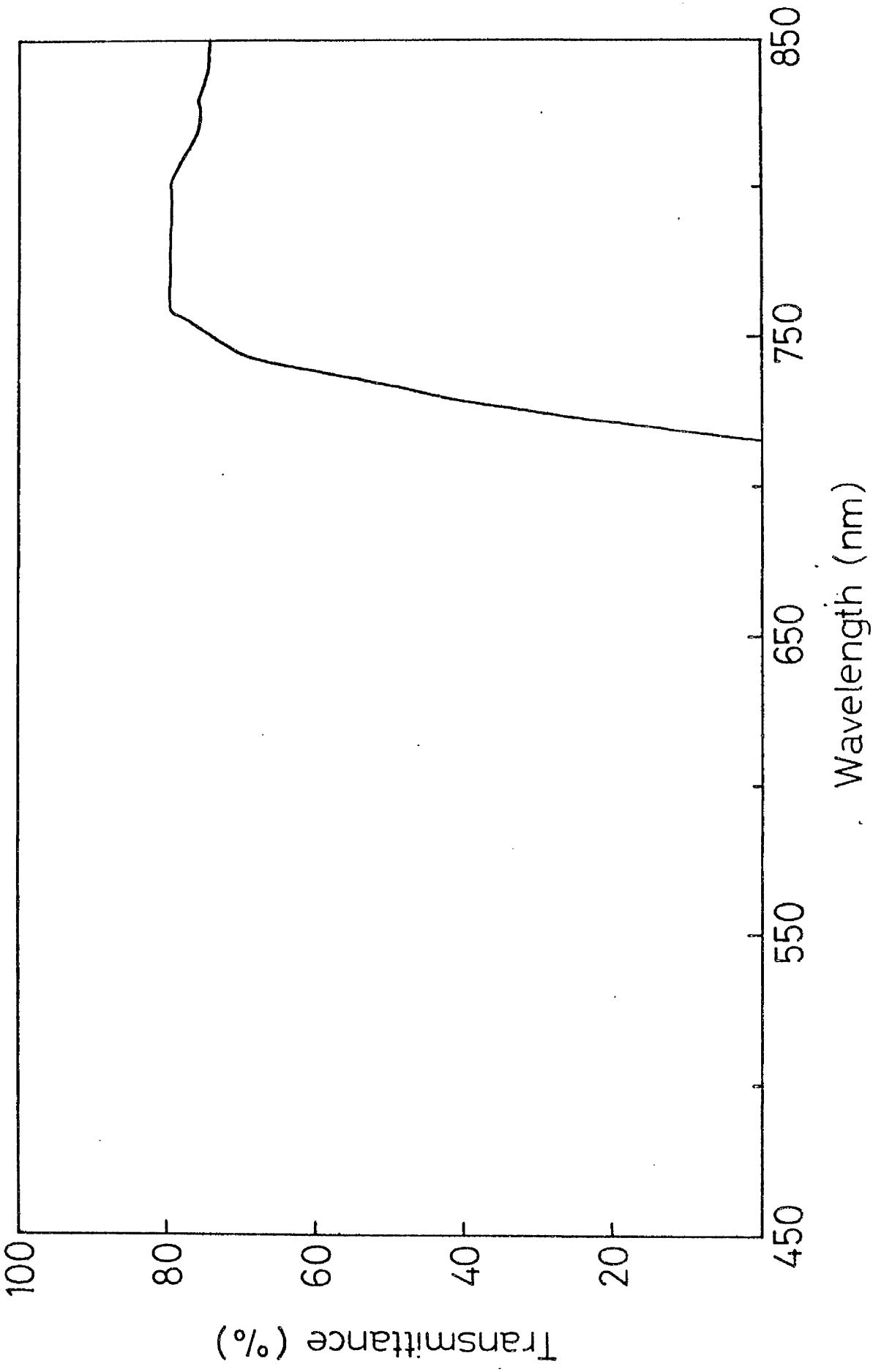
A higher intensity of radiant energy in the far-red region of the spectrum was obtained by passing the ~~collimated~~ collimated beam from a monochromator tungsten light source through a Corning 7-69 far-red filter (Corning Glass Works, Corning, N.Y., U.S.A.). The transmittance of this filter in the 450-850 nm region of the spectrum was measured with a Pye Unicam UV recording spectrophotometer and is shown in Figure 6. Wavelengths of radiant energy between 200 and 450 nm were not transmitted. The quality of radiant energy reaching the leaves was also modified to some extent by the absorption due to the 150 mm path of water in the water bath.

The radiant flux density emitted by the tungsten and monochromatic light sources was measured directly with a Kipp Zonen Compensated Thermopile and Preamplifier (model 11330) and a Scalamp galvanometer (model 7902/T) (W.G. Pye and Co. Ltd., Cambridge U.K.). Because of the unidirectional nature of the thermopile this instrument was not suitable for measuring the radiant emittance of the fluorescent lamp. This was measured with an EEL 'Light Master' photometer (Evans Electro Selenium

FIGURE 6

The transmittance of a Corning 7-69 far-red filter as a function of wavelength.

FIGURE 6


Transmittance (%)
100
80
60
40
20
450
550
650
750
850
Wavelength (nm)

Ltd., Halstead, Essex U.K.). The photometer had previously been calibrated against a thermopile for the radiant energy ~~emitted~~ emitted by a fluorescent lamp covered with black polythene so only a small portion of the tube, approximating to a point source, was exposed.

Irradiation of the leaves during experiments was controlled by time switches. Irradiations of 15 min or multiples of 15 min were controlled by Venner 'multiset' time switches. When two light sources were to be operated successively for 15 min each, this was achieved with a 1-h cam timer adjusted to give the two pulses, connected in series to a Venner time switch. A sequential timer was constructed which enabled light pulses to be applied in rapid alternation with darkness or with other wavelengths of radiant energy. The two outputs of the sequential timer could be set independently to deliver a pulse ranging from 7 s to 14 min. Full details of this timing device are given in Appendix 1. The sequential timer was operated at the required time and for the required duration by a Venner 24-h time clock to which it was connected. The operation of the monochromator lamps during short and alternating pulse experiments was monitored with EEL selenium photovoltaic cells mounted inside the lamp housing. The photovoltaic cells were connected directly to a potentiometric chart recorder in such a way that when one of the lamps was operated the recorder pen was deflected to one side of a base line and when the other was operated the pen was deflected to the other side of the base line (Figure 7).

4. Experimental procedure.

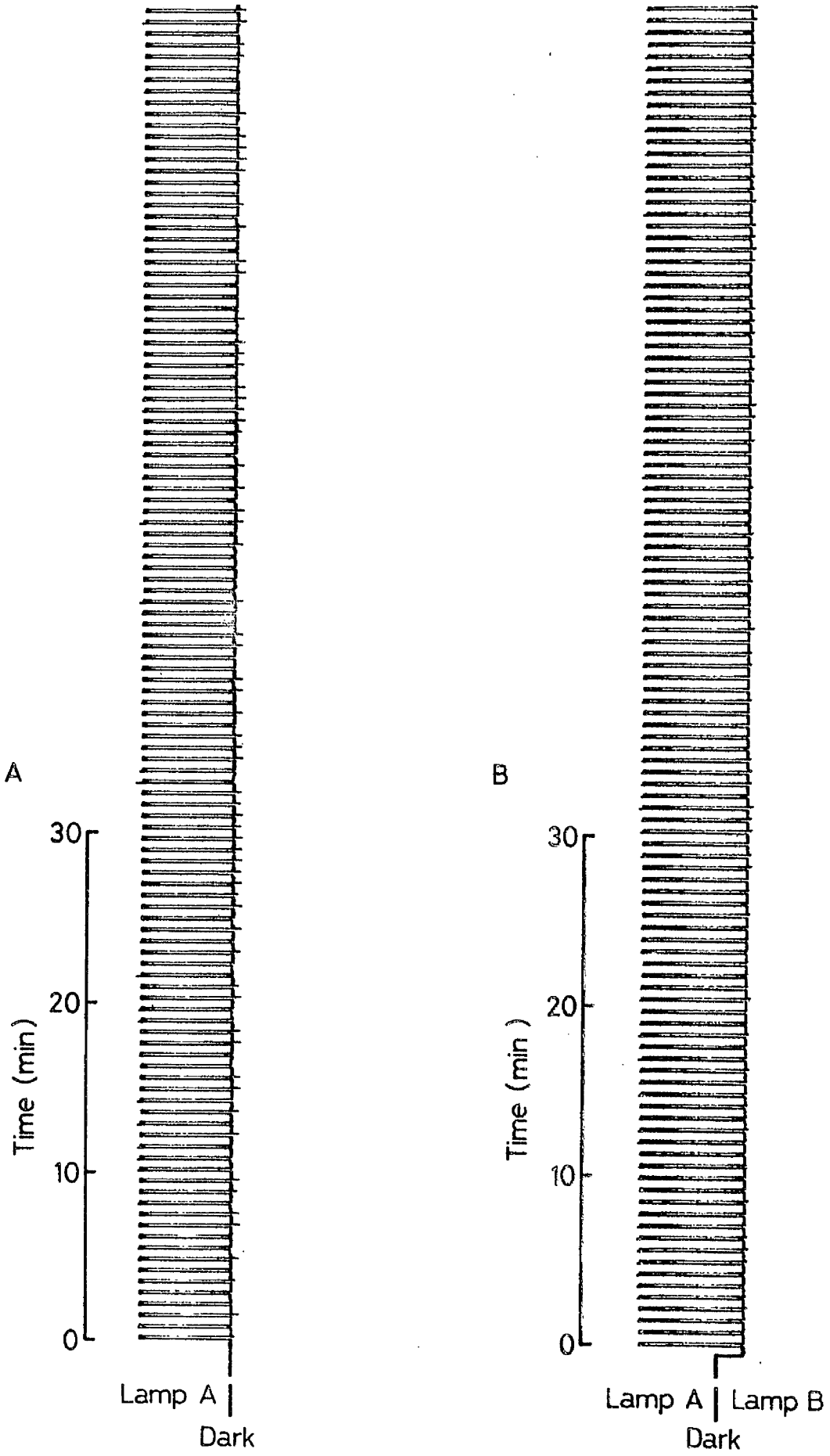
Leaves were excised from plants at the end of the photoperiod. They were weighed, placed singly in plant chambers and transferred to the water baths. Unless otherwise stated all experiments were performed at 15°C on plants from the controlled environment room. The gas flow rate

FIGURE 7

Examples of traces obtained with the apparatus used to monitor the operation of lamps in experiments where leaves were exposed to:-

A. 10s irradiation from lamp A alternating with 30s darkness.

B. 10s irradiation from lamp A alternating with 30s irradiation from lamp B.


A
30
20
10
0
Time (min)
Lamp A
Dark
B
30
20
10
0
Time (min)
Lamp A
Lamp B
Dark

was 1.5 l h^{-1}. There was a delay of 2 min between a change in the carbon dioxide concentration occurring in the plant chambers and the commencement of the corresponding recorder pen deflection. A new steady level was recorded 6 min after the solenoid valves altered the gas flow reaching the IRGA from normal air passing through one plant chamber to carbon-dioxide-free air passing through a second.

5. Treatment of data.

One value for the carbon dioxide content of gas passing through each of the plant chambers was obtained in each hour. The sequence in which the chambers were sampled during experiments which included both darkened and irradiated leaves was, zero check, irradiated leaf chamber, dark leaf chamber, irradiated leaf chamber, zero check etc.. The zero line, while the gas flowing through the plant chambers was being sampled, was taken as the mean of preceding and following values obtained for the zero check gas stream. In practice very little variation of the zero level occurred. The carbon dioxide output, calculated from calibration data, was expressed as µg carbon dioxide h^{-1} g (fresh weight)$^{-1}$. This was plotted as an hourly value and a trace was obtained by joining all the values obtained. Unless stated any breaks in the traces are due to the carbon dioxide output exceeding the maximum value which could be recorded at the sensitivity setting of the IRGA or chart recorder.

The phase of the rhythm, taken for comparative purposes, was the time of occurrence of peaks of carbon dioxide output. The time of occurrence of a peak was estimated by measuring the distance across the peak at equidistant points from near the base to near the apex. The mean time of occurrence of the mid-points of these lines was the time at which a peak was deemed to occur. The period of the rhythm was assessed as the mean time between successive peaks of the first four

cycles of the rhythm. The time from the start of the experiment to the first peak was termed the transient.

Where examples of traces are given to illustrate the results, these are representative of the data obtained in at least two, and usually many more, experiments. Where data are presented graphically, the mean of a number of observations is given together with the standard error. This is represented on the graphs by vertical lines drawn symmetrically about the data points and equal to twice the standard error. The Student's t-test was used to ascertain whether rhythms in leaves exposed to two treatments differed significantly. Calculation of the standard error and t-values was performed as described by Bailey (1959). The probability levels were obtained from the Student's t-distribution (Fisher and Yates, 1963). Differences between means were referred to as significant when the probability value $p < 0.05$. The probability levels for significant differences are quoted as $p < 0.05$, $p < 0.02$, $p < 0.01$ or $p < 0.001$. Where one of these values is given to the significance of the difference between two means, it implies that p lies below the quoted value but above the next lowest value.

RESULTS

1. Rhythmicity in light-dark cycles.

1.1 Short photoperiods of white light.

Preliminary experiments were carried out using plants grown in an unheated greenhouse with the natural photoperiod extended to give LD 16:8. Figure 8 shows the carbon dioxide output from leaves excised at 2200 h, the end of the photoperiod, and maintained in darkness at 15°C. The rate of carbon dioxide output from these leaves was initially low but began to increase after 8-12 h. A peak of carbon dioxide output occurred 20 h after transfer to darkness. Some leaves displayed a second peak of carbon dioxide emission approximately 24 h after the first. Thus plants grown under these conditions did not exhibit a persistent circadian rhythm when placed in continuous darkness. The pattern of carbon dioxide emission under these conditions may be interpreted as a weak circadian rhythm which faded out after 1 or 2 cycles.

Rhythmicity may frequently be induced or maintained by exposure to light-dark cycles, even when the photoperiod is of only a few minutes duration. The effects of exposing leaves to short photoperiods ending at 0600 h each day are shown in Figure 8. A rhythm in the rate of carbon dioxide output was induced by a 1-h exposure to white fluorescent light at a radiant flux density of 0.6 J m^{-2} s^{-1} (Figure 8A). An exposure to a radiant flux density of 4.7 J m^{-2} s^{-1} from a tungsten lamp for 1 h was also effective in inducing rhythmicity (Figure 8B). Daily irradiations of 30, 15 and 5 min duration from tungsten lamps also induced rhythmicity (Figure 8 C-E). No rhythm was apparent however when the duration of irradiation was reduced to 1 min (Figure 8F). A large increase in the rate of carbon dioxide output which lasted for 2-3 h occurred following irradiations of from 15 min to 1 h.

FIGURE 8

The carbon dioxide output of Bryophyllum leaves exposed once every 24 h to radiant energy from:-

A. White fluorescent lamp (0.6 J m^{-2} s^{-1}) for 1 h.

B. Tungsten lamp(4.7 J m^{-2} s^{-1}) for 1 h.

C. " " " " 30 min.

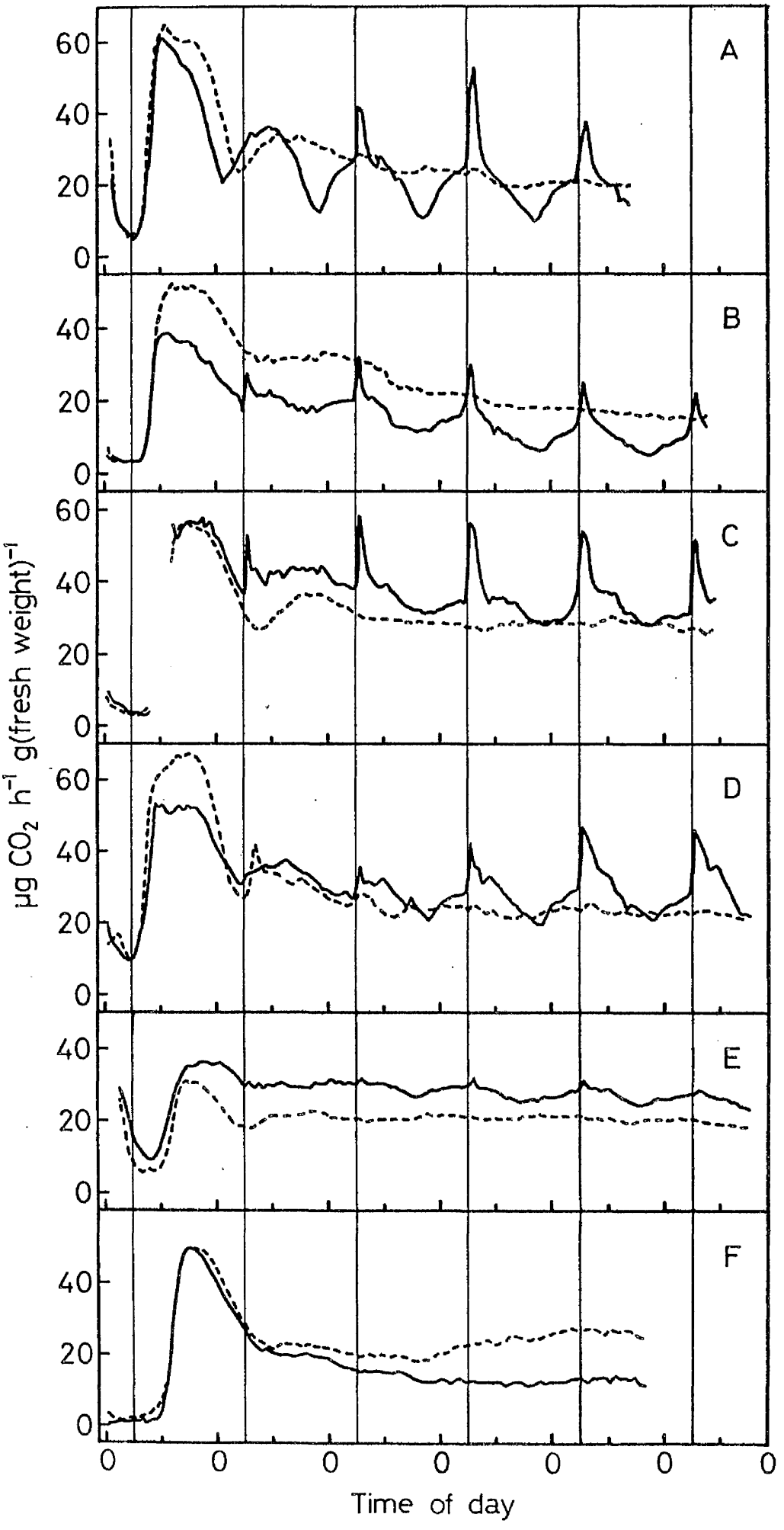
D. " " " " 15 min.

E. " " " " 5 min.

F. " " " " 1 min.

The vertical lines indicate the times of irradiation. Rhythms in control leaves in darkness are shown by the broken lines and those in irradiated leaves by the continuous lines.

0 = midnight.

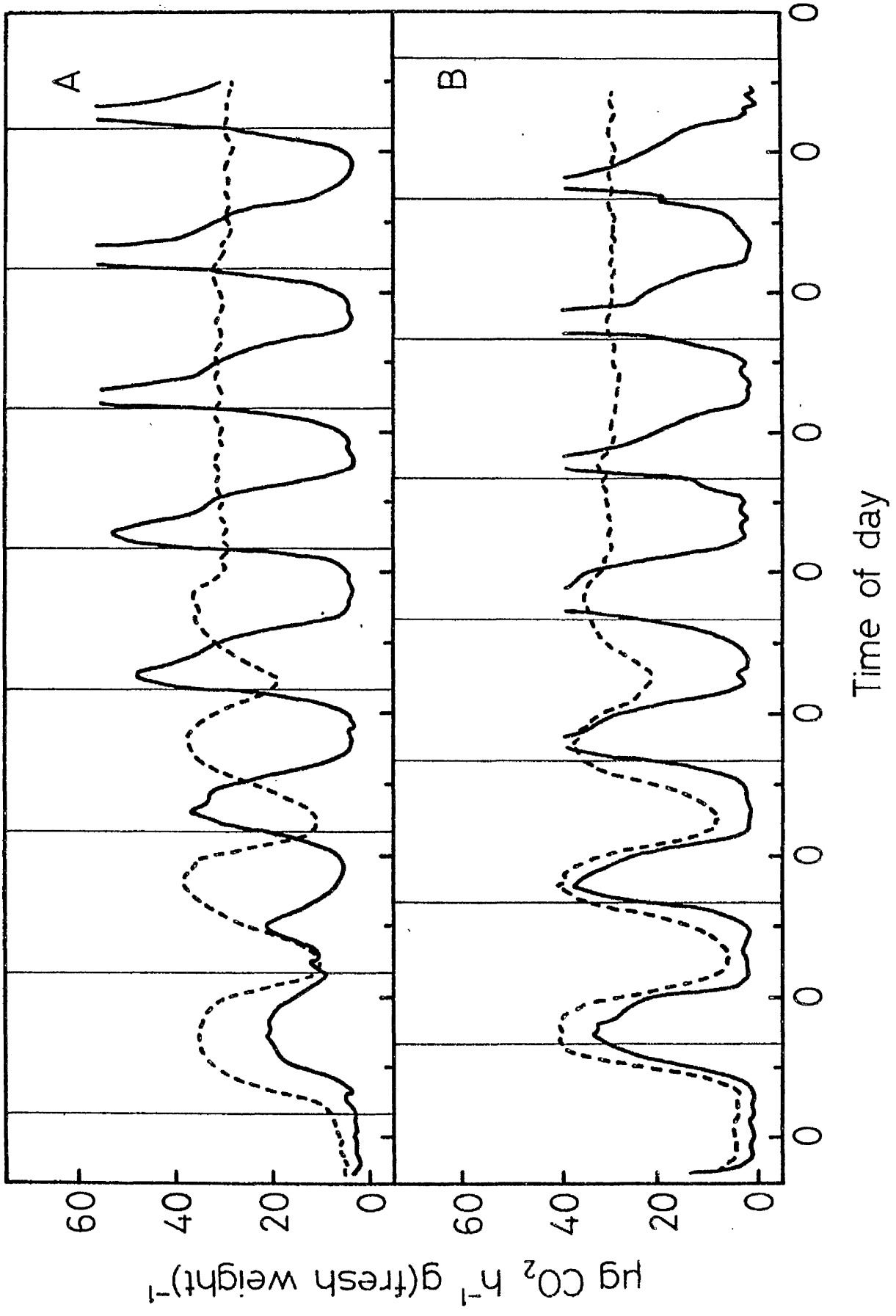

A
B
C
D
E
F
60
40
20
0
μg CO2 h-1 g(fresh weight)-1
0 0 0 0 0 0 0
Time of day

All subsequent experiments were carried out using plants which had been grown in LD 8:16 in a controlled environment room. Leaves from these plants showed a circadian rhythm of carbon dioxide output which persisted in darkness at 15°C for 3-5 cycles. The first peak of carbon dioxide output occurred 26 h after transfer to darkness and the rhythm persisted with a period of approximately 24 h. The influence of light-dark cycles on the rhythm of carbon dioxide output was further examined using these plants. Exposure of Bryophyllum leaves to cycles consisting of 0.25 h of white fluorescent light and 23.75 h of darkness are shown in Figure 9A and B. The phase of the light-dark cycles in Figure 9A was 12 h different from that in Figure 9B. In each case the rhythm was entrained and locked on to a new phase after a number of cycles. In Figure 9A the 0.25-h photoperiod began at 0400 h and the new stable phase was attained after 3 cycles, whereas in Figure 9B the light period began at 1600 h and entrainment was complete after 2 cycles. The course of the rhythm during entrainment to a new stable phase is clearly determined by the phase of the entraining cycles and the initial phase of the free-running rhythm. In Figure 9A the final phase determined by the entraining cycles is very different from that displayed by the free-running rhythm during the first few cycles. The phase of the rhythm is progressively shifted to that of the entraining cycle and consequently two transient cycles with short periods occur. In Figure 9B the phase of the free-running rhythm corresponds rather closely during the first few cycles to the final phase developed in relation to the entraining cycle. Thus, transient cycles are not immediately apparent. However, while the entrained rhythm persists with a period of exactly 24 h, the free-running rhythm has a period slightly less than 24 h. As a result of this the two rhythms become progessively out of phase with each other. In both cases the phase relationship between the entrained and free-running rhythms is constantly changing. It is clear that exposure to light-dark

FIGURE 9

Entrainment of the rhythm in Bryophyllum leaves to 0.25 h of white fluorescent light and 23.75 h of darkness every 24 h. The vertical lines indicate the times of irradiation which differ by 12h in A and B. The broken lines show the control rhythms in unirradiated leaves. 0 = midnight.

FIGURE 9


A
B
0
20
40
60
0
0
0
0
0
0
Time of day
µg CO2 h-1 g(fresh weight)-1

cycles is not necessary for the occurrence of rhythmicity but that such exposures influence the endogenous circadian rhythm of the leaves by increasing its persistence and entraining it in a specific phase relationship to the external cycles.

1.2 Short photoperiods of radiant energy in defined spectral bands.

A more detailed analysis of the mechanism of entrainment can be achieved by exposing the leaves to 24-h cycles of light and darkness in which the photoperiod is both of very short duration and of defined spectral band. Leaves of Bryophyllum were exposed daily to 0.25 h of monochromatic radiation at a quantum flux density of 47 pE cm^{-2} s^{-1}. A comparison was made of the effect of regions of the spectrum most often involved in the photocontrol of circadian rhythms in plants.

Periodic exposure to a 25-nm spectral band centred on 450 nm had no effect on either the phase or the persistence of the rhythm (Figure 10). Periodic exposure to red light (660nm) for 0.25 h caused the rhythm to persist for much longer than in leaves in continuous darkness, and to assume a specific phase relationship to the entraining cycles (Figure 11). Entrainment with red light was very similar to that obtained with white fluorescent light, although slower when red light was applied between 0400 and 0415 h. The persistence and amplitude of rhythms in leaves periodically exposed to red light were somewhat variable. In general, the greatest persistence and amplitude were found in leaves used from plants known to exhibit clear rhythmicity in darkness.

A spectral band centred on 730 nm (Figure 12) acted in a similar way to red light, although 5-7 cycles were required before the new stable phase was established. When leaves were exposed to light-dark cycles of red light which were 12 h out of phase (Figure 11) the phases of the

FIGURE 10

The effectiveness, in entraining the circadian rhythm in Bryophyllum, of exposing leaves to a quantum flux density of 47 pE cm^{-2} s^{-1} at 450 nm. The vertical lines indicate the times of irradiation which differ by 12h in A and B. The broken lines show the control rhythms in unirradiated leaves. 0 = midnight.

FIGURE 10

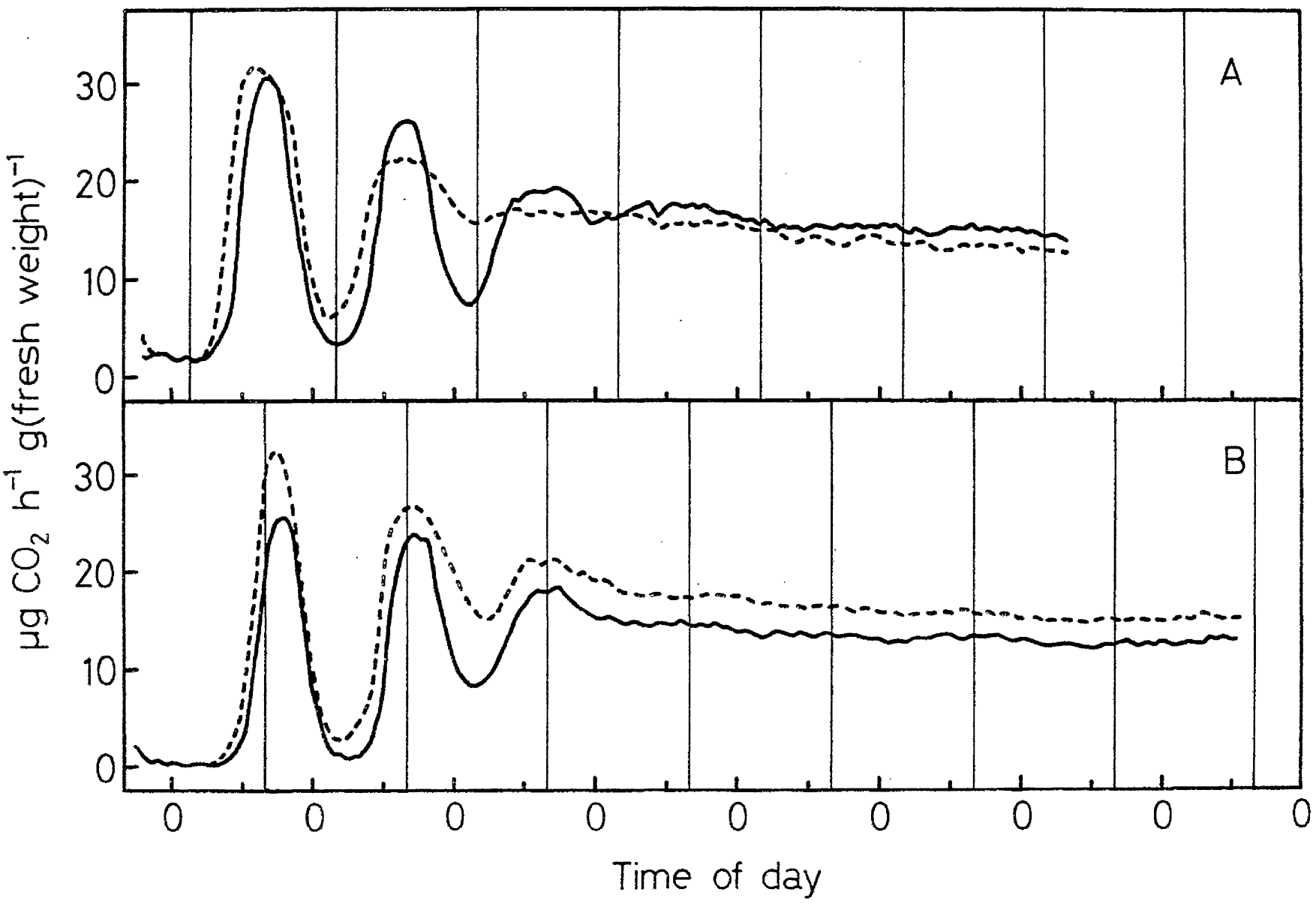

A
B
30
20
10
0
μg CO_2 h^{-1} g(fresh weight)$^{-1}$
0 0 0 0 0 0 0 0 0
Time of day

FIGURE 11

The effectiveness, in entraining the circadian rhythm in Bryophyllum, of exposing leaves to a quantum flux density of 47 pE cm^{-2} s^{-1} at 660 nm. The vertical lines indicate the times of irradiation which differ by 12 h in A and B. The broken lines show the control rhythms in unirradiated leaves. 0 = midnight.

FIGURE 11

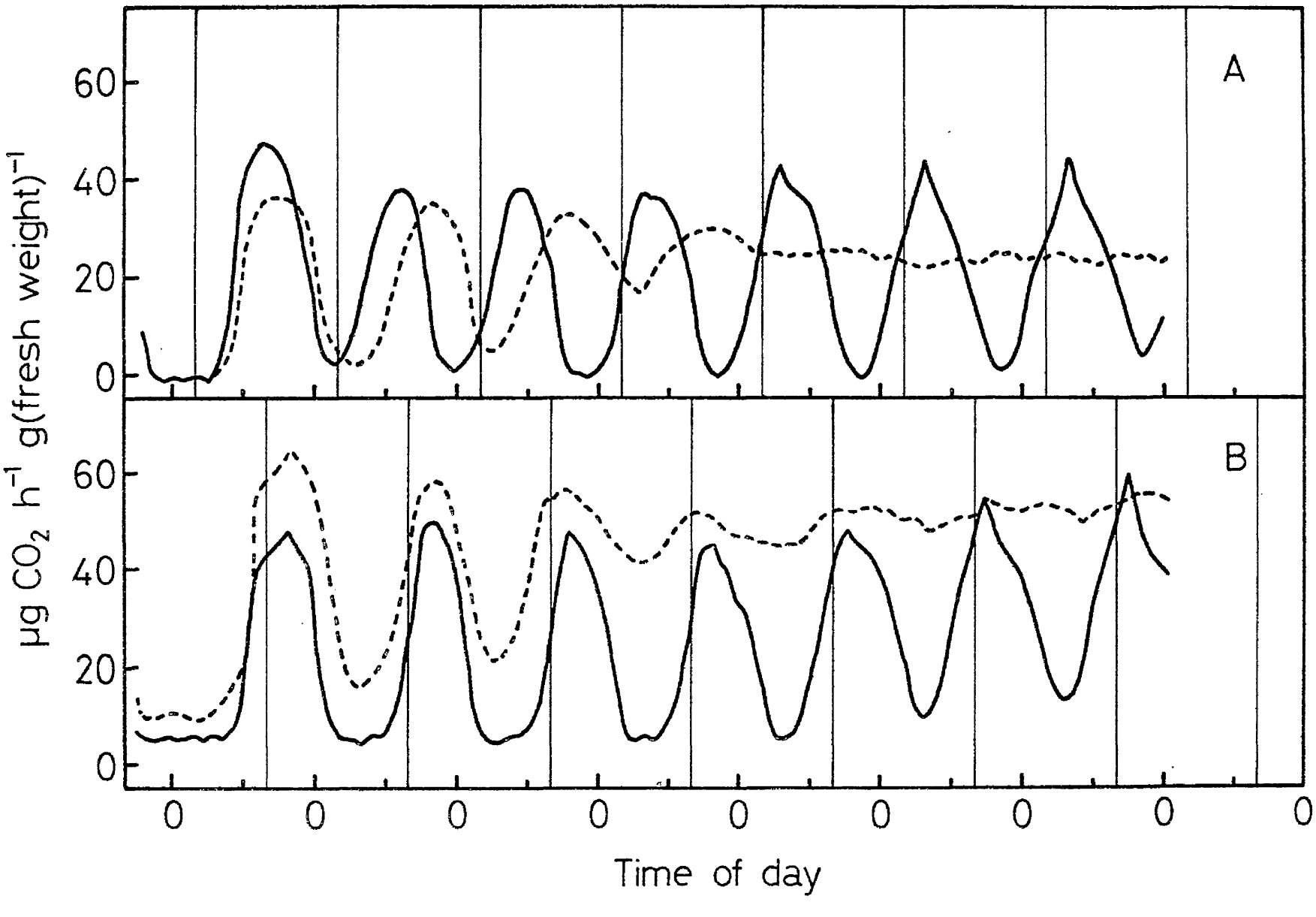
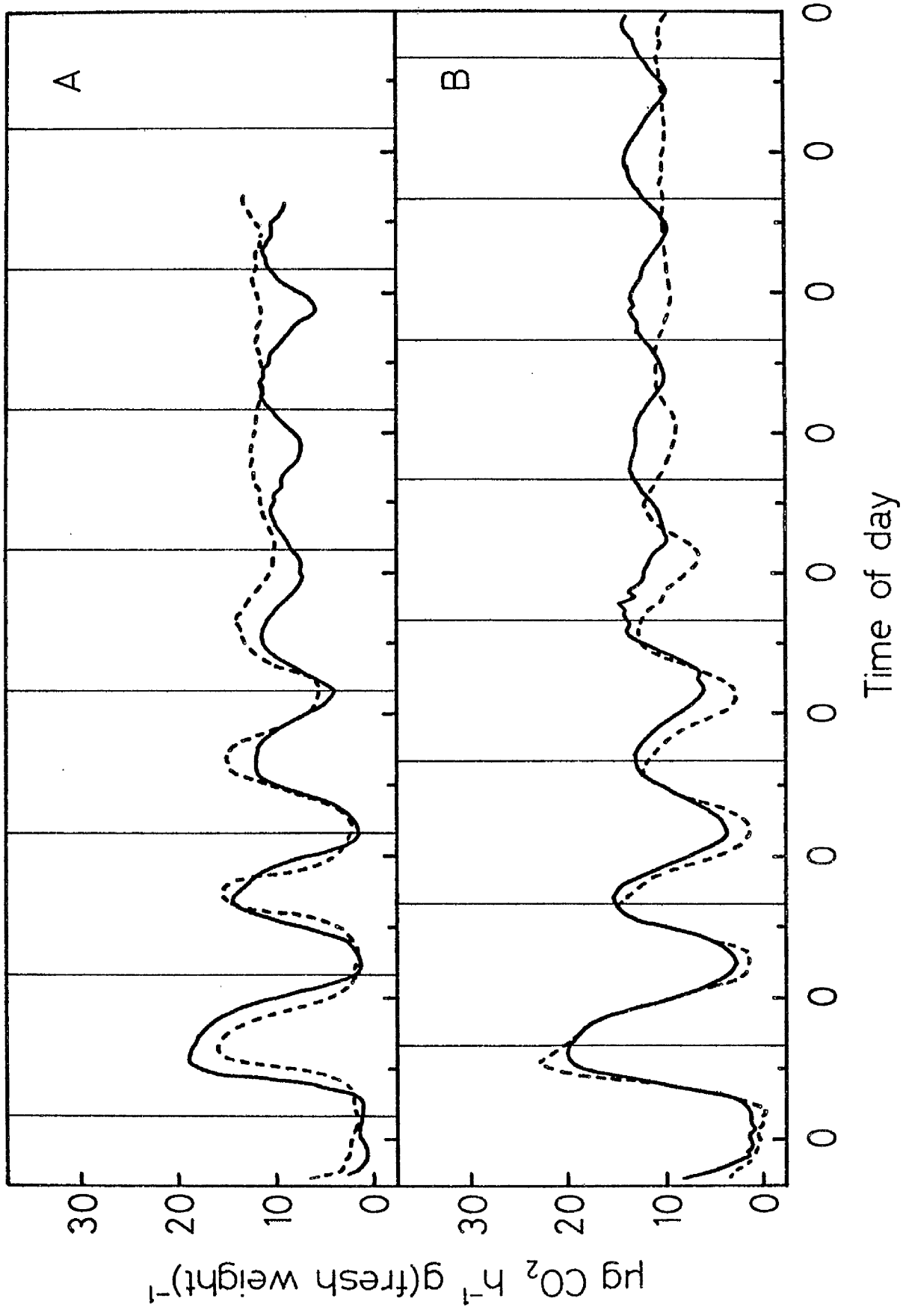

A
B
60
40
20
0
μg CO2 h-1 g(fresh weight)-1
0 0 0 0 0 0 0 0 0
Time of day

FIGURE 12

The effectiveness, in entraining the circadian rhythm in Bryophyllum, of exposing leaves to a quantum flux density of 47 pE cm^{-2} s^{-1} at 730 nm. The vertical lines indicate the times of irradiation which differ by 12 h in A and B. The broken lines show the control rhythms in unirradiated leaves. 0 = midnight.

FIGURE 12


A
B
30
20
10
0
0
0
0
0
0
0
Time of day
μg CO2 h-1 g(fresh weight)-1

entrained rhythms were clearly different by the second cycle. Using the same criterion, exposure to radiant energy at 730 nm could be seen to have little or no effect when applied during the first three cycles, but to entrain the rhythm to a new stable phase rapidly during the fourth and subsequent cycles. Despite the variability noted in the amplitude of rhythms entrained by red light, the effects of red and far-red could also be distinguished with respect to this parameter. The difference in the amplitude in irradiated leaves and comparable leaves in darkness was always greater with red than with far-red radiation.

1.3 The interaction of red and far-red radiation.

The effectiveness of far-red radiation in entraining the rhythm of carbon dioxide output in leaves of Bryophyllum was further investigated using radiant energy obtained with a Corning 7-69 far-red filter. Rhythms in leaves exposed to cycles consisting of 0.25 h of far-red radiation and 23.75 h of darkness are shown in Figure 13. Clear entrainment of the rhythm was not achieved with this treatment. Although a small difference in the phase of the rhythms in irradiated and unirradiated leaves was apparent, the rhythm faded out rapidly in either condition. When applied at 0400 h, periodic exposure to far-red radiation tended to damp the rhythm compared with that in the unirradiated control leaves.

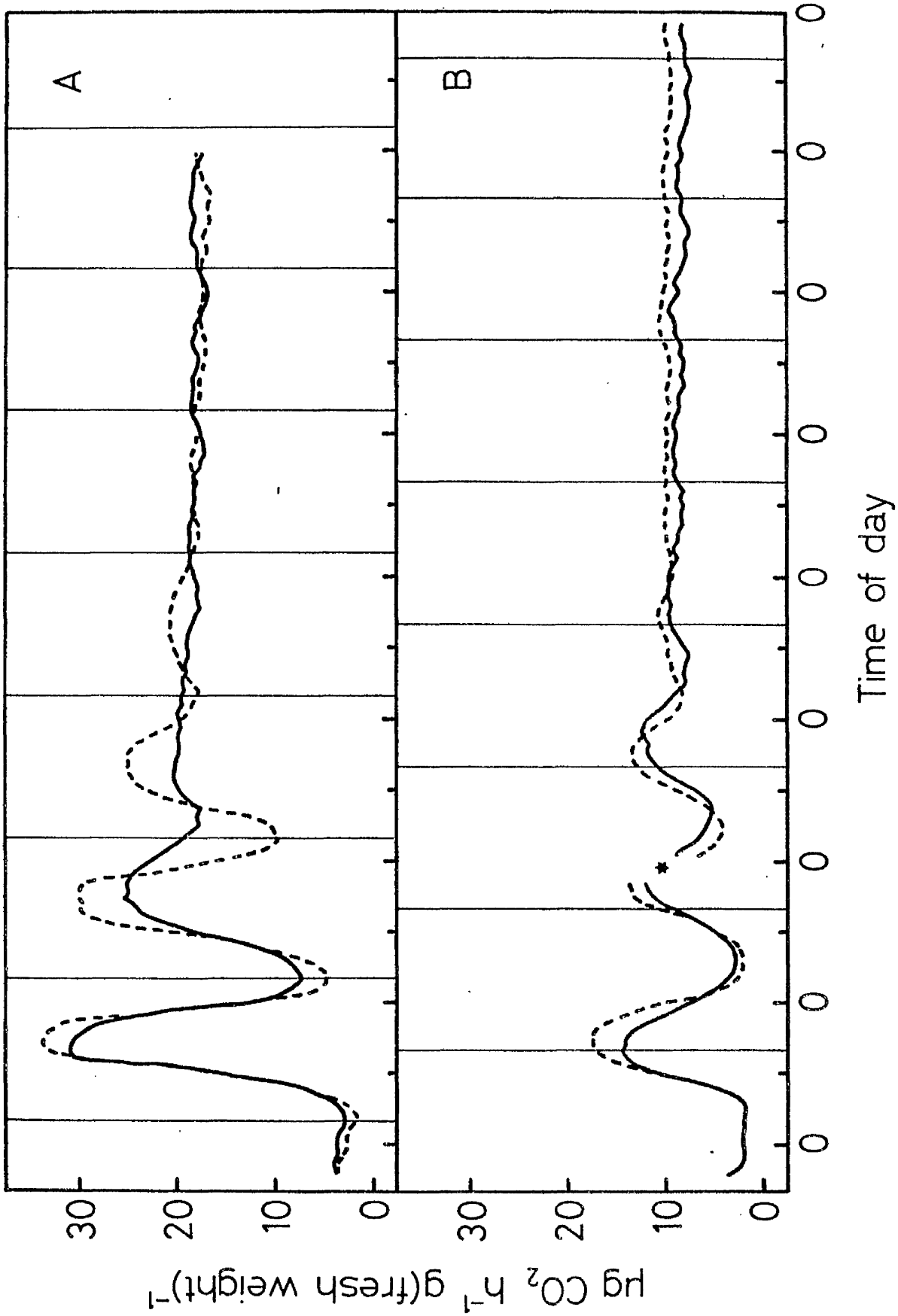
The possible involvement of phytochrome, as the pigment responsible for photoreception in the entrainment of the rhythm to light-dark cycles, was investigated. As both red and far-red radiation at 730 nm entrained the rhythm, a complete reversal of the effect of an exposure to red light with an exposure to far-red radiation was considered unlikely. However, as the effects of red and far-red radiation were different in terms of rapidity of entrainment and persistence of the rhythm, experiments were performed to investigate their possible interaction.

FIGURE 13.

The effectiveness in entraining the circadian rhythm in Bryophyllum of exposing leaves to far-red radiation at a radiant flux density of 7.8 J m^{-2} s^{-1}. The vertical lines indicate the times of irradiation which differ by 12 h in A and B. The broken lines show the control rhythms in unirradiated leaves. 0 = midnight.

* trace lost through equipment failure.

FIGURE 13


A
B
30
20
10
0
0
0
0
0
0
0
Time of day
μg CO2 h-1 g(fresh weight)-1

When monochromatic red light (660 nm) at a radiant flux density of 85 mJ m^{-2} s^{-1} was applied each day between 0400 and 0415 h (Figure 14B) the rhythm was entrained to a phase different from that of the free-running rhythm in darkness (Figure 14A). Far-red radiation (Corning 7-69 filter, 7.8 J m^{-2} s^{-1}) applied at this time each day appeared to abolish the rhythm (Figure 14C). When 0.25 h of far-red radiation was given immediately following an exposure to red light the rhythm damped rapidly. With this treatment a carbon dioxide output pattern similar to that with far-red radiation alone was obtained. However, in the example given (Figure 14D) rhythmicity of a very low amplitude may have persisted. If, however, 0.25 h of far-red radiation immediately preceded the exposure to red light (Figure 14E), the rhythm was entrained and persisted in a manner very similar to that in leaves exposed to red light alone (Figure 14B).

The persistence and entrainment of the rhythm thus depends on whether red or far-red radiation is experienced by the leaves immediately before the end of the short photoperiod. This result provides some evidence for an interaction of red and far-red radiation in the photocontrol of the circadian rhythm by light-dark cycles.

1.4 Skeleton photoperiods of white light.

The study of the carbon dioxide output rhythm in leaves exposed to cycles of light and darkness was extended to examine the effects of 24-h cycles containing either a single photoperiod of 8-12 h duration or two 0.25-h irradiations. Figures 15A and B show the rhythm in leaves exposed to LD 8:16. The photoperiod was provided by a fluorescent lamp giving an incident radiant flux density of 0.6 J m^{-2} s^{-1}. An example of the rhythm obtained when this schedule began with the 16-h dark period is given in Figure 15A, while Figure 15B illustrates the rhythm

FIGURE 14

Effect on the circadian rhythm of carbon dioxide output in *Bryophyllum* leaves of:-

A. Continuous darkness

B. Cycles of 0.25 h red light and 23.75 h darkness

C. Cycles of 0.25 h far-red radiation and 23.75 h darkness

D. An exposure to 0.25 h far-red radiation immediately after the exposure to red light in a schedule otherwise as in B.

E. An exposure to 0.25 h far-red radiation immediately before the exposure to red radiation in a schedule otherwise as in B.

Vertical lines indicate the times of irradiation. 0 = midnight.

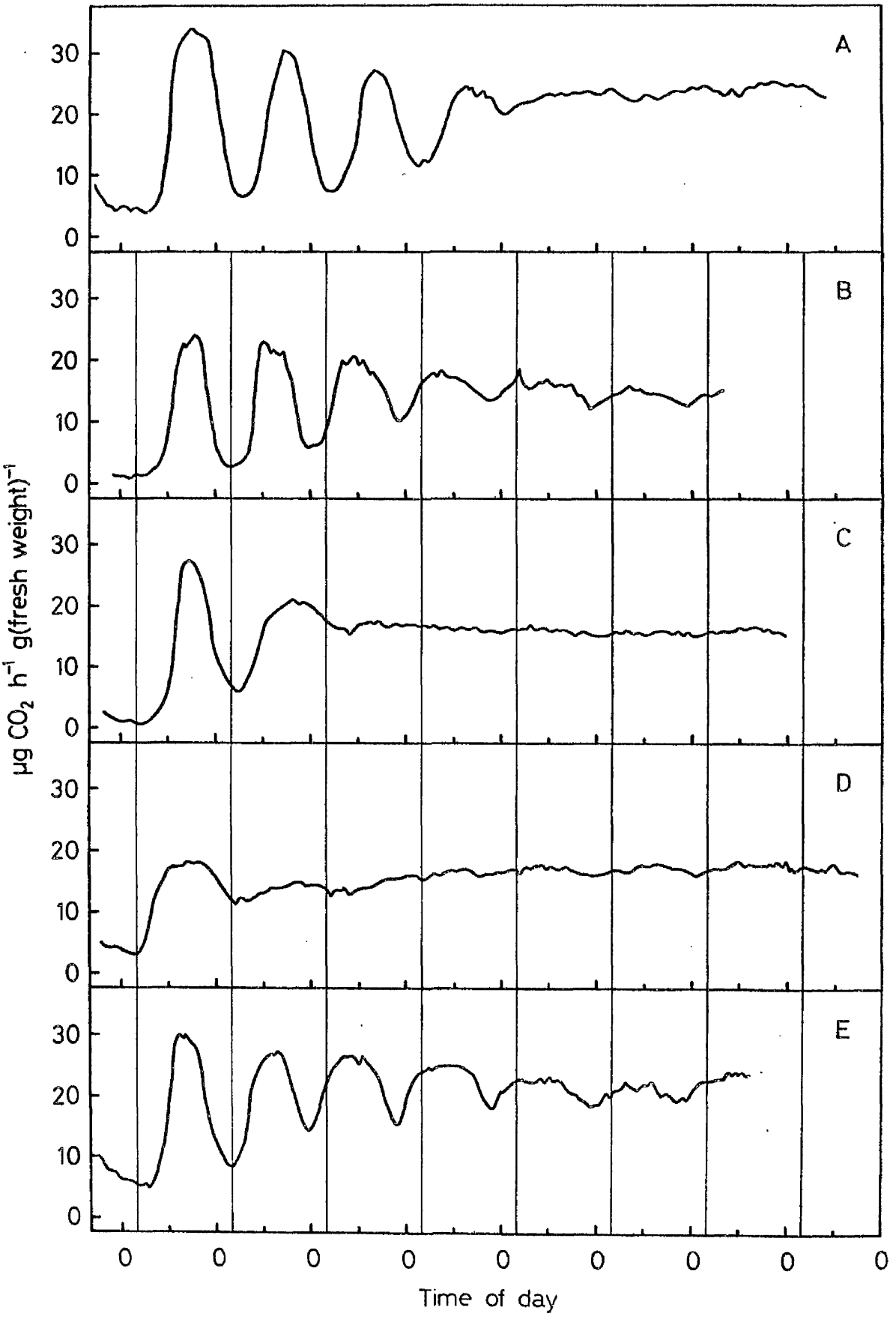
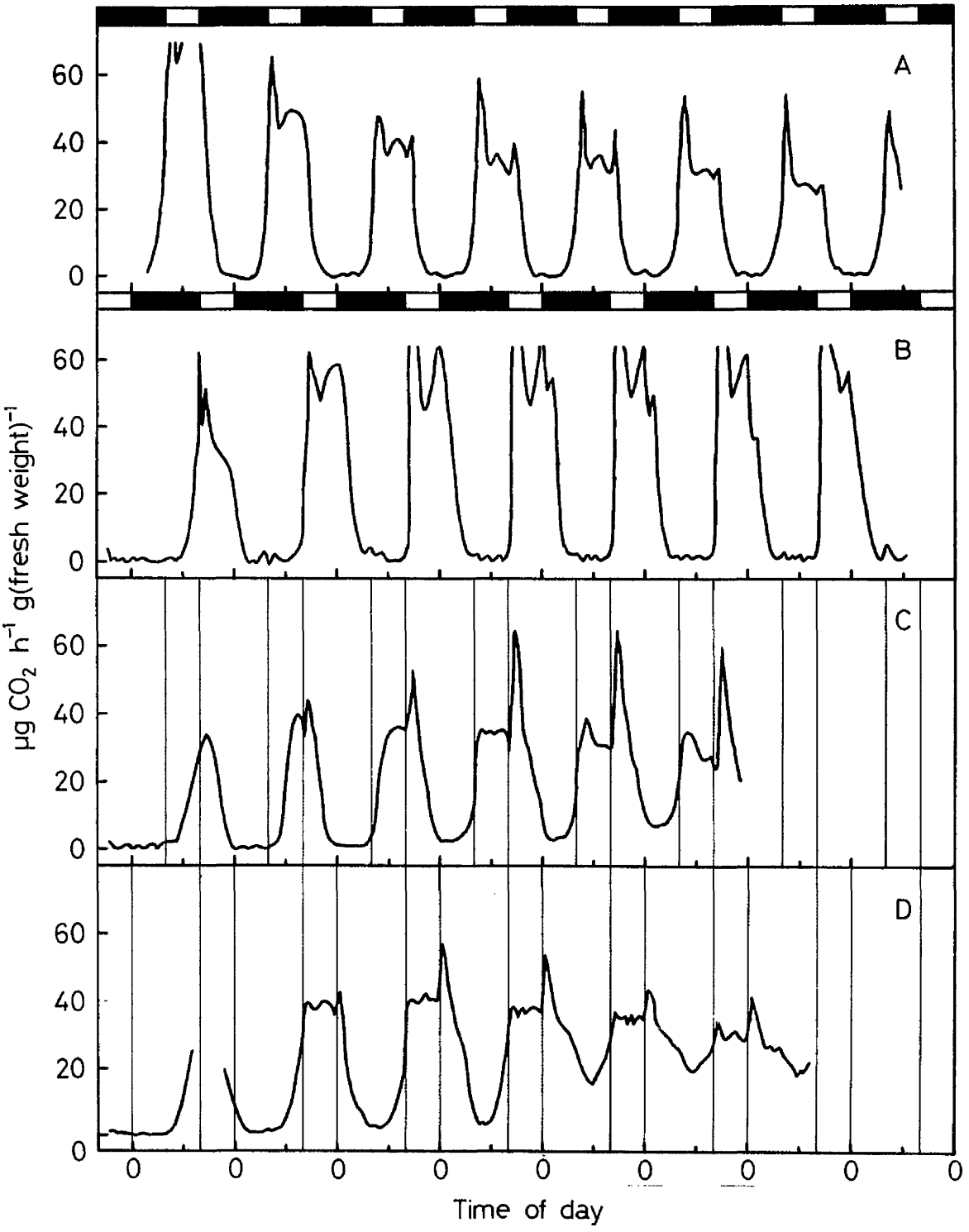

A
B
C
D
E
30
20
10
0
μg CO_2 h^{-1} g(fresh weight)$^{-1}$
0 0 0 0 0 0 0 0 0
Time of day

FIGURE 15

The effectiveness of LD 8:16 and corresponding skeleton photoperiods in entraining the circadian rhythm in Bryophyllum.

In A and B, dark periods are indicated by shaded bars above the trace. In C and D, vertical lines indicate the times of 0.25-h irradiations. 0 = midnight.


A
B
C
D
60
40
20
0
μg CO_2 h^{-1} g(fresh weight)$^{-1}$
0 0 0 0 0 0 0 0 0
Time of day

obtained when leaves were first exposed to the 8-h photoperiod. The rhythms were rapidly entrained, adopting an identical phase relationship to the entraining cycles irrespective of the order in which the leaves experienced light and darkness. The entraining cycle in Figure 15A was 8 h out of phase with that in Figure 15B. Consequently, the rhythms in leaves exposed to these schedules persisted throughout the experiment 8 h out of phase with one another. In each case the peaks of carbon dioxide release were confined almost entirely to the photoperiod. Little or no carbon dioxide was evolved by the leaves during the ~~peak~~ dark period.

The circadian rhythms of plants such as Lemna (Hillman, 1970) and of some insects (Minis, 1965; Pittendrigh, 1965) are entrained by 24-h cycles which include two brief irradiations. With such cycles, entrainment often corresponds closely to that obtained with a single photoperiod, the beginning and end of which occur at the same time as the two light pulses. The two light pulses are referred to as the skeleton of the corresponding complete photoperiod.

In order to determine whether or not the carbon dioxide output rhythm of Bryophyllum may be entrained by both complete and skeleton photoperiods, the schedules in Figures 15A and B were replaced with 24-h light (L) - dark(D) sequences of either 16hD:0.25hL:7.5hD:0.25hL or 0.25hL:7.5hD:0.25hL:16hD (Figure 15C and D). The leaves were thus exposed to 0.25-h light pulses marking the beginning and end of the corresponding complete photoperiods shown in Figure 15A and B.

The same phase relationship was established between the rhythm and the entraining cycle irrespective of whether the 7.5-h or the 16-h dark period was experienced first. The peak of carbon dioxide emission occurred in each case, between the two light pulses separated by 7.5 h

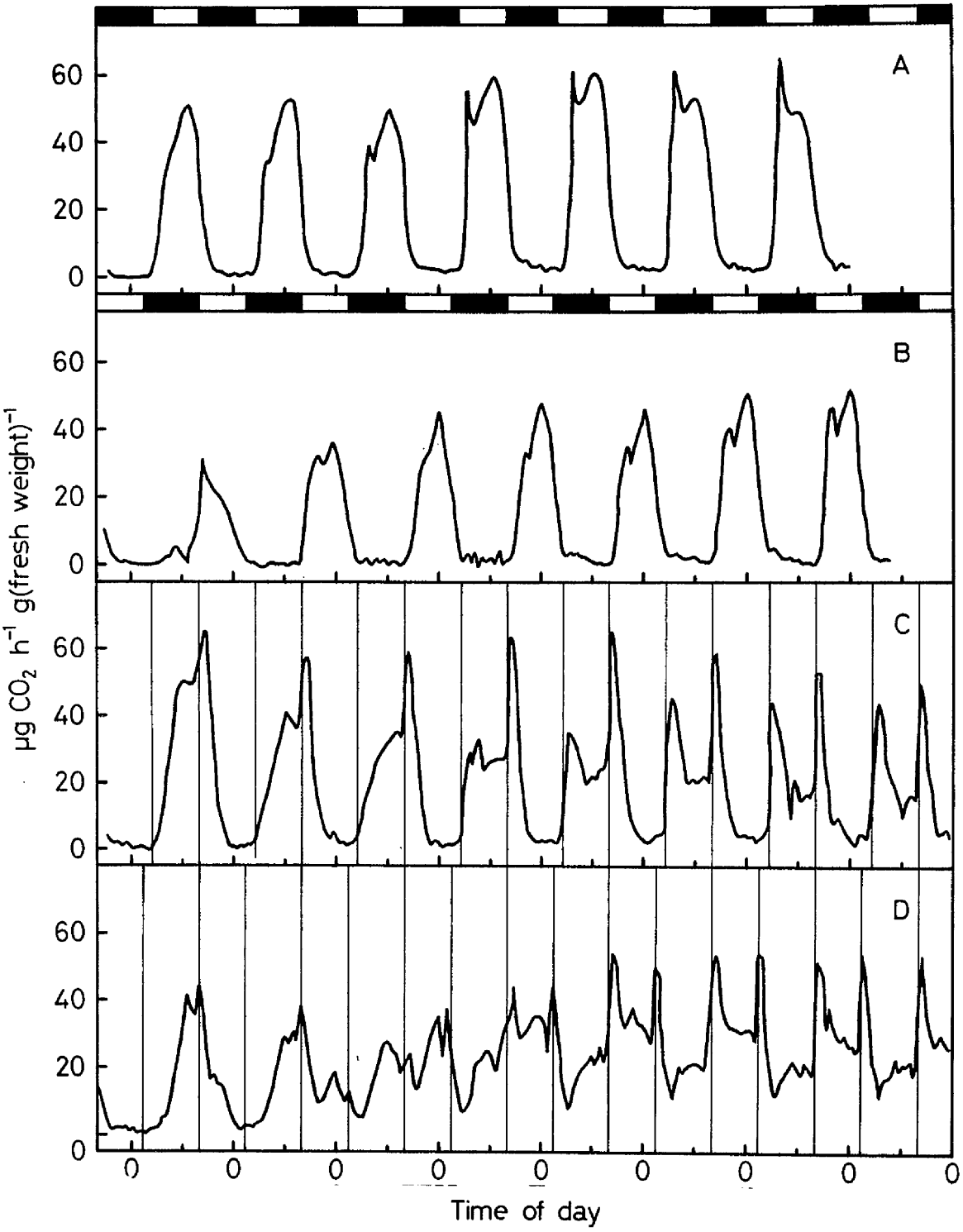
of darkness. The rhythm was clearly entrained by the skeleton photoperiod in the same general manner as by LD 8:16. The schedules employed were interpreted as LD 8:16 rather than LD 16.5:7.5 for which they may also be considered skeleton photoperiods. The rhythms in leaves exposed to the skeleton photoperiods were not however identical to those in leaves exposed to complete photoperiods. Entrainment was complete by the end of the first 24-h cycle with 8-h photoperiods, but required 3-4 cycles of skeleton photoperiods. The peaks of carbon dioxide emission were broader when leaves were exposed to skeleton photoperiods than when they were exposed to complete photoperiods. In particular, the rate of carbon dioxide output continued at a high level for several hours after the irradiation marking the end of the corresponding complete photoperiod.

Figure 16 shows the results of a similar series of experiments in which leaves were exposed to LD 11:13. As with LD 8:16, complete photoperiods rapidly entrained the rhythm and maintained it in the same phase relationship to the entraining cycles, irrespective of whether the 13-h dark period (Figure 16A) or the 11-h photoperiod (Figure 16B) was experienced first by the leaves. Carbon dioxide emission was again confined largely to the photoperiod. Figure 16C and D show the effectiveness in entraining the rhythm of skeleton photoperiods corresponding to the complete photoperiods shown in Figure 16A and B. These skeleton photoperiods entrained the rhythm, although, as was found with the 8-h photoperiods, entrainment was slower than with complete photoperiods. The peaks of carbon dioxide output occurred principally during the 10.5-h dark period, suggesting that the light pulses were interpreted as the skeleton of LD 11:13 rather than the alternative LD 13.5:10.5. Differences are apparent however, between the rhythms in Figure 16C and D. Unlike the effects of skeleton photoperiods representing LD 8:16, those of photoperiods representing LD 11:13 differed, depending on which of

FIGURE 16

The effectiveness of LD 11:13 and corresponding skeleton photoperiods in entraining the circadian rhythm in *Bryophyllum*.

In A and B, dark periods are indicated by shaded bars above the trace. In C and D, vertical lines indicate the times of 0.25-h irradiations. 0 = midnight.


A
B
C
D
60
40
20
0
μg CO2 h-1 g(fresh weight)-1
0 0 0 0 0 0 0 0 0
Time of day

the dark periods was given first. When the 10.5-h dark period was given first the rhythm passed through a number of transient cycles. In its final steady state the rhythm exhibited a distinct shoulder or sub-peak during the 13-h dark period in addition to the major peak during the 10.5-h dark period. This did not occur with the schedule in which the 13-h dark period preceeded that of 10.5-h.

When leaves were exposed to LD 12:12 (Figure 17A and B), the rhythm was entrained. Thus all cycles tested which contained a complete photoperiod entrained the rhythm in a similar manner. Neither the schedule 12hD:0.25hL:11.5hD:0.25hL (Figure 17C) nor 0.25hL:11.5hD:0.25hL: 12hD (Figure 17D) entrained the rhythm, although they may be considered the skeletons of either LD 12:12 or LD 12.5:11.5. This result differs from that obtained with the skeletons of shorter photoperiods. Exposure of the leaves to two daily light pulses in these schedules resulted in a loss of the circadian nature of the carbon dioxide output. The brief irradiations induced small temporary increases in the rate of carbon dioxide release and additional, regular small peaks were observed in some experiments during each of the dark periods.

1.5 Skeleton photoperiods of red light.

Figure 18 shows the effect on the Bryophyllum rhythm of two 0.25-h exposures to monochromatic radiation of 660 nm at a radiant flux density of 85 mJ m^{-2} s^{-1}. These were arranged in the same sequence as in Figure 17, representing the skeleton photoperiods of LD 12:12 or LD 12.5:11.5. When the 12-h dark period was given first (Figure 18A) there was little modification of the phase of the rhythm compared with that in leaves in darkness or exposed to a single red pulse at 1600h each day (Figure 11B). However, the rhythm did not persist after the fifth peak of carbon dioxide emission. When the 11.5-h dark period was given first the results were

FIGURE 17

The effectiveness of LD 12:12 and corresponding skeleton photoperiods in entraining the circadian rhythm in Bryophyllum.

In A and B, dark periods are indicated by shaded bars above the trace. In C and D, vertical lines indicate the times of 0.25-h irradiations. 0 = midnight.

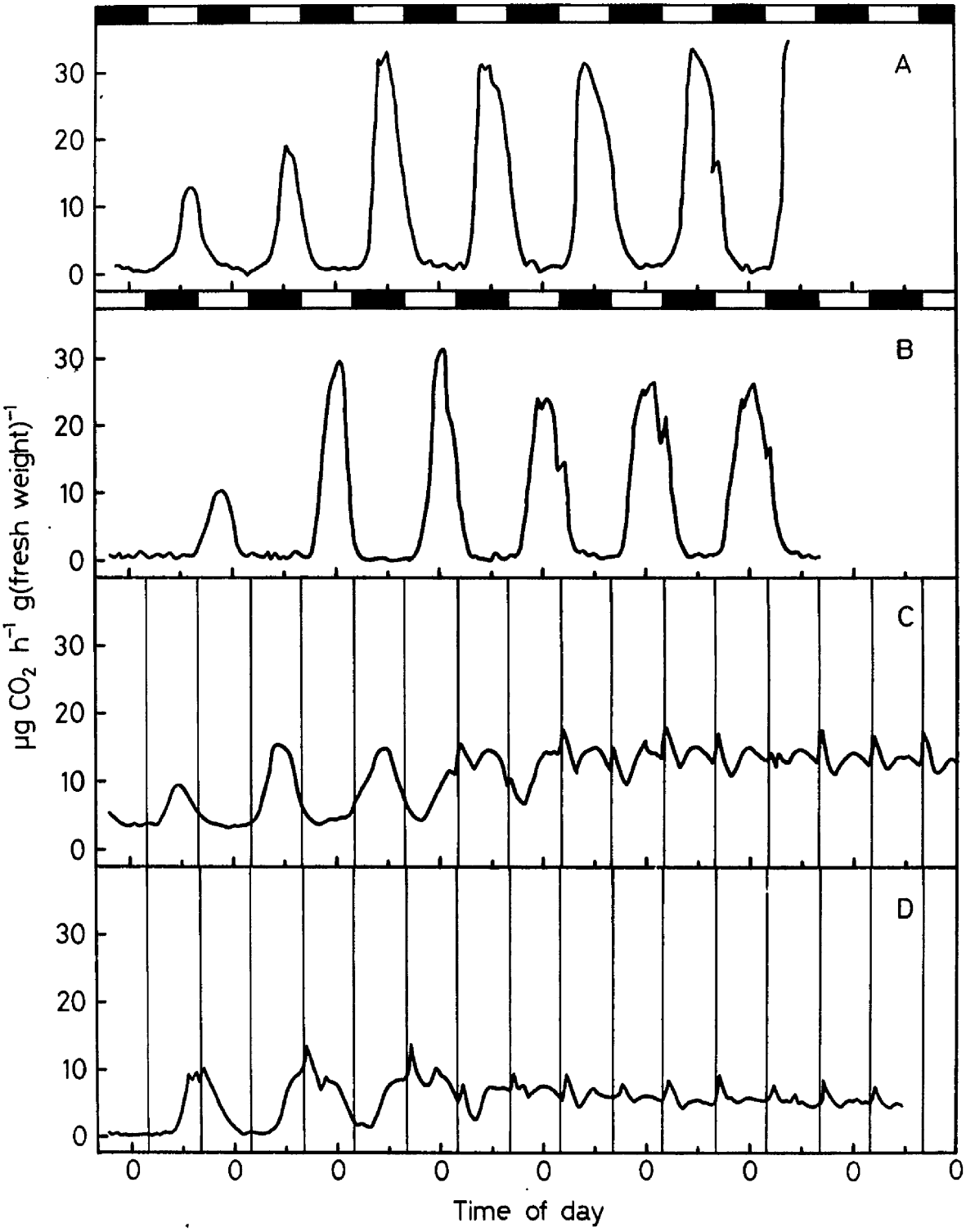
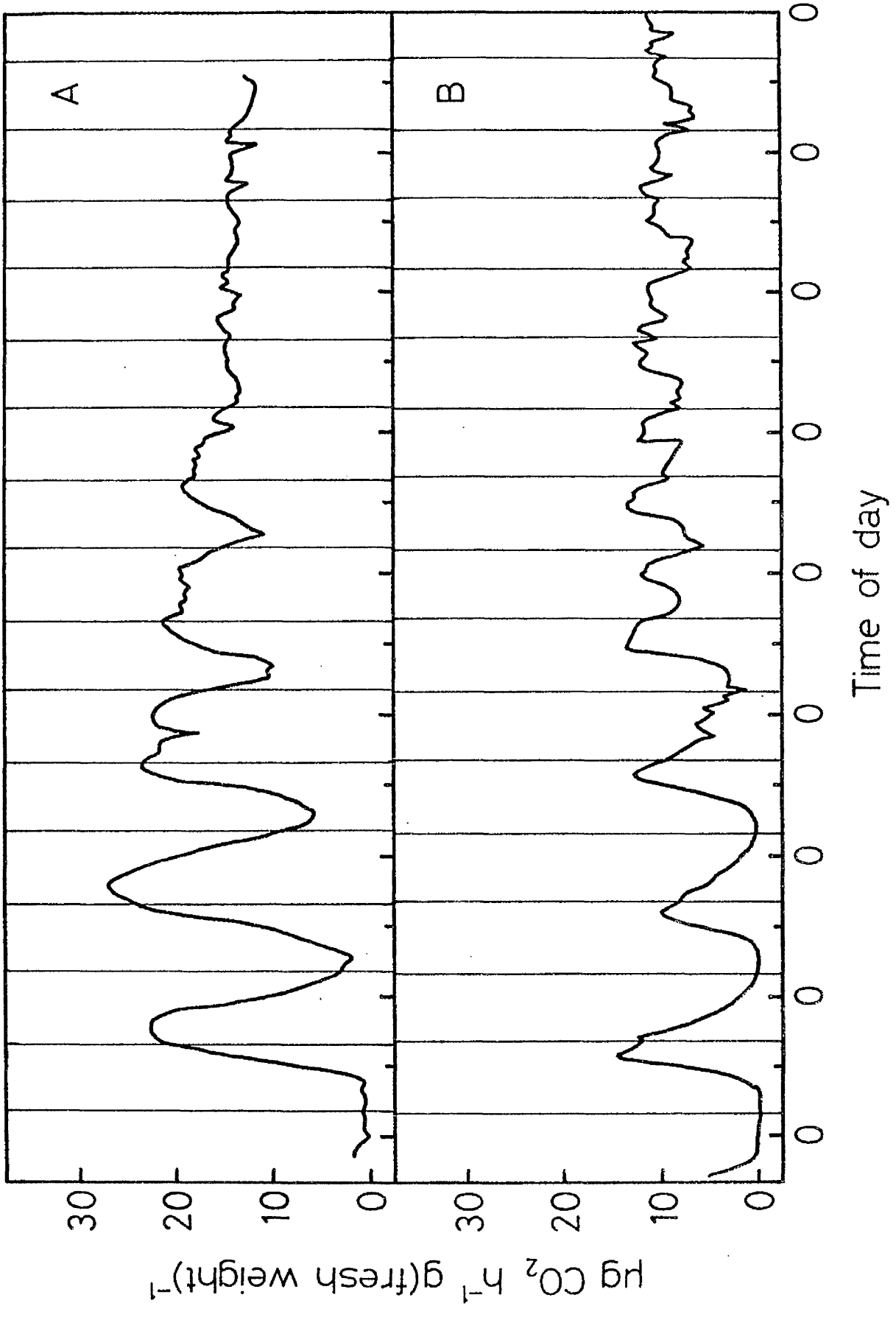

A
B
C
D
30
20
10
0
μg CO2 h-1 g(fresh weight)-1
0 0 0 0 0 0 0 0 0
Time of day

FIGURE 18

The effect on the circadian rhythm of carbon dioxide output in Bryophyllum of 0.25-h exposure to monochromatic (660 nm) radiation, arranged as the skeleton photoperiod of LD 12:12.

Vertical lines indicate the times of 0.25-h irradiations. 0 = midnight.

FIGURE 18


A
B
30
20
10
0
0
0
0
0
0
Time of day
μg CO2 h-1 g(fresh weight)-1

somewhat variable although the carbon dioxide output appeared to be of a diurnal nature, with a fairly regular pattern of sub-peaks superimposed on a 24-h rhythm. It is clear however that skeleton photoperiods of red light are ineffective in replacing the complete photoperiod of LD 12:12 obtained with white light.

1.6 Interaction of red and far-red radiation in skeleton photoperiods.

The modification of the circadian rhythm of carbon dioxide output in leaves exposed to two 0.25-h irradiations, given as the skeleton photoperiod of LD 12:12, presented an opportunity to further test the red/far-red reversibility of the entrainment phenomenon. If the effect of one of two such pulses was negated, entrainment of the rhythm by the other pulse should occur. This might be expected to produce clearer evidence for red/far-red reversal than the abolition or modification of the rhythm obtained with far-red radiation applied after a single red pulse (Figure 14D).

The inhibition of the rhythm was more marked when two pulses of red light, of the same quality and incident radiant flux density as used in the experiment shown in Figure 18, were applied between 0400 and 0415 h and 1600 and 1615 h each day (Figure 19A). If, in a similar schedule, the 0.25-h pulse of red light commencing at 1600 h was immediately followed by a 0.25-h exposure to far-red radiation at a radiant flux density of 7.8 J m^{-2} s^{-1} (Figure 19B) the rhythm was not lost, but persisted for as long, and with the same phase, as when leaves received only one exposure to red light at 0400 h each day (Figure 11A, 14B).

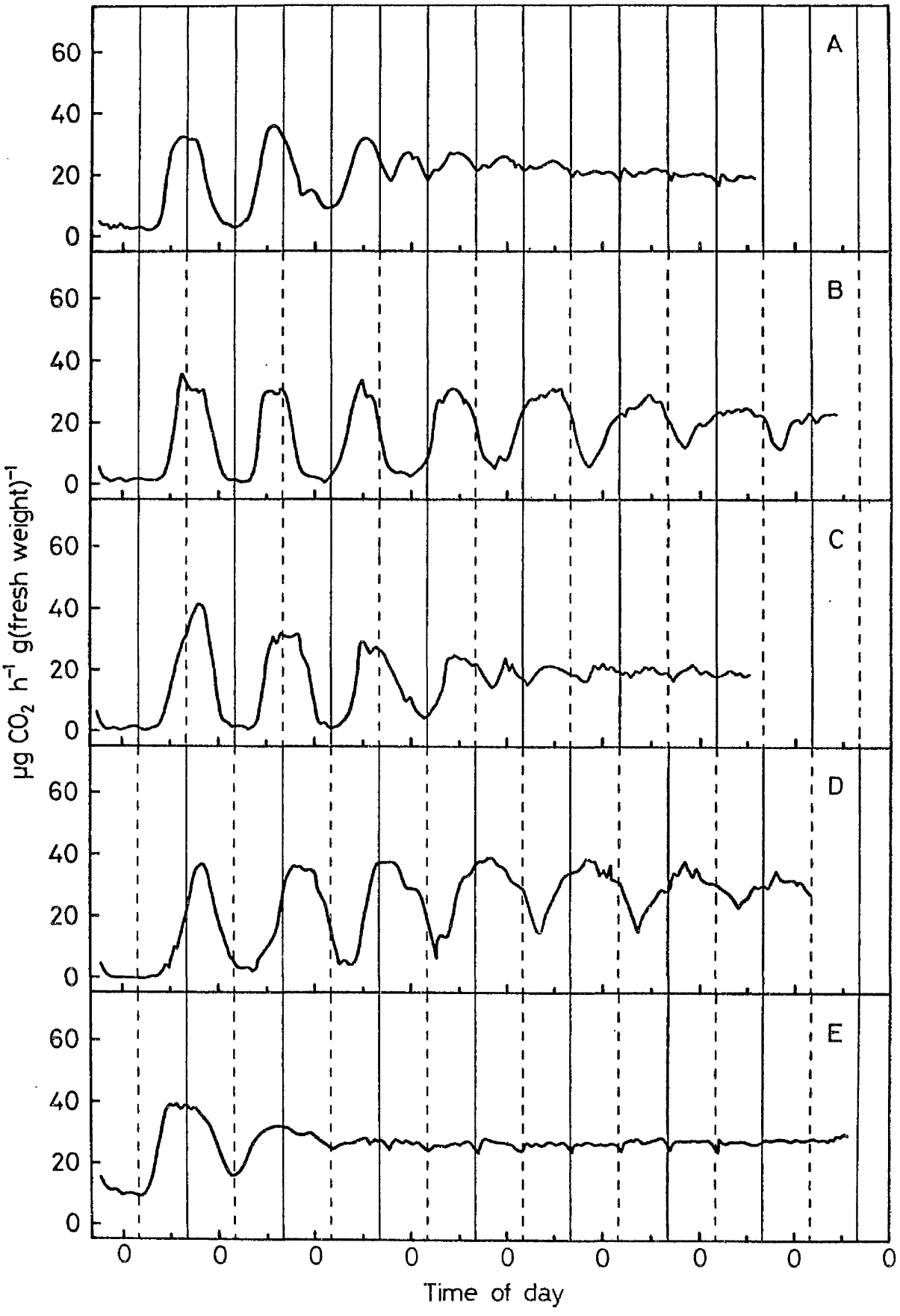
When the leaves were exposed to two pulses of red light each day, and the exposure at 1600 h was immediately ~~preceded~~ preceded by far-red radiation, the circadian rhythm was either abolished after three or four cycles,

FIGURE 19

The effect on the circadian rhythm of carbon dioxide output in Bryophyllum of exposures to 0.25 h red light (vertical lines) commencing at 1600 and 0400 h (A).

The effect of a 0.25-h exposure to far-red radiation immediately after or immediately before one of the exposures to red light used in A are shown in B - E. In each case the red light pulses are shown by solid vertical lines, while the broken lines indicate either red followed by far-red radiation (B and D) or far-red followed by red radiation (C and E).

0 = midnight.


A
B
C
D
E
60
40
20
0
μg CO_2 h^{-1} g(fresh weight)$^{-1}$
0 0 0 0 0 0 0 0 0
Time of day

or continued with a low amplitude 12-h periodicity (Figure 19C), a result which was similar to that in Figure 19A.

Similarly, if leaves were exposed to two pulses of red light each day and the pulse at 0400 h was immediately followed by a 0.25-h exposure to far-red radiation (Figure 19D), the rhythm was entrained in the same way and with the same phase as if the leaves had received only one pulse of red light at 1600 h (Figure 11B). If the irradiation at 0400 h was immediately ~~preceded~~ preceded by far-red radiation, a result was again obtained which was similar to that from leaves exposed to red light at both 0400 and 1600 h.

Thus there is clear evidence from the data presented in Figure 19 for reversal of the effectiveness of red, by far-red radiation and hence for the involvement of phytochrome ~~in~~ as the photoreceptor pigment. Only in this kind of experiment, in which entrainment of the rhythm was studied by using two 0.25-h exposures of red light every day was it possible to observe a totally reversible red/far-red effect on the circadian rhythm in Bryophyllum leaves.

2. Initiation of the rhythm by transfer from light to darkness.

The rhythm in the rate of carbon dioxide output in leaves of Bryophyllum can be initiated or reset by a transfer from light to darkness (Wilkins, 1959), or by a reduction in the incident radiant flux density (Wilkins, 1960a). In the experiments reported here rhythms were initiated by transferring the leaves from the growth room, in which the plants were held prior to the experiment, to darkness or low intensity white light, at the end of the photoperiod. In constant conditions of either light or darkness the amplitude of the rhythm became progressively smaller and the carbon dioxide output eventually became arhythmic. Figure 20 shows that after the rhythm had faded out in white light at a radiant flux density of 0.6 J m^{-2} s^{-1}, it could be reinitiated by switching off the light. The first peak of this new rhythm occurred after 21 h and although only two peaks could be recorded, the rhythm was clearly of a circadian nature with a period of approximately 24 h.

Figure 21 A-D shows the activity of various regions of the spectrum in this response. Leaves were exposed to a quantum flux density of 47 pE cm^{-2} s^{-1} in spectral bands centred on 450, 530, 660, and 730 nm for 5-7 d until the rhythm had damped. The monochromator lamps were then switched off. Figure 21C shows that the transition from continuous irradiation with a spectral band centred on 660 nm to darkness re-established the rhythmic nature of the carbon dioxide output. The first peak of this rhythm occurred 19 h after the transfer to darkness, a second peak occurring 24 h after the first. Some evidence of one peak of low amplitude was found after transfer of the leaves from continuous exposure to radiant energy of wavelength 530 (Figure 21B) or 730 nm (Figure 21D) to darkness. The spectral band centred on 450 nm was without effect in this respect. It is therefore principally the red region of the spectrum which is responsible for the effects observed with white light.

FIGURE 20

The effect, on the rate of carbon dioxide output, of transferring to darkness Bryophyllum leaves which had been exposed to continuous white light for 107 h. Darkness is indicated by a shaded bar above the trace. The rhythm in a leaf in darkness throughout the experiment is shown by the broken line.

0 = midnight.

FIGURE 20

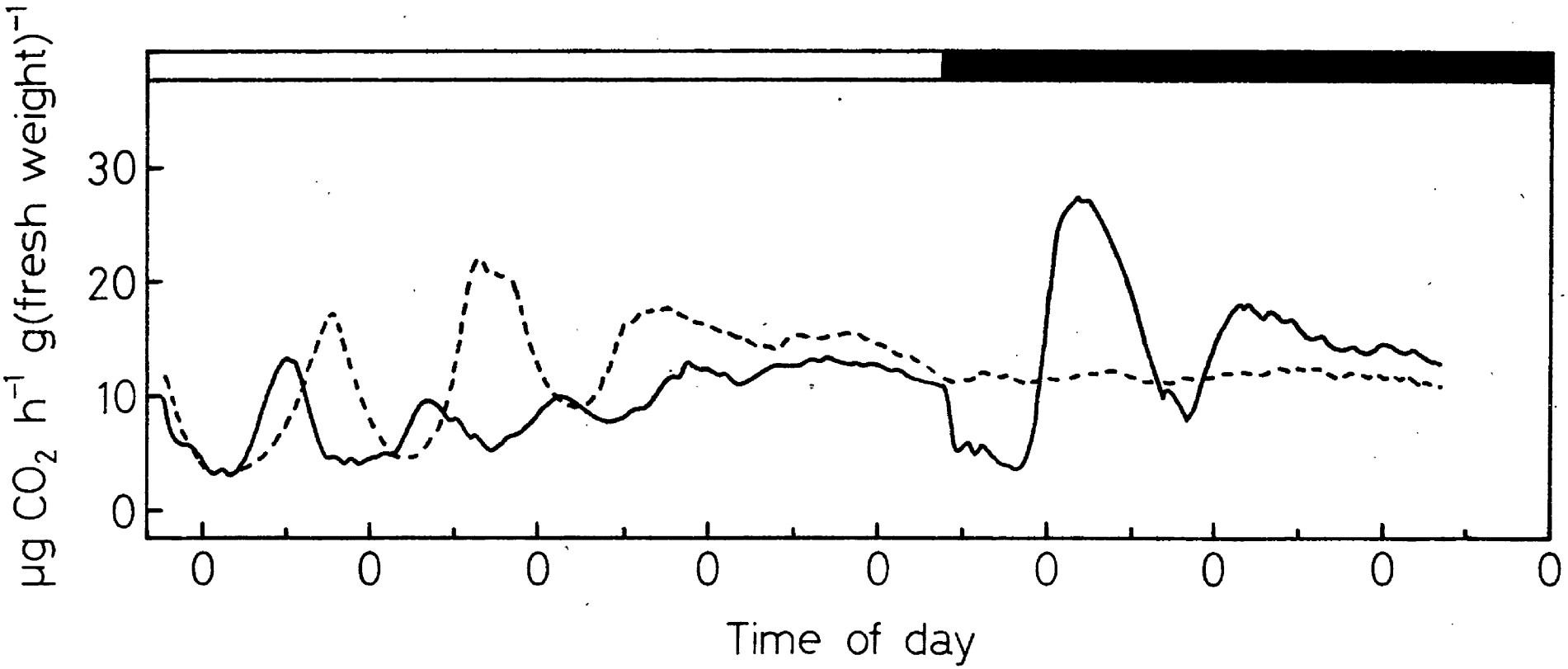
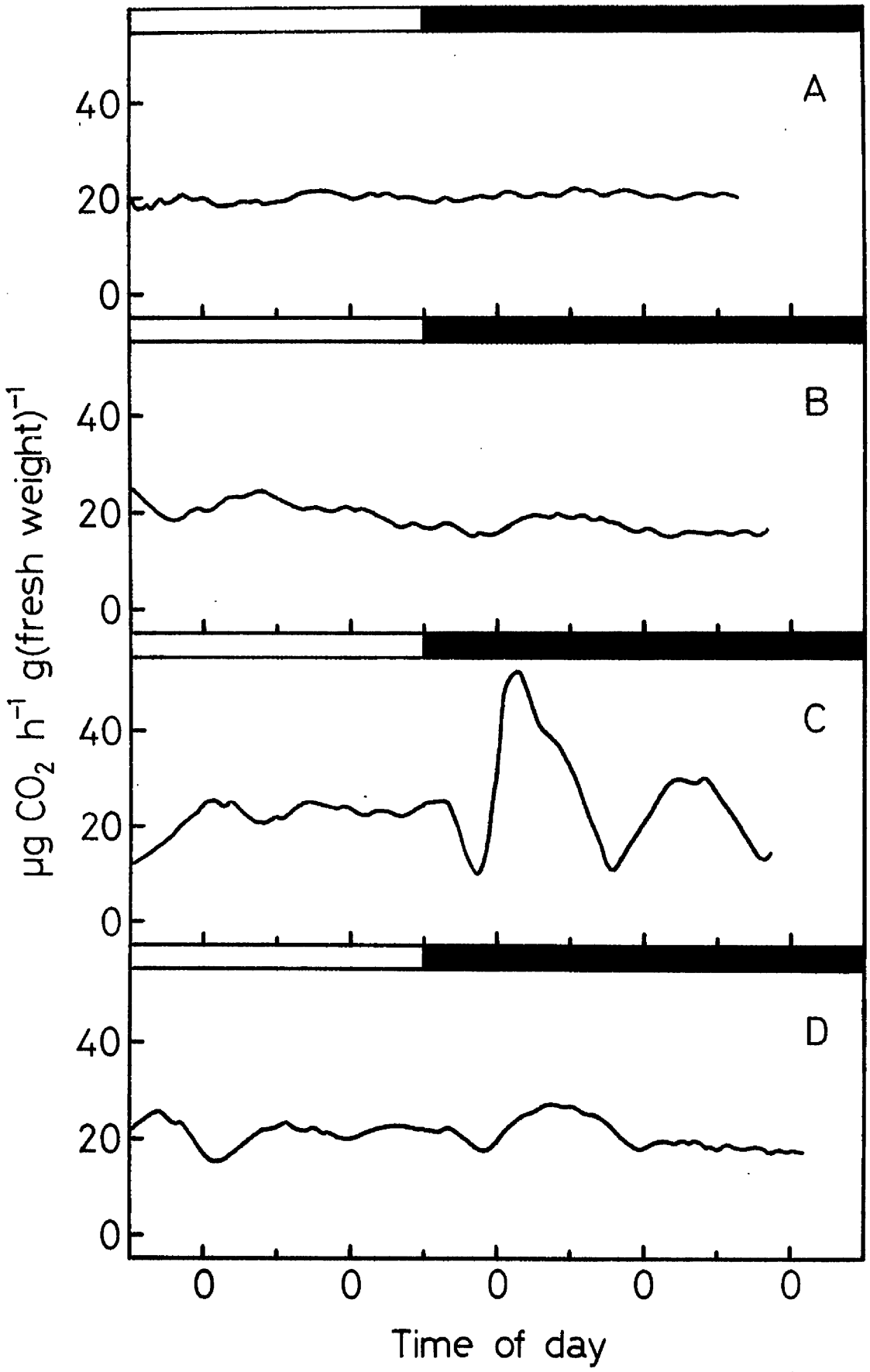

μg CO_2 h^{-1} g(fresh weight)$^{-1}$
30
20
10
0
0 0 0 0 0 0 0 0 0
Time of day

FIGURE 21

The effect, on the rate of carbon dioxide output, of transferring to darkness, Bryophyllum leaves which had been exposed to an equal quantum flux density in spectral bands centred on 450 (A), 530 (B), 660 (C) and 730 nm (D) for 5-7 d. Shaded bars above the trace indicate darkness.
0 = midnight.


A
B
C
D
40
20
0
μg CO_2 h^{-1} g(fresh weight)$^{-1}$
0 0 0 0 0
Time of day

3. Phase control by single exposures to radiant energy.

3.1 Phase shifts induced by white light.

The relative effectiveness of a standard exposure to radiant energy in shifting the phase of the rhythm was assessed as a function of the time in the cycle at which it was applied. Leaves in darkness were exposed once to a 4-h interruption with white fluorescent light at a radiant flux density of 0.6 J m^{-2} s^{-1}. One and a half cycles of the rhythm were scanned at intervals of 1 or 2 h beginning at 0400 h, 12 h after the leaves had been transferred from the growth room to continuous darkness. The results of a number of individual experiments are shown in Figures 22-25 to illustrate the types of results obtained.

When the light interruption was given before the first peak of carbon dioxide output, at the end of the period of minimum carbon dioxide release, the first and subsequent peaks occurred a few hours earlier (Figure 22 A-C). With interruptions, the mid-points of which occurred 16 or 18 h after the start of the experiment, no effect on the phase of the rhythms was observed. Light interruptions applied after the first peak of carbon dioxide output, delayed the occurrence of the second major peak. However, a small peak or shoulder occurred soon after the end of the light period.(Figure 24 A-C). Figure 24 shows that as the light interruptions were given later,during this the most sensitive part of the cycle, the minor peak became larger until it clearly constituted the advance second peak (Figures 24D and 25A). Interruptions during the second cycle had essentially the same effect as those applied during the first cycle.

Figure 26 shows the combined results of a series of experiments of the type illustrated in Figures 22-25. Each point is the mean of at least two, and in most cases more, individual measurements. The times of

FIGURE 22

Effect of a 4-h exposure to white fluorescent light on the phase of the circadian rhythm of carbon dioxide output in Bryophyllum leaves, otherwise kept in continuous darkness. The positions of the light treatments in the cycle are shown by the shaded bars. The rhythms in control, unirradiated leaves are shown by the broken lines.

0 = midnight.

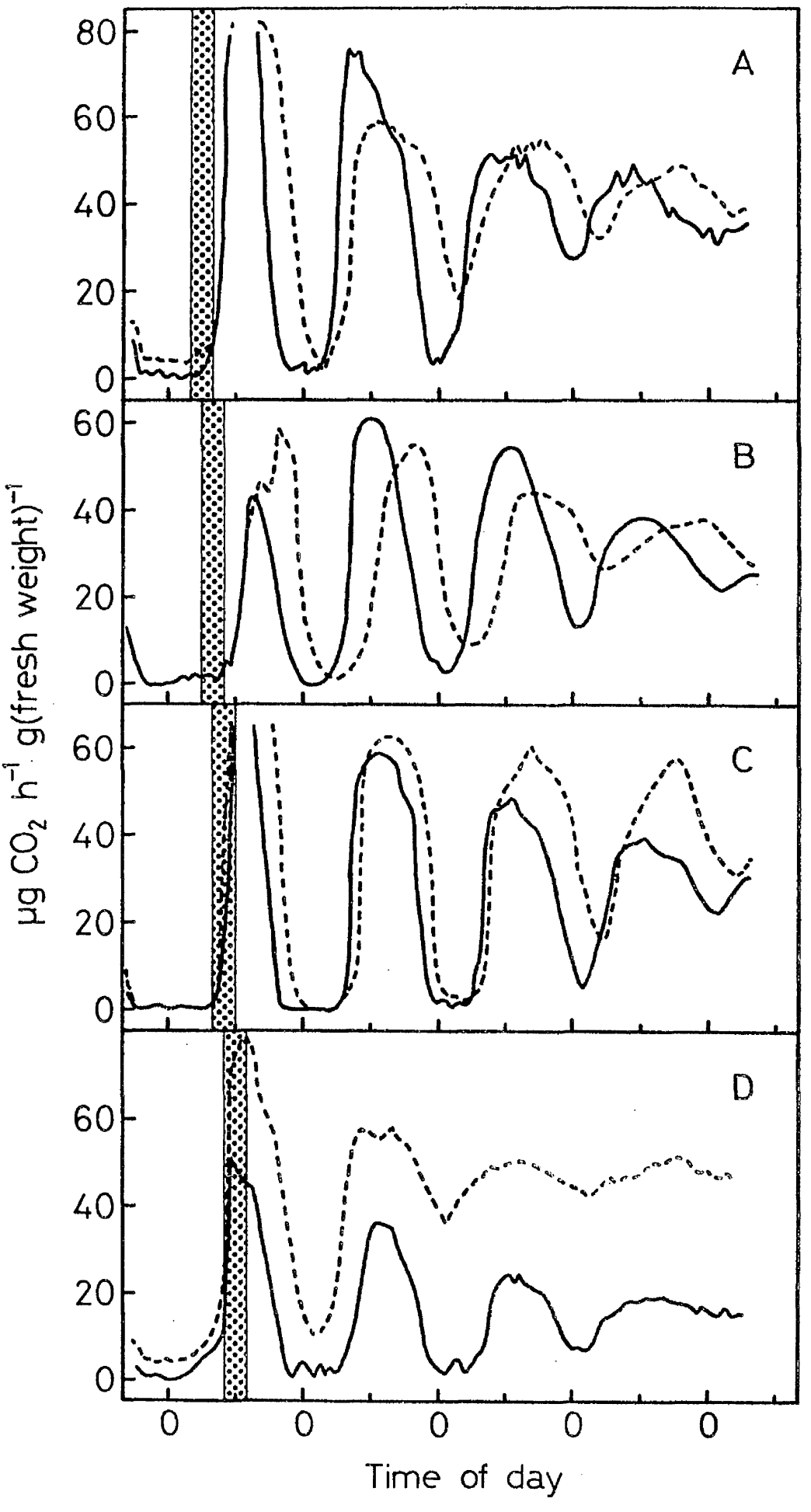

A
B
C
D
80
60
40
20
0
μg CO_2 h^{-1} g(fresh weight)$^{-1}$
0
0
0
0
0
Time of day

FIGURE 23

Effect of a 4-h exposure to white fluorescent light on the phase of the circadian rhythm of carbon dioxide output in Bryophyllum leaves, otherwise kept in continuous darkness. The positions of the light treatments in the cycle are shown by the shaded bars. The rhythms in control, unirradiated leaves are shown by the broken lines.
0 = midnight.

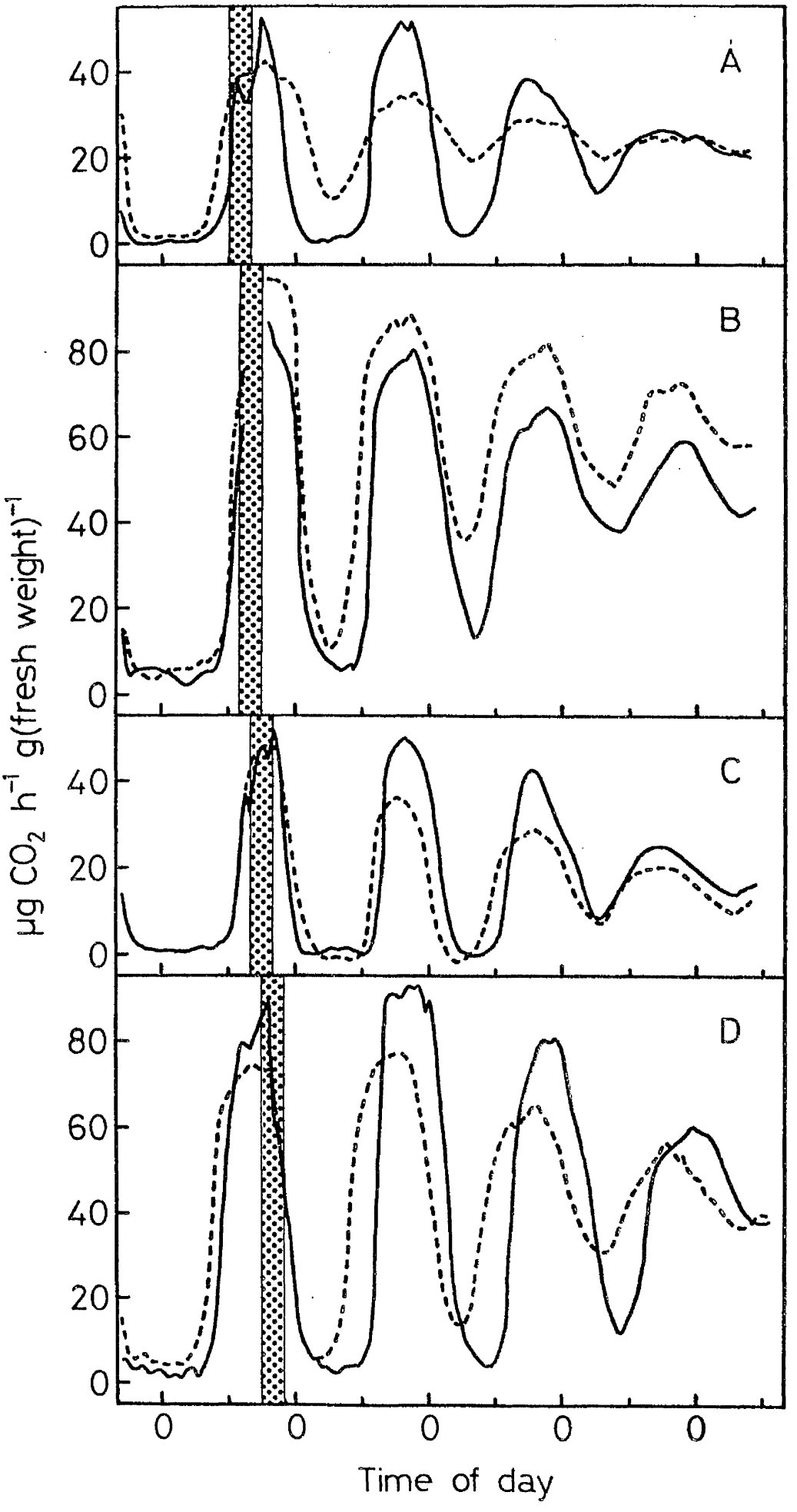

A
B
C
D
40
20
0
80
60
40
20
0
40
20
0
80
60
40
20
0
0
0
0
0
0
μg CO_2 h^{-1} g(fresh weight)$^{-1}$
Time of day

FIGURE 24

Effect of a 4-h exposure to white fluorescent light on the phase of the circadian rhythm of carbon dioxide output in Bryophyllum leaves, otherwise kept in continuous darkness. The positions of the light treatments in the cycle are shown by the shaded bars. The rhythms in control, unirradiated leaves are shown by the broken lines.

0 = midnight.

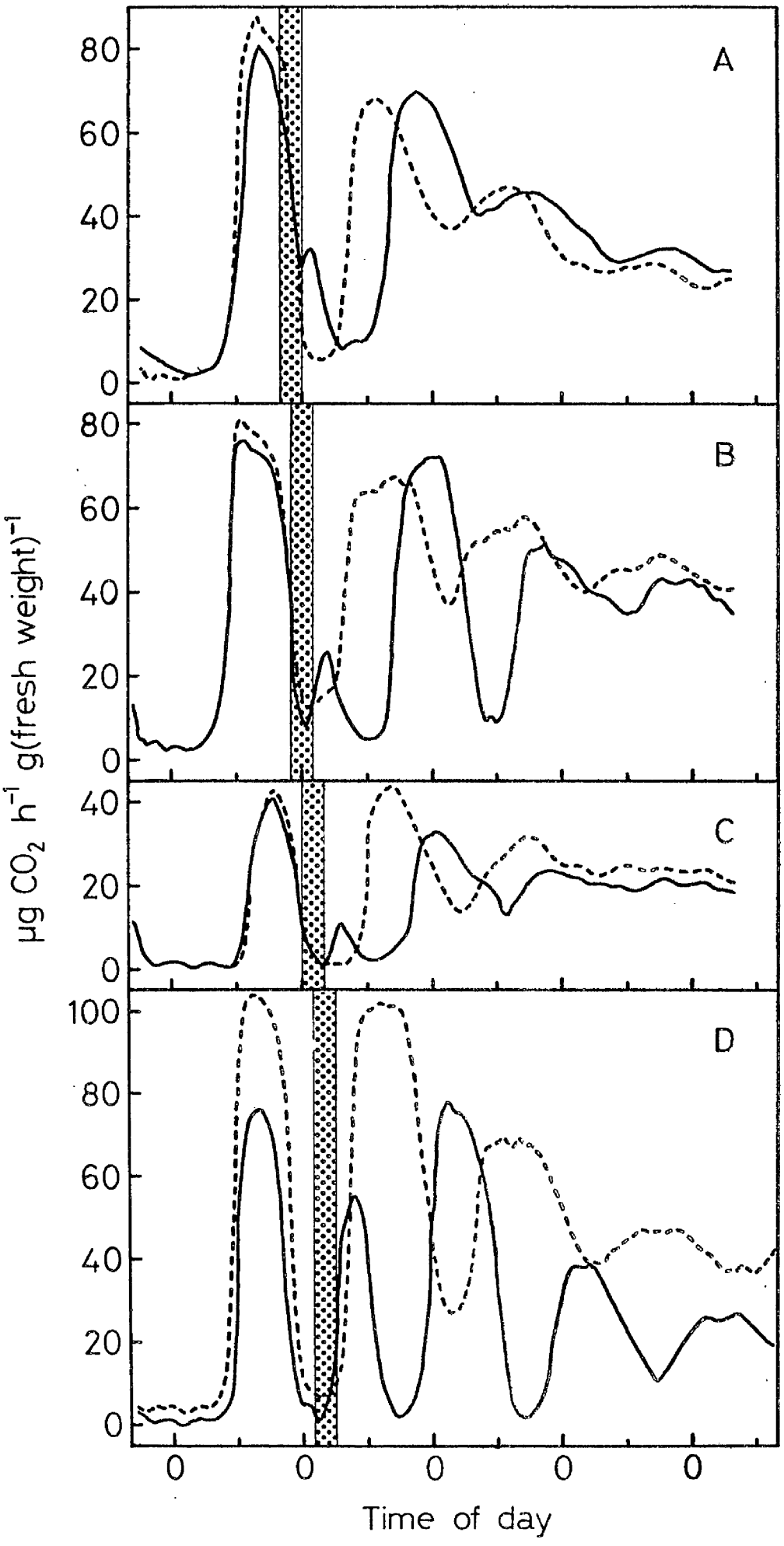

A
B
C
D
80
60
40
20
0
100
μg CO_2 h^{-1} g(fresh weight)$^{-1}$
0
0
0
0
0
Time of day

FIGURE 25

Effect of a 4-h exposure to white fluorescent light on the phase of the circadian rhythm of carbon dioxide output in Bryophyllum leaves, otherwise kept in continuous darkness. The positions of the light treatments in the cycle are shown by the shaded bars. The rhythms in control, unirradiated leaves are shown by the broken lines.

0 = midnight.

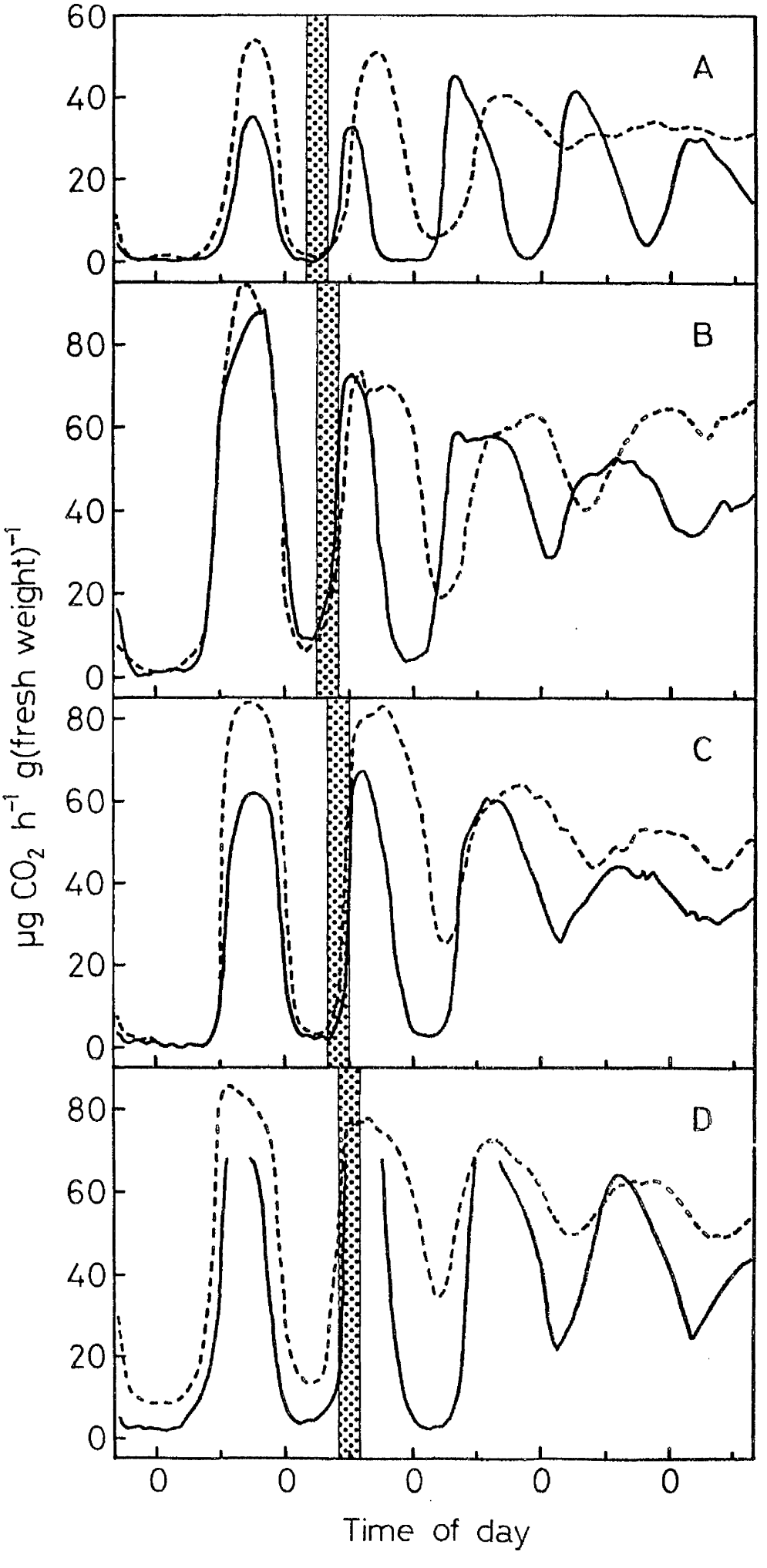
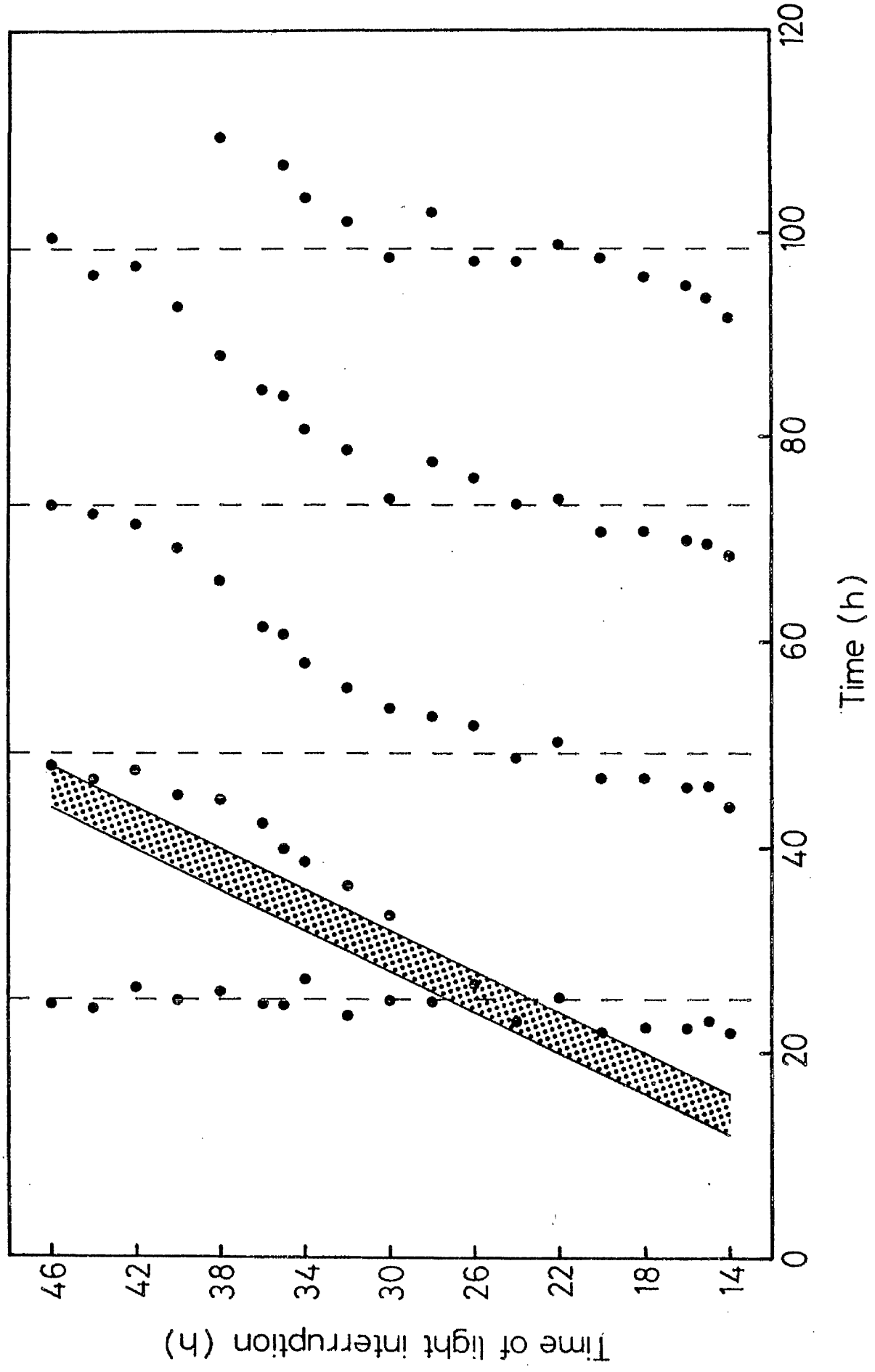

A
B
C
D
60
40
20
0
80
60
40
20
0
80
60
40
20
0
80
60
40
20
0
μg CO_2 h^{-1} g(fresh weight)$^{-1}$
0
0
0
0
0
Time of day

FIGURE 26

The time of occurrence of peaks of carbon dioxide output from Bryophyllum leaves exposed to 4 h of white light at the times indicated by the shaded bar. The times of the mid-points of irradiations are also shown on the vertical axis. The times at which peaks occurred in control, unirradiated leaves are shown by the broken lines. Time 0 is the time at which the rhythms were initiated by transfer to darkness.

FIGURE 26


Time of light interruption (h)
46
42
38
34
30
26
22
18
14
0
20
40
60
80
100
120
Time (h)

occurrence of peaks of the rhythm in leaves in darkness shown in Figure 26 are the mean values for 24 rhythms in leaves in darkness. Small peaks which occurred after the end of light interruptions, given between the first and second peaks of the rhythm in darkness, have been included in Figure 26.

In many experiments, in which leaves were exposed either once or periodically to radiant energy, a temporary increase in the rate of carbon dioxide output occurred during or soon after irradiation. As this may have represented a direct effect of radiant energy on the biochemical process being monitored, rather than on the underlying circadian oscillation, it is difficult to unequivocally distinguish between phase advances and delays in this series of experiments. It is clear, however, that in each experiment in which the phase of the rhythm was changed by irradiation of the leaves, the rhythm had been reset with a peak of carbon dioxide emission occurring 21.5 - 29 h after the end of the light interruption. On the basis of this observation it can be concluded that whether or not a phase shift is induced and its magnitude are determined by the time of application of the light treatment. The time between the end of the treatment and the occurrence of a peak is not precisely the same following irradiation of the leaves at various times in the cycle. This is apparent from the fact that a line joining the times of occurrence of peaks would not be parallel to that marking the end of the light treatment.

From these conclusions one can predict that at one point in the cycle, 21.5 - 29 h before a peak, an interruption of darkness with white light would be ineffective in shifting the phase of the rhythm. This was confirmed in this series of experiments, an interruption ending 26 h before the expected time of occurrence of the second peak of the rhythm

in darkness having no effect on the phase of that rhythm. It is also apparent that the first peak of the rhythm occurs a similar time after the initiating transfer from light to darkness, in this case 25.2 h after the start of the experiment.

The magnitude of the phase shifts induced by the light treatments are virtually the same in three succesive cycles after the light interruption. Thus the new steady state is reached rapidly, at least by the time of the peak which occurs 21.5 - 29 h after the light interruption.

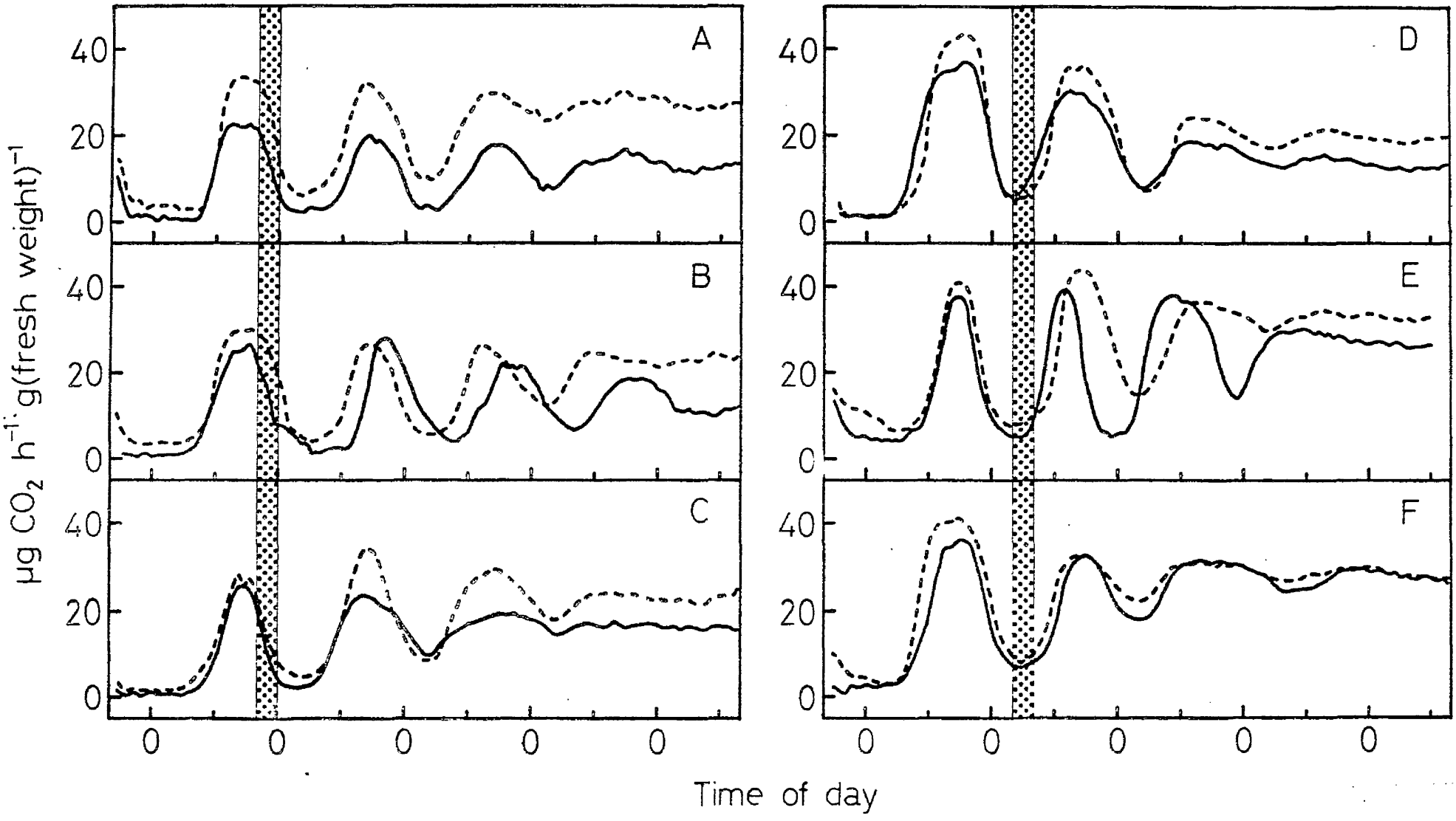
3.2 Phase shifts induced by monochromatic radiation.

Leaves in darkness were exposed for 4 h to monochromatic radiation at a quantum flux density of 47 pE cm^{-2} s^{-1}. Spectral bands centred on 450, 660, and 730 nm were used. These were selected to include wavelengths fr the red region of the spectrum, previously found to be active in shifting the phase, and radiant energy of shorter and longer wavelength reported to be ineffective in this respect (Wilkins, 1973).

Radiant energy in these bands was applied at several different times during the cycle to assess its effectiveness in inducing phase shifts. The effect on the phase of the rhythm, of exposures at two different times in the cycle, are shown in Figure 27. When applied between 2000 and 0000 h during the second day in darkness, the spectral band centred on 660 nm induced a phase delay of 3-4 h, a peak occurring 21.5 h after the end of the irradiation. An exposure to radiant energy in this band between 0400 and 0800 h in the same cycle induced a steady state phase advance of 6 h, peaks occurring 6 and 28 h after the end of the irradiation. In all parts of the cycle tested, the spectral band centred on 660 nm induced a phase shift similar to that induced by white light. Spectral bands centred on 450 and 730 nm had no measurable effect

FIGURE 27

Effectiveness, in inducing phase shifts, of a 4-h exposure to monochromatic radiation in spectral bands centred on 450 (A and D), 660 (B and E) and 730 nm (C and F). Times of exposure are shown by the shaded bars. Rhythms in leaves in continuous darkness are shown by the broken lines.
0 = midnight.


A
B
C
D
E
F
40
20
0
0
0
0
0
0
0
0
0
0
μg CO_2 h^{-1} g(fresh weight)$^{-1}$
Time of day

on the phase of the rhythm at any time in the cycle. It is therefore, principally radiant energy from the red region of the spectrum which is responsible for the phase control exerted by white light.

3.3 The interaction of red and far-red radiation in phase control.

The dependence of phase-shifting activity on wavelength found in this investigation and by Wilkins (1973) suggested the possible involvement of phytochrome in the reception of the light stimulus. In order to test this hypothesis evidence of red/far-red reversibility was sought. Exposing leaves for 4 h to red light (660 nm) at a radiant flux density of 85 mJ m^{-2} s^{-1} resulted in a phase shift of about 12 h (Figure 28A) whereas an exposure to far-red radiation at a radiant flux density of 7.8 J m^{-2} s^{-1} was without effect (Figure 28B). An exposure to red light for 4 h, followed immediately by exposure to far-red radiation for 4 h, resulted in a marked phase shift, although its magnitude was 4-5 h less than that attained with red light alone (Figure 28C). When leaves were exposed simultaneously to red and far-red radiation for 4 h (Figure 28D) the circadian rhythm of carbon dioxide output was virtually abolished. Although the phase appeared to be shifted to some extent by this treatment, it was not possible to accurately assess the magnitude of this shift. The lack of complete reversibility in these experiments might be attributed to the fact that the exposures were sufficiently long for any effect to proceed beyond the reversible photochemical stages.

Attempts to overcome this difficulty were made by exposing leaves for 4 h to rapid alternations of red light and darkness. When this treatment involved exposure to 5 min red light alternating with 5 min of darkness for 4 h, a distinct phase shift was observed (Figure 29A). In a second experiment (Figure 29B), leaves were exposed to 5 min of red light alternating with 5 min of far-red radiation for 4 h. The result

FIGURE 28

Effect of exposure to 4-h red light (A), 4-h far-red radiation (B), 4-h of red light followed by 4-h of far-red radiation (C), and red and far-red radiation simultaneously for 4-h (D), on the phase of the circadian rhythm in Bryophyllum leaves. The times of the irradiations are shown by the shaded bars, and control rhythms in leaves in continuous darkness by the broken lines.

0 = midnight.

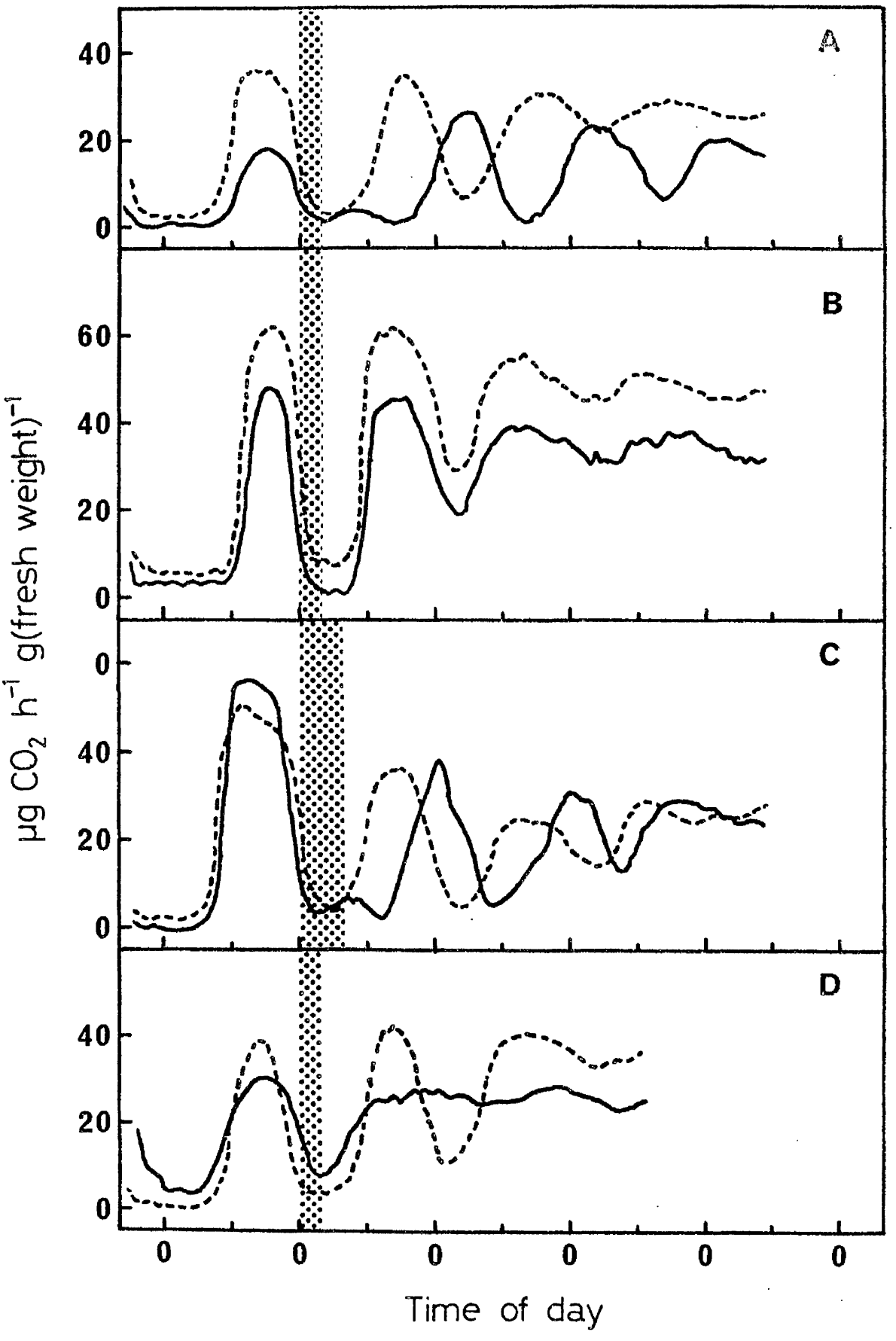
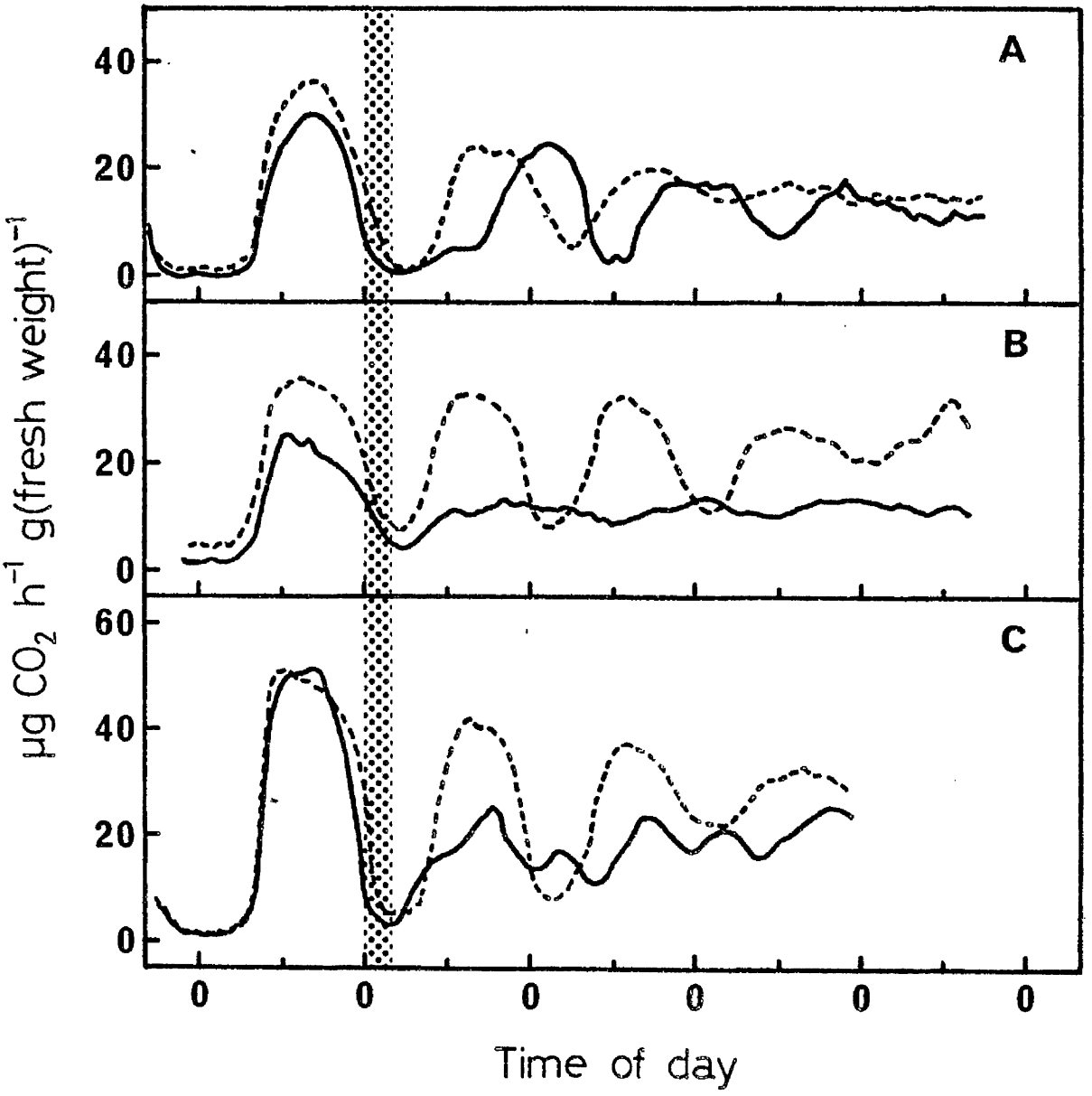

A
B
C
D
40
20
0
60
40
20
0
0
40
20
0
40
20
0
0 0 0 0 0 0
Time of day
μg CO_2 h^{-1} g(fresh weight)$^{-1}$

FIGURE 29

Effects of exposing leaves of Bryophyllum for 4 h, to 5 min of red light alternating with 5 min of darkness (A), to 5 min of red alternating with 5 min of far-red radiation (B), and to the regime in A superimposed on a 4-h continuous exposure to far-red radiation (C). Times of irradiation are shown by the shaded bars. The broken lines show the rhythm in unirradiated control leaves.

0 = midnight.


A
B
C
40
20
0
40
20
0
60
40
20
0
0
0
0
0
0
0
Time of day
μg CO_2 h^{-1} g(fresh weight)$^{-1}$

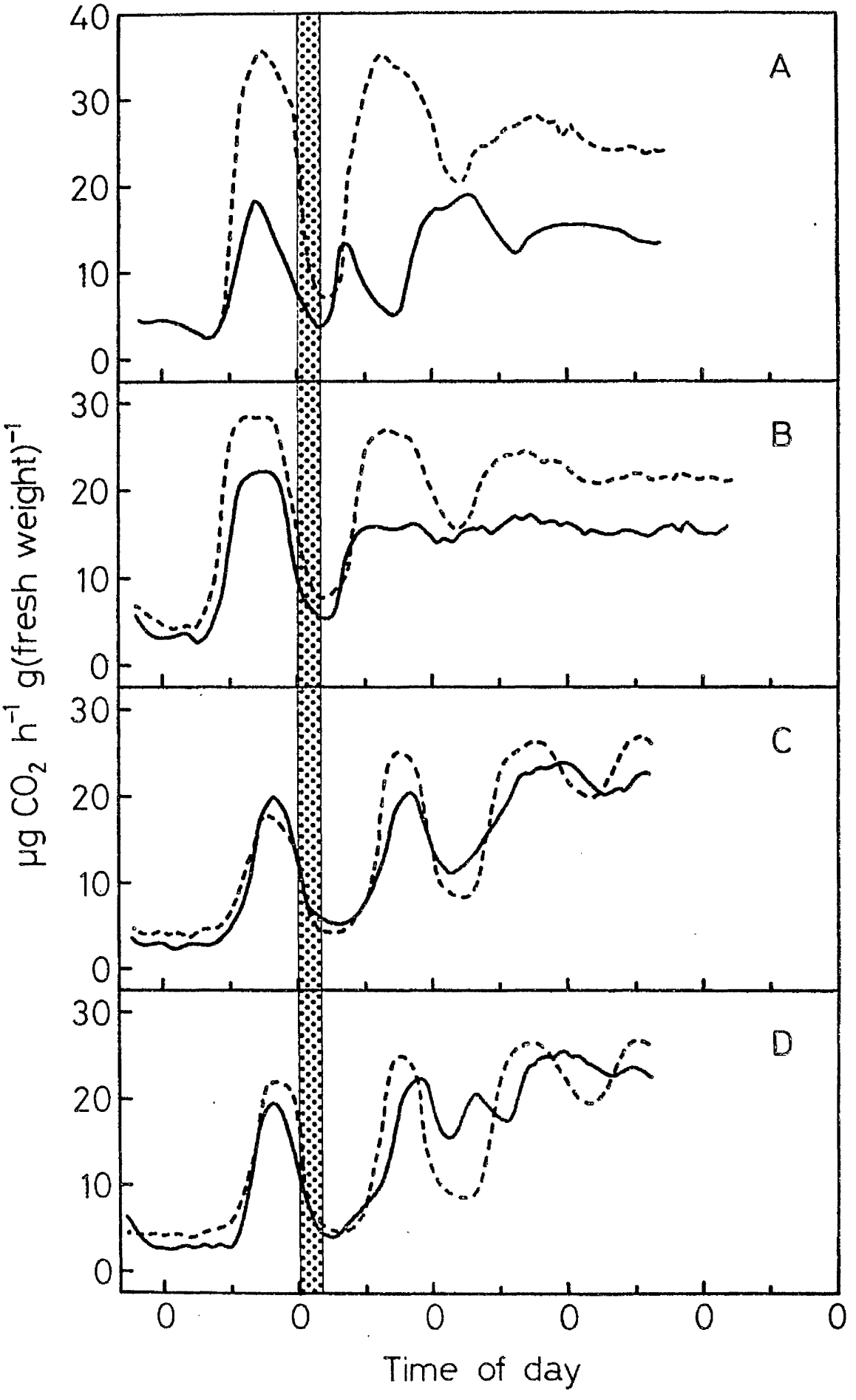
of this treatment was not a clear reversal of the phase shift induced by red light alone, but a considerable reduction of the amplitude of the rhythm in subsequent darkness. When the leaves were exposed to continuous far-red radiation for 4 h superimposed on the 5 min red/ 5 min darkness experimental regime, the circadian nature of the rhythm was lost and small peaks tended to occur at 12 h intervals (Figure 29C). This technique, thus, also fails to demonstrate a clear reversal of the effectiveness of red light by exposure to far-red radiation, in so far as phase shifts were concerned. There is no doubt however, that far-red radiation interacts in some way with the red and leads to a loss of the circadian nature of the rhythm.

Some variation in the test regimes to which the leaves were exposed for a period of 4 h was examined for evidence of red/far-red reversibility. A sequential regime of 10 s of red light followed by 30 s of darkness led to the induction of a substantial phase shift (Figure 30A), but a regime of 10 s of red followed by 30 s of far-red radiation led to the virtual abolition of the circadian rhythm (Figure 30B). When the regime of 10 s red light and 30 s of darkness was applied to leaves for 4 h with a simultaneous and continuous exposure to far-red radiation, the experimental result was somewhat variable. Either, a very slight phase shift was induced in the rhythm (Figure 30C), or the rhythm appeared to lose its circadian nature (Figure 30D).

The alternation of either red light and darkness or of red and far-red radiation therefore gave some evidence for the occurrence of red/far-red reversibility. There is no doubt, however, that red and far-red radiation interact under certain circumstances and lead to a modification of the effects on the circadian rhythm of carbon dioxide output in Bryophyllum leaves, obtained with red light alone.

FIGURE 30

Effects of exposing leaves of Bryophyllum for 4 h to 10 s of red light alternating with 30 s of darkness (A), 10 s of red alternating with 30 s of far-red radiation (B), and to the regime used in A superimposed upon a continuous exposure to far-red radiation for 4 h (C and D). Times of irradiation are shown by the shaded bars. The broken lines show the rhythm in unirradiated leaves.
0 = midnight.


40
30
20
10
0
A
30
20
10
0
B
30
20
10
0
C
30
20
10
0
D
μg CO_2 h^{-1} g(fresh weight)$^{-1}$
0
0
0
0
0
0
Time of day

4. The rhythm in continuously irradiated leaves.

4.1 The rhythm in white light.

A rhythm in the rate of carbon dioxide output was induced by transferring the leaves from the controlled environment room to darkness or a low radiant flux density of white light at 15°C. Examples of the rhythm in darkness, a radiant flux density of 0.6 J m^{-2} s^{-1} from white fluorescent lamps and of 2.5 J m^{-2} s^{-1} from tungsten lamps are shown in Figure 31 A-C. The period of the rhythm was reduced by both irradiation treatments. Radiant energy from the fluorescent lamp reduced the period from approximately 24 h in darkness to 20.3 h, the first peak occurring 22 h after the start of the experiment. Radiant energy from the tungsten lamp reduced the period to 19.1 h while the transient was decreased from 26 h in darkness to 19.3 h.

4.2 The effect of continuous monochromatic radiation.

In order to determine the region of the spectrum responsible for the effects of white light, and to assess whether any wavelengths lengthened the period as has been reported for *Phaseolus* (Lörcher, 1958) and *Coleus* (Halaban, 1969), the rhythm was recorded in leaves exposed to a number of different wavelengths of monochromatic radiation at 15°C. Examples of the effects of continuous exposure to a quantum flux density of 47 pE cm^{-2} s^{-1} in several 25 nm wide spectral bands are shown in Figure 32. In continuous darkness the peaks of the rhythm occur at approximately the same time each day indicating a period close to 24 h. In contrast, the peaks of rhythms in leaves irradiated with spectral bands centred on 530, 600, 660 and 730 nm occurred earlier each day indicating periods less than 24 h. The period-shortening effect of these treatments is also apparent from the fact that the rhythms in irradiated leaves became progressively more out of phase with those in unirradiated, control leaves. The peaks of the rhythm in leaves exposed

FIGURE 31

The circadian rhythm of carbon dioxide output from Bryophyllum leaves at 15°C in darkness (A), 0.6 J m^{-2} s^{-1} from white fluorescent lamps (B) and 2.5 J m^{-2} s^{-1} from tungsten lamps (C).

0 = midnight.

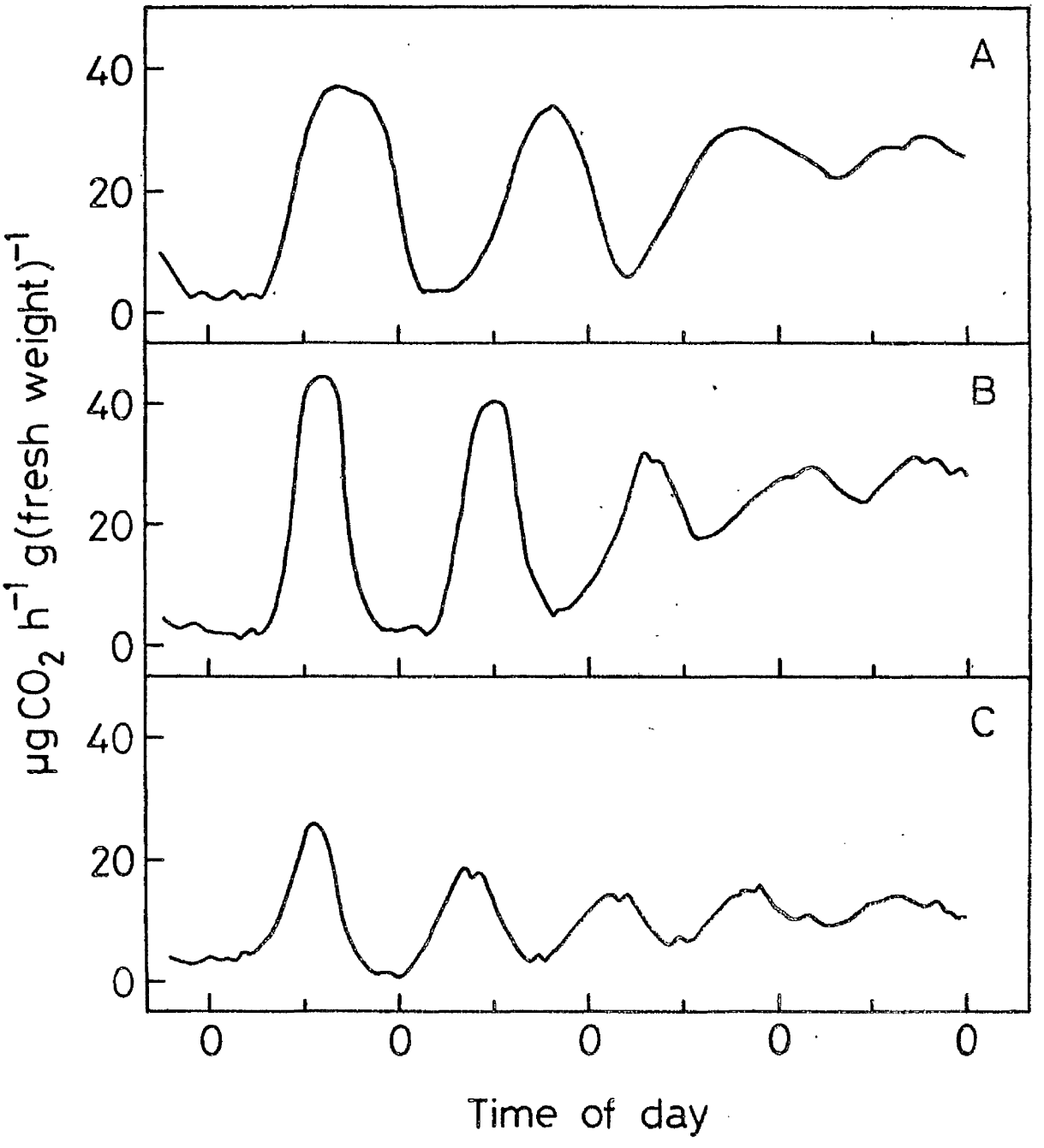
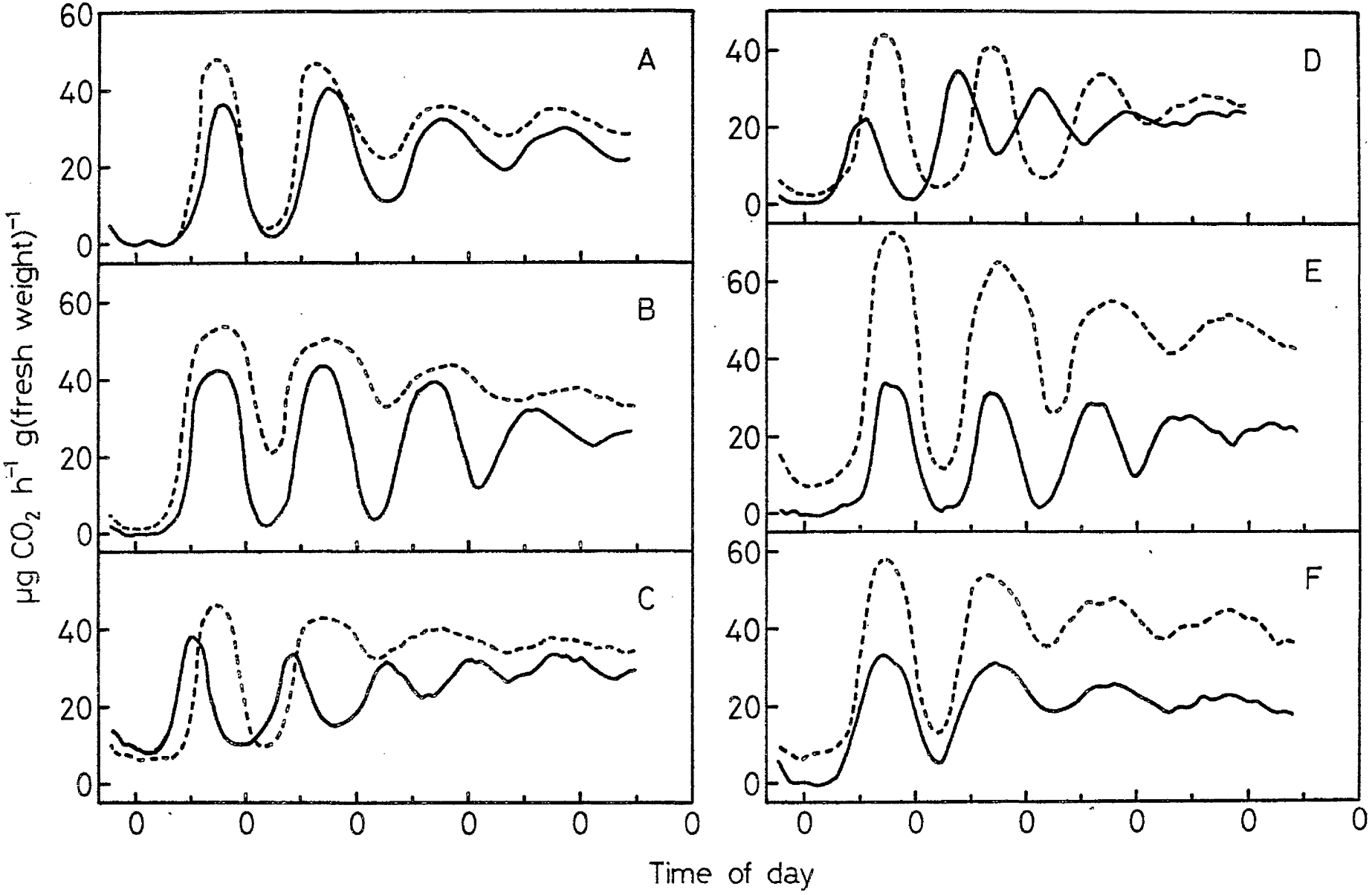

A
B
C
40
20
0
μg CO2 h-1 g(fresh weight)-1
0
0
0
0
0
Time of day

FIGURE 32

Effect of continuous exposure to monochromatic radiation, in spectral bands centred on 450 (A), 530 (B), 600 (C), 660 (D), 730 (E) and 760 nm (F), on the rhythm of carbon dioxide output in Bryophyllum leaves. The broken lines show the rhythms in leaves held in darkness throughout the experiment. 0 = midnight.

FIGURE 32


A
B
C
D
E
F
60
40
20
0
μg CO2 h-1 g(fresh weight)-1
0 0 0 0 0 0
Time of day

to spectral bands centred on 450 and 760 nm occurred at the same time as those of rhythms in leaves in darkness, demonstrating the ineffectiveness of these wavelengths in modifying the period.

The combined results of a number of such experiments are shown in Figure 33. Further details of these results are given in Appendix 2, tables 1 and 2. Continuous irradiation with spectral bands centred on 660, 600, 730 and 530 nm significantly reduced the period compared with that in darkness. Maximum reduction occurred at 660 and 600 nm while least reduction occurred at 530 nm. None of the wavelengths tested significantly lengthened the period. The transient was also shortened by the 660, 600 and 530 nm bands of radiant energy by an amount proportional to the reduction of the period. However, although the spectral band centred on 730 nm reduced the period, the transient in this case was not reduced. No significant difference in either the period or the transient was found between unirradiated leaves and leaves continuously exposed to radiant energy of 450 or 760 nm. It is clear from these results that radiant energy principally from the red region of the spectrum is responsible for determining the period and transient observed with white light.

4.3 The effect of quantum flux density.

The dependence of the period and transient on the incident quantum flux density was determined by exposing leaves to five flux densities of monochromatic radiation in the spectral band (660 nm) found to be most active in shortening the period and transient. The results of individual experiments from this series are shown in Figure 34. Figure 35 shows the combined results from a number of such experiments. Further details of these results are given in Appendix 2, tables 3 and 4. All the quantum flux densities tested significantly reduced the period compared with that

FIGURE 33

The effect of continuous exposure to an incident quantum flux density of 47 pE cm^{-2} s^{-1} in 25 nm wide spectral bands on the period (closed circles) and the transient (open circles) of the rhythm of carbon dioxide output from Bryophyllum leaves. Lines A and B show the values of the transient and period respectively of rhythms in darkness. The vertical lines are the standard errors of the means.

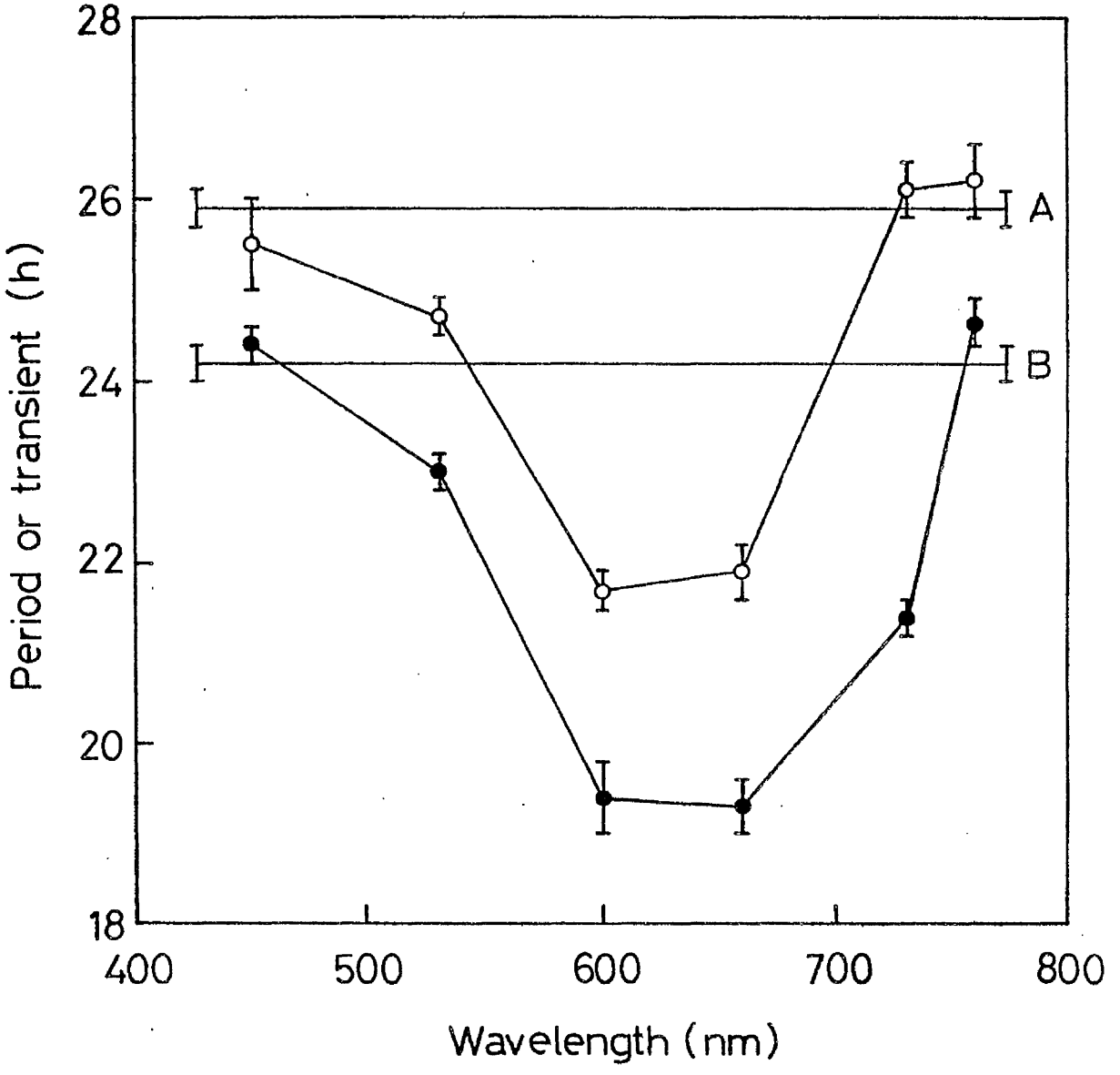

28
26
24
22
20
18
Period or transient (h)
400
500
600
700
800
Wavelength (nm)
A
B

FIGURE 34

The circadian rhythm in Bryophyllum leaves continuously exposed to monochromatic radiation (660 nm) at quantum flux densities of:-

A. 0.98pE cm^{-2} s^{-1}

B. 3.3 " "

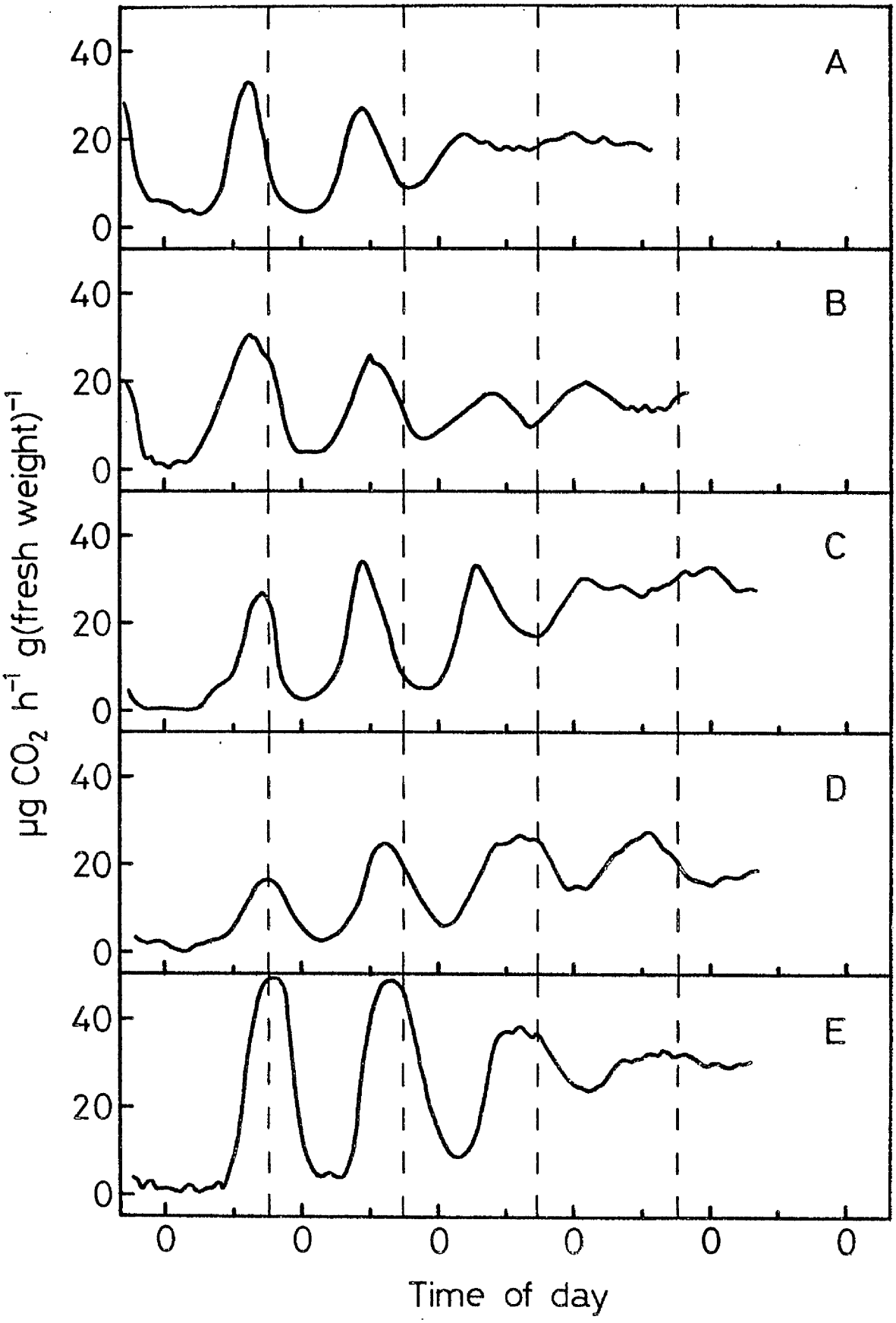
C. 13.0 " "

D. 22.0 " "

E. 47.0 " "

The mean times of occurrence of the peaks of rhythms in darkened control leaves are shown by the broken lines.

0 = midnight.


40
20
0
A
B
C
D
E
μg CO_2 h^{-1} g(fresh weight)$^{-1}$
0 0 0 0 0 0
Time of day

observed in leaves held in darkness. An approximately linear relationship exists between the magnitude of this reduction and the logarithm of the quantum flux density. The transient also decreased with increasing flux density, the magnitude of the reduction being approximately proportional to that of the period for a given flux density. The lowest quantum flux density, however, significantly reduced the period but not the transient. There was again an approximately linear relationship between the logarithm of the quantum flux density and the magnitude of the reduction of the transient.

4.4 The interaction of light and temperature.

In continuous darkness the period of the rhythm in *Bryophyllum* leaves shows a small but significant dependence on the ambient temperature (Wilkins, 1962b). Rhythms in leaves continuously irradiated with monochromatic radiation (660 nm) at a quantum flux density of 47 pE cm^{-2} s^{-1} were compared with those in leaves held in darkness throughout the experiment, to determine whether or not the effectiveness of radiant energy in shortening the period and transient was influenced by temperature.

The carbon dioxide output of leaves was recorded at 6 different temperatures. The results of individual experiments are shown in Figures 36 and 37. Figure 36A shows that the rate of carbon dioxide output of both irradiated and unirradiated leaves at 10°C was initially low, increasing slightly towards the end of the experiment, and arhythmic. A circadian rhythm in the rate of carbon dioxide output was apparent in both irradiated leaves and leaves in darkness at temperatures within the range 15-30°C (Figure 36B and C, Figure 37A and B). At 35°C the rate of carbon dioxide output was arhythmic, decreasing during the first 20 h of the experiment, increasing for a further 24 h in irradiated leaves and 32 h in leaves in darkness, and then decreasing throughout the remainder

FIGURE 35

The effect, of the incident quantum flux density of a 25 nm wide spectral band centred at 660 nm, on the period (closed circles) and transient (open circles) of the rhythm of carbon dioxide output from leaves of Bryophyllum. The vertical lines are the standard errors of the means.

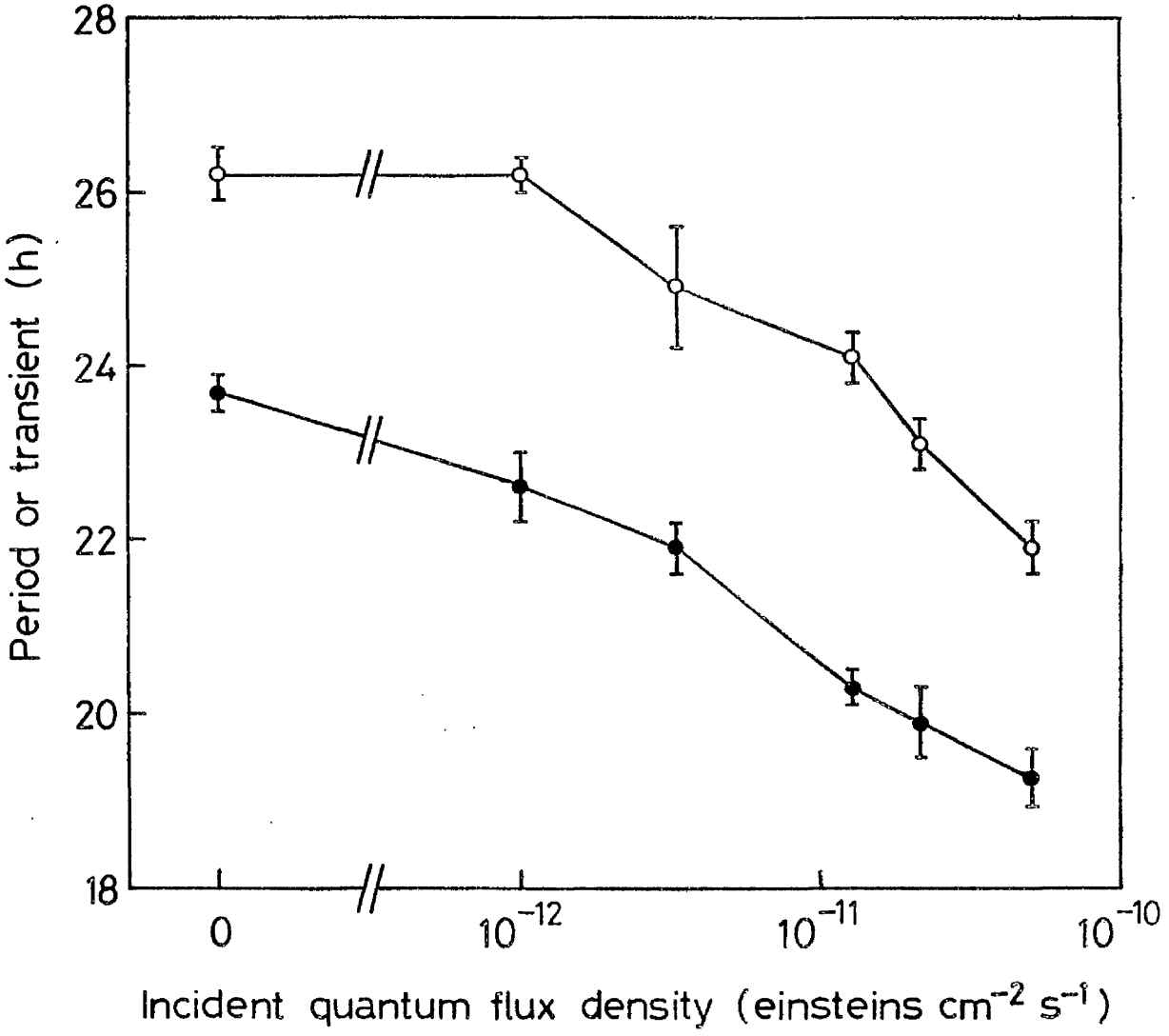

28
26
24
22
20
18
Period or transient (h)
0
10^-12
10^-11
10^-10
Incident quantum flux density (einsteins cm^-2 s^-1)

FIGURE 36

The rate of carbon dioxide output of Bryophyllum leaves in darkness, or exposed to monochromatic radiation (660 nm) at a quantum flux density of 47 pE cm^{-2} s^{-1} at 10°C (A), 15°C (B) and 20°C (C).

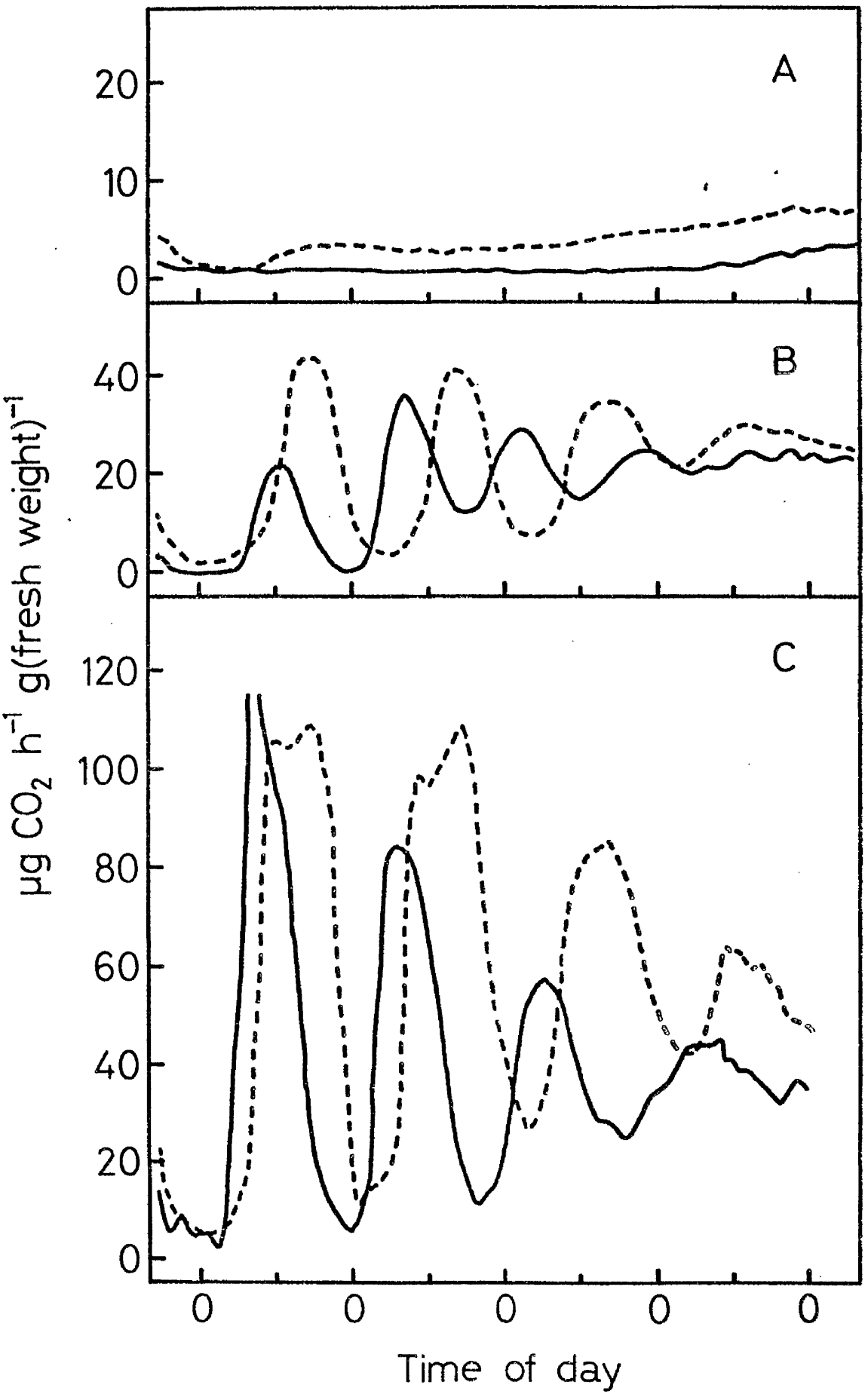
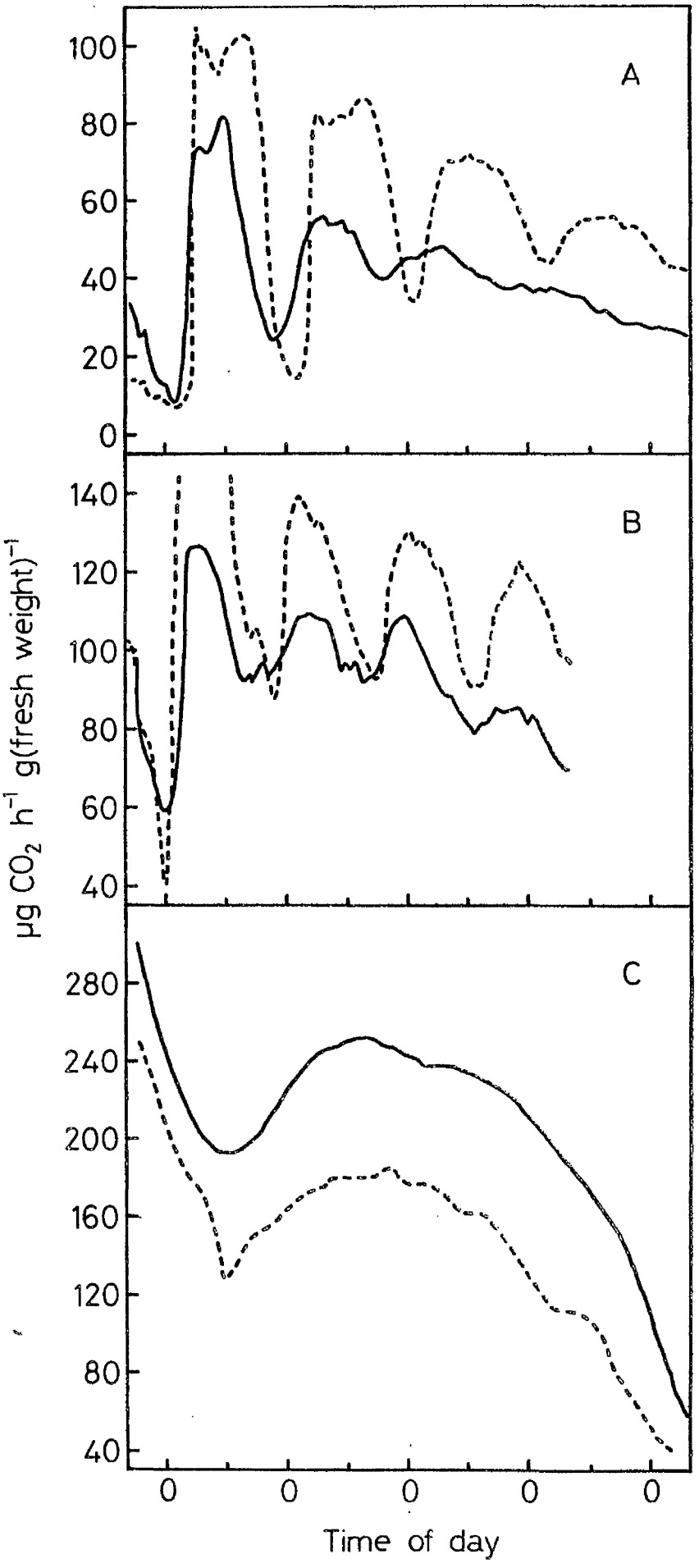

A
B
C
20
10
0
40
20
0
120
100
80
60
40
20
0
0
0
0
0
0
μg CO_2 h^{-1} g(fresh weight)$^{-1}$
Time of day

FIGURE 37

The rate of carbon dioxide output of Bryophyllum leaves in darkness, or exposed to monochromatic radiation (660 nm) at a quantum flux density of 47 pE cm^{-2} s^{-1} at 25°C (A), 30°C (B) and 35°C (C).

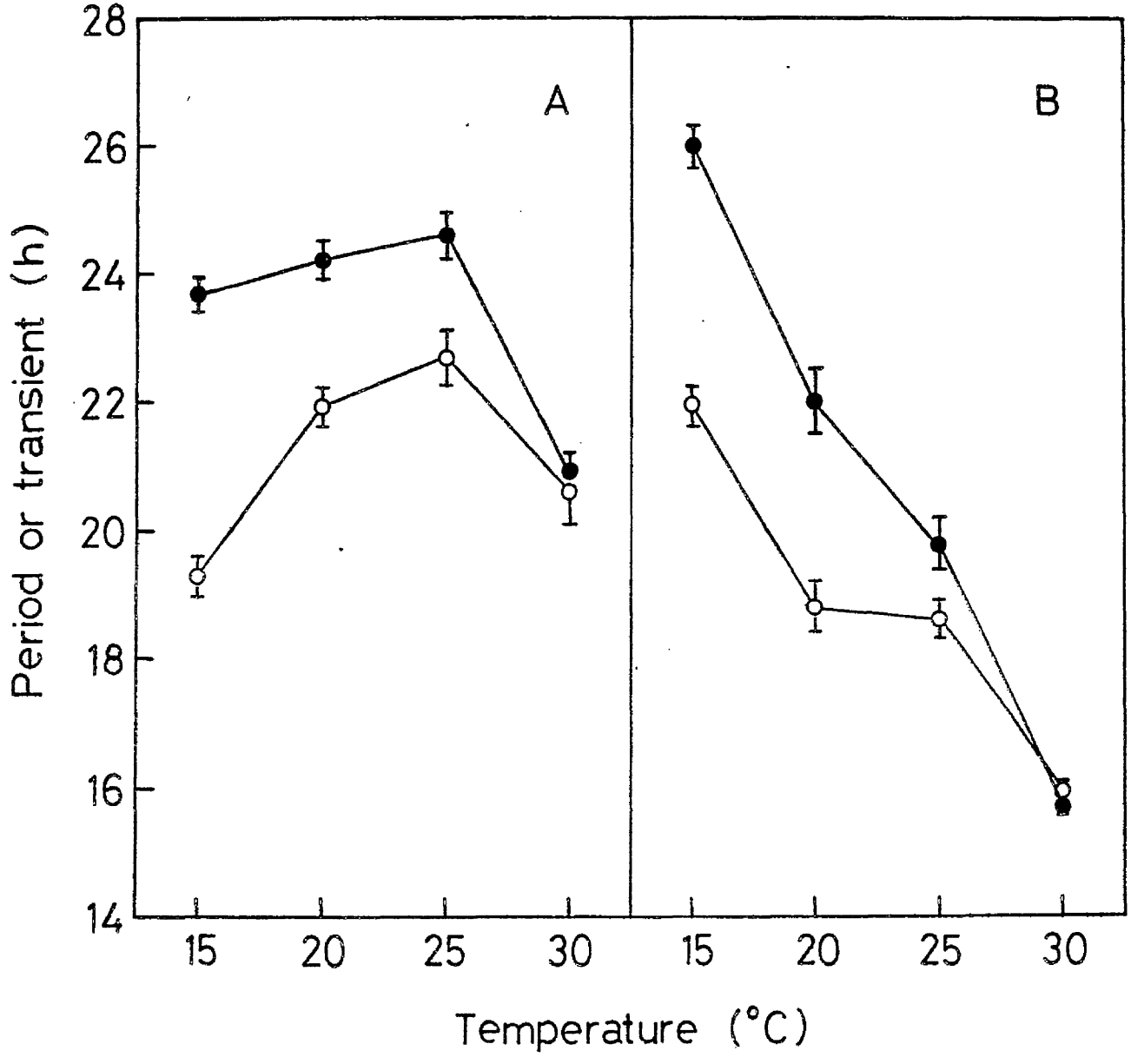

A
B
C
100
80
60
40
20
0
140
120
100
80
60
40
280
240
200
160
120
80
40
0
0
0
0
0
Time of day
µg CO_2 h^{-1} g(fresh weight)$^{-1}$

of the experiment. There was a general tendency for an increased rate of carbon dioxide output with increased temperature. The rhythm tended to persist for rather longer in leaves in darkness than in leaves which were irradiated.

Figure 38 shows the combined results of this series of experiments. Further details of these results are given in Appendix 2, tables 5-10. The period of rhythms in both darkened and irradiated leaves increased between 15° and 25°C and decreased between 25° and 30°C. However, while irradiation reduced the period at 15° C by 4-5 h, this difference decreased with increasing temperature and at 30°C was not significant. The transient decreased with increasing temperature over the range 15-30° C whether leaves were in constant light or darkness. Whether or not the transient was shortened by the irradiation treatment depended on the ambient temperature in a similar way to that observed for the reduction of the period. The magnitude of the reduction of the transient by irradiation decreased between 15° and 30°C and at the latter temperature there was no significant difference between the transients of rhythms in darkened and irradiated leaves. Thus, although the ambient temperature influenced the period and transient in quite distinct ways, irradiation exerted an effect on the transient which was approximately proportional to the effect on the free-running period of the rhythm.

FIGURE 38

The effect of temperature on the period (A), and the transient (B), of the rhythm of carbon dioxide output in leaves of Bryophyllum in darkness (closed circles) or irradiated with 47 pE cm^{-2} s^{-1} in a 25 nm wide spectral band centred on 660 nm (open circles). The vertical lines are the standard errors of the means.


A
B
Period or transient (h)
28
26
24
22
20
18
16
14
15
20
25
30
15
20
25
30
Temperature (°C)

DISCUSSION

The carbon dioxide output of leaves of Bryophyllum fedtschenkoi into air initially free of carbon dioxide is rhythmic over a range of ambient temperatures and irradiation treatments. That this represents an endogenous circadian rhythm has previously been demonstrated, in so far as it is possible to obtain conclusive evidence of the endogenous origin of rhythmicity (Wilkins, 1959, 1960a, 1967). All the data obtained in this investigation are consistent with this conclusion and four of the five basic properties which a rhythm should possess to be considered endogenous have again been demonstrated.

As reported previously (Wilkins, 1962a), the rhythm in leaves of Bryophyllum can, in common with all circadian rhythms, be entrained by cycles of light and darkness. In some organisms, whether or not a circadian rhythm is entrained by a 24-h cycle of light and darkness, depends on the length of the light period. Entrainment occurs if the photoperiod falls within specific limits, characteristic for that organism. A 0.25-h exposure to white light every 24 h rapidly entrains the rhythm in Bryophyllum and maintains it in a precise phase relationship to the entraining cycle. In this respect, the rhythm in Bryophyllum resembles those in Lemna perpusilla (Hillman, 1971) and Drosophila pseudoobscura (Pittendrigh and Minis, 1964). On the other hand, the leaf movement rhythm of Pharbitis nil is entrained only by 24-h cycles containing a photoperiod of between 4 and 20 h (Bollig, 1974).

Skeleton photoperiods consisting of 0.25-h irradiations marking the beginning and end of a corresponding 8-h photoperiod were effective in replacing the complete photoperiod in entraining the Bryophyllum rhythm. The ability to entrain to skeleton photoperiods, shown by the

Bryophyllum rhythm, is also possessed by rhythms in Lemna (Hillman, 1970, 1972), Drosophila (Pittendrigh, 1966), Pectinophora (Minis, 1965) and Portulaca (Karvé and Jigajinni, 1966a), although some differences between Bryophyllum and the other species are apparent. In Bryophyllum, and also in Lemna and Drosophila, 0.25-h exposures are sufficient to act as components of the skeleton photoperiods. Entrainment of the leaf opening rhythm of Portulaca grandiflora can be achieved by irradiations of 1 or 2 h duration, but not by irradiations of 0.5 h, applied as a skeleton photoperiod.

Although the skeleton photoperiod of LD 8:16 may be considered ambiguous, in that it also represents the skeleton photoperiod of LD 16.5:7.5, Bryophyllum leaves exposed to this schedule always interpret the shorter of the two dark periods in each 24-h cycle as the photoperiod This is a general feature of entrainment to skeleton photoperiods in which the lengths of the alternate dark periods differ considerably. When skeleton photoperiods are used with alternating dark periods of more similar duration however, differences in the responses of organisms become apparent. When exposed to the skeletons of photoperiods of between 11 and 13 h, the eclosion rhythm of Drososphila displays the phenomenon of bistability. Organisms which show bistability interpret the second dark period of some skeleton regimes as the complete photoperiod, irrespective of whether it is the longer or shorter of the two (Pittendrigh, 1966). In contrast, investigations of the rhythm of carbon dioxide output in Lemna have provided no evidence for bistability with skeleton photoperiods in the 11 to 13-h range (Hillman, 1972). When exposed to the skeleton photoperiods of LD 11:13, the rhythm in Bryophyllum differs, depending on the irradiation sequence. This difference is in the appearance of a sub-peak in the 13-h dark period when this period follows, but not when it preceeds the 10.5-h dark period. It is not clear however, whether or not this should be considered as bistability,

as it does not represent a complete change in the phase relationship of the rhythm to the entraining cycle. The rhythm retained the major features of entrainment to the skeleton of LD 11:13 rather than that expected of LD 13.5:10.5. When exposed to the skeleton photoperiod of LD 12:12, no clear entrainment was observed, in contrast to the entrainment obtained with the schedules containing the complete photoperiods.

The mechanism of entrainment of a circadian rhythm to cycles of light and darkness has been the subject of detailed investigation in Drosophila (Pittendrigh, 1965: 1966). The data obtained with this organism suggest that entrainment is the net result of individual phase shifts induced by the successive exposures to light. Entrainment of a rhythm with a free-running period of exactly 24 h, to 24-h light-dark cycles, would involve a series of phase shifts, advances, delays, or both, until the irradiation occurred at the time in the cycle at which no phase shift was induced. In this way a precise phase relationship between the rhythm and the entraining cycle would be achieved. Entrainment of a rhythm with a free-running period differing from 24 h, by a single daily photoperiod, would be rather more complex. In this case, a light interruption occurring on one particular day, at the time in the cycle at which no phase shift is induced, would coincide with an earlier or later part of the cycle on the next day, depending on whether the period of the rhythm was more or less than 24 h. Steady-state entrainment of such rhythms would therefore require a series of phase shifts resulting finally in the organism being irradiated at the point in the cycle at which the phase advance or delay induced was such that the same point recurred exactly 24 h later.

One can therefore reasonably conclude that 0.25-h periods of white light and of certain wavelengths of monochromatic radiation induce

phase shifts of the Bryophyllum rhythm. The rhythm in Bryophyllum leaves exposed to 0.25 h of white light at 0400 h each day rapidly becomes 12 h out of phase with the rhythm in leaves in constant darkness. This is achieved after only three 24-h cycles and hence exposure to a total of 0.75 h of white light. A previous investigation of the duration of exposure to light necessary to induce a phase shift revealed that even at the most sensitive part of the cycle a single 1-h exposure to white light induced only a slight phase shift, or a reduction in the period of the rhythm by 1-2 h, in the two subsequent cycles (Wilkins, 1960a). However, the former investigation was carried out at a higher temperature, and using a different radiant energy source. Further investigation would thus be required to ascertain whether this accounts for the apparent difference in the sensitivity of the rhythm to phase shifting treatments. It is also possible that one light interruption increases the sensitivity of the rhythm to a second pulse applied 24 h later.

From phase-response curves determined for the phase shift induced by 0.25-h exposures to white light, Pittendrigh (1965) was also able to predict the entrainment pattern of the rhythm of eclosion in Drosophila pupae exposed to two such irradiations each day. The experimental results confirmed his predictions and were consistent with entrainment resulting from a series of phase shifts. Each irradiation appeared to cause an immediate, stable phase shift of the basic oscillator, even though the overt rhythm normally passed through a number of transient cycles before reaching a new stable phase.

These results also supported the concept, advanced by Pittendrigh et. al. (1958) of coupled 'A' and 'B' oscillators. The 'A' oscillator is considered to be the ultimate oscillator, to be light sensitive and to be immediately reset by a light exposure. The 'B' oscillator is

thought to be light insensitive, and to require several cycles to be reentrained by 'A', after the latter is reset by a light treatment. If it is assumed that the overt rhythm follows oscillator 'B' it is possible to explain why the phase shift induced by a second light pulse is precisely that predicted for a rhythm, immediately reset by the first pulse, irrespective of the time in the transient cycles of the overt rhythm at which the second exposure is given. Evidence for the occurrence of coupled oscillators can also be inferred from the phase shifts induced by exposing Coleus (Halaban, 1968b) and Kalanchoë (Engelmann and Honegger, 1967) plants to successive light exposures, although in the latter case this is not the only possible interpretation.

An analysis of the phase response of the Bryophyllum rhythm to a single 0.25-h irradiation at different times in the cycle is not available. It is thus not possible to make a detailed comparison between the Bryophyllum and Drosophila rhythms exposed to two light interruptions every 24 h. However, in view of the fact that the overt rhythm in Bryophyllum is rapidly, and possibly immediately, reset by light treatments of several hours duration, it is unlikely that any evidence for the occurrence of coupled 'A' and 'B' oscillators would be found in this organism.

The phase response of the rhythm of carbon dioxide output in Bryophyllum leaves in darkness to 4-h exposures to white fluorescent light has been examined. Whether or not a phase shift occurs, and its magnitude, depend on the time in the circadian cycle at which the light interruption is given. Both phase advances and phase delays were induced, the direction of the phase shift also depending on the time of exposure to light. The phase-response of Bryophyllum is basically similar to that reported for a number of other organisms (Aschoff, 1965; Pittendrigh, 1965).

Phase response curves which express the magnitude of the phase shift as a function of the time in the cycle at which the light interruption is given, may be constructed from phase shift data. The phase-response curves of most organisms show a relationship between phase shift and time of exposure to light which is clearly not linear. In order to plot the results obtained for the Bryophyllum rhythm in a comparable way, it is necessary to make a rather arbitrary distinction between phase advances and delays. This is because it is not always clear whether some small, relatively brief increases in the rate of carbon dioxide emission reflect similar changes in the basic oscillating mechanism, or are simply the result of a direct effect of the light stimulus on the biochemical processes monitored as the overt rhythm. This difficulty should not however detract from the value of such a curve in assessing the linearity of the relationship between the steady-state phase shift and the time of application of the resetting stimulus. On the basis of such a graph, the relationship in Bryophyllum would appear to be approximately linear. This conclusion supports a previous report by Wilkins (1960a) that the magnitude of the phase shift induced by a single white light perturbation is determined by the fact that a peak of carbon dioxide emission occurs a fixed time after the end of the perturbation. If the data obtained in this investigation is expressed in a slightly different way by plotting the time of occurrence of the peaks, rather than the magnitude of the phase shift, against the time of the perturbation (Figure 26), it is apparent that the response is not linear. The time between the end of the light interruption and the peak in the next circadian cycle is not constant, but varies between 21.5 and 29 h depending on when in the cycle the light treatment is given. In addition, a peak of carbon dioxide output may occur a few hours after the end of the light treatment, but still within the same circadian cycle.

The overt rhythms of organisms such as Drosophila (Pittendrigh and

Bruce, 1957), which are phase shifted by a single exposure to light, pass through several transient cycles before attaining a new steady-state. Commonly, advancing phase shifts pass through transient cycles while delaying phase shifts often reach their final values immediately (Aschoff, 1965). In other organisms such as Gonyaulax the new stable phase is reached immediately after either delaying or advancing phase shifts (Hastings and Sweeney, 1958). Bryophyllum resembles more closely the latter type, the phase difference between the rhythm reset by a light perturbation and the rhythm persisting in darkness being virtually the same in each of the three cycles following the stimulus. This indicates that the new stable phase is reached rapidly.

The overt rhythm in the carbon dioxide output of Bryophyllum leaves fades out after prolonged exposure to light or darkness. Furthermore, results obtained using the monitoring techniques employed in this investigation, previously suggested that the rhythm is inhibited by high irradiance white light (Wilkins, 1960a). Using a different measuring technique, Jones and Mansfield (1970, 1972) have detected a rhythm in the carbon dioxide compensation point of Bryophyllum fedtschenkoi leaves exposed to high incident radiant flux densities. These authors consider that the same underlying mechanism may be responsible for rhythmicity in both darkness and high irradiance white light. It is thus uncertain whether the basic oscillating mechanism, or simply the overt rhythm, is inhibited by light in the experiments reported by Wilkins (1960a). It is clear, however, that a rhythm appears after a transfer from light to darkness or a decrease in the radiant flux density. This rhythm persists with a phase determined by the time of reduction of the flux density rather than by the previous phase of the oscillation (Wilkins, 1960a). This observation is consistent with either the induction of a new rhythm or with the reappearance of a previously undetected rhythm, reset by the

change in radiant flux density. The results of this investigation confirm the recurrence in darkness of a rhythm in leaves held in constant light until the rhythm initiated at the start of the experiment has damped.

The first peak of the rhythm initiated by transfer from the controlled environment room at the end of the normal photoperiod occurred after 25.2 h. When the photoperiod was extended by an additional 107-h exposure to white light at a radiant flux density of 0.6 J m^{-2} s^{-1} at 15°C, a rhythm occurred during this time and faded out after 96 h. The first peak after an eventual transfer to darkness occurred after 21 h. Wilkins (1959) reported a decrease in the time between the transfer to darkness and the occurrence of the first peak with increasing duration of exposure to light, additional to an 8-h photoperiod, experienced by the plants immediately before transfer to the experimental conditions. A 4-h reduction of this transient was obtained with 44 h additional exposure to white light. It is possible that the 4.2-h reduction of the transient observed in this investigation is a further manifestation of this phenomenon. However, as the previous investigation was carried out at 26°C while the latter was at 15°C, and it is known that the ambient temperature itself exerts a marked effect on the transient, the magnitudes of the reductions are not strictly comparable. The results of part of this investigation, designed to study the phase shift induced by an interruption of darkness with white light, show that between 21.5 and 29 h may elapse between the end of the light treatment and the occurrence of a peak. Thus, if the basic oscillator continues during prolonged exposure to light, even though the rhythm may no longer be apparent, the length of the transient may be a function either of the time in the cycle at which the transfer back to darkness is made, or simply of the duration of the additional light treatment. If the former possibility represents

the true situation the length of the transient should vary rhythmically between 21.5 and 29 h with increasing duration of additional irradiation. Such experiments have not been performed under the conditions of this investigation. However, the data presented by Wilkins (1959) gives no indication of such a cyclic response. The decrease in the transient shown in these results is thus almost certainly a function of the increased duration of exposure to light.

Once the steady-state oscillation of a circadian rhythm has been established, the period remains stable. This is true not only of rhythms entrained to environmental periodicities, but also of free-running oscillations. Under constant conditions the period remains stable until the rhythm fades out. Period stability is clearly an essential feature for accurate time measurement, as is a relative independence from small fluctations of the temperature of irradiance. The periods of most rhythms are not completely ~~independant~~ independent of the environment and small but significant differences in the period are found under different constant conditions. Periods both longer and shorter than 24 h are found, most frequently within the range 20 - 28 h.

It had previously been concluded that continuous irradiation did not modify the period of the carbon dioxide output rhythm of Bryophyllum at 26°C (Wilkins, 1960a). In this investigation it was found that increasing the radiant flux density resulted in a decrease of the period at 15°C. In this respect the Bryophyllum rhythm is similar to the rhythms of luminescence of Gonyaulax polyedra (Hastings and Sweeney, 1959) and petal movement of Kalanchoë blossfeldiana (Bünsow, 1953). In contrast, the periods of the leaf movement rhythms of Coleus blumeii x C. frederici (Halaban, 1968a) and Pharbitis nil (Bollig, 1974) increase with increasing radiant flux density, and the period of the leaf movement rhythm of

Canavalia ensiformis remains unchanged (Kleinhoonte, 1932).

Aschoff (1960) proposed a general rule, which has some exceptions (Hoffmann, 1965), that the period of rhythms in animals active in the light decreases with increasing light intensity while in animals active in the dark it increases with increasing light intensity. Although plants can clearly be divided into different response types there is no apparent basis for this division. The Bryophyllum rhythm does, however, correspond rather closely to the second part of Aschoff's rule which states that the period changes linearly with the logarithm of the light intensity.

The rhythmic growth rate of Avena coleoptiles (Ball and Dyke, 1954) and the pigment migration rhythm in the fiddler crab, Uca (Brown and Webb, 1948), are among the few oscillations for which there is no detectable effect of temperature on the period. In most organisms the period shows a small but significant dependence on the ambient temperature. In the majority of cases this appears as a slight decrease in the period with increasing temperature. The rhythm of sporulation in Oedogonium cardiacum however, displays longer periods as the temperature is increased (Bühnemann, 1955b). A lengthening of the period of the rhythm of stimulated luminescence in Gonyaulax polyedra also occurs over the lower part of the temperature range tested (Hastings and Sweeney, 1957). Over the upper part of the temperature range however, the period decreases with increasing temperature. The high degree of temperature compensation reported for Bryophyllum (Wilkins, 1962b) is borne out by the present results. The period of the rhythm in leaves in darkness at 30°C was shorter than in those at 15°C. The magnitude of the reduction in the period observed between these temperatures in the present investigation is very similar to that reported by Wilkins (1962b). However, some difference in the

effect of temperature on the period was found over part of this range. A small but significant increase in the period between 15°C and 25°C was observed in the present investigation whereas previously a small decrease between 16°C and 26°C was reported. The Bryophyllum rhythm thus resembles the Gonyaulax rhythm in having a maximum period length which decreases with either increasing or decreasing temperatures.

It is evident from the results of the present investigation that the effects of radiant energy and temperature on the period are not entirely independent of one another. Although at none of the temperatures tested was the period increased by irradiation, the extent to which the period was decreased depended upon the temperature. Thus, any analysis of the effect of irradiation on the period of a rhythm should also take into account the possibility of different responses at different temperatures. Conversely, the effect of temperature on the period clearly varies with the irradiance. The absence of a modification of the period by radiant energy, reported by Wilkins (1960a), may therefore be due to the fact that the periods which were obtained in darkness were shorter than those found in the present investigation. Also, a temperature was employed at which, on the basis of the present results, only a relatively small reduction of the period by radiant energy would be expected.

As a result of the greater reduction of the period by radiant energy at 15°C than at higher temperatures, the period of the rhythm in irradiated leaves shows a greater dependence on the ambient temperature than does the period of the rhythm in darkened leaves, over the 15 - 25°C range. A further consequence of this is that in irradiated leaves the period at 30°C is longer than at 15°C. The effect of temperature on the rhythm in irradiated Bryophyllum leaves resembles closely that on the rhythm of stimulated luminescence in Gonyaulax. The experiments which

revealed an increased period with increased temperature in both Gonyaulax and Oedogonium were also performed in the light.

The water balance within a plant may also modify the period of a rhythm. Bünning and Moser (1968) have reported that withholding water from Phaseolus seedlings results in an effect similar to that obtained with red light in that the period of the leaf movement rhythm is lengthened. In this investigation some loss of turgor was noted in detached Bryophyllum leaves after several days at 30°C and to a lesser extent at 25°C, but not at lower temperatures. The sharp decrease in the period between 25°C and 30°C may therefore be due, at least in part, to this loss of turgor. Thus, differences in the resistance to water loss, particularly between leaves grown under different conditions may also account for the slight difference in the results found in this investigation and by Wilkins (1960a, 1962b).

The mechanism by which the period is altered by temperature or radiant energy has not been elucidated for any organism. Bünning (1973) has suggested that, "a factor producing short periods and also causing the rhythm to fade out quickly, indicates that this factor suppresses one part of the oscillation before the normal final value is reached. The opposite part of the oscillation is thereby also shortened." In view of the considerable influence temperature exerts on biochemical processes generally it is more surprising that the period at different temperatures varies so little than that it varies at all. Again, little is known of how organisms compensate so effectively for substantial differences in temperature. Some of the hypotheses advanced to explain the occurrence of rhythmicity have also attempted to account for the phenomenon of temperature compensation. It has been suggested (Bünning, 1973; Sweeney, 1969) that this might result from the interaction of opposing processes

with different temperature coefficients. In Bryophyllum the rhythm results from the periodic fixation of carbon dioxide. The enzyme PEP carboxylase catalyses the fixation of carbon dioxide into oxaloacetate which is converted to malate by malate dehydrogenase. During decarboxylation malate is converted to pyruvate by malic enzyme. It has been demonstrated that the opposing processes of carboxylation and decarboxylation have very different temperature coefficients (Brandon, 1967). It is thus possible that the processes responsible for the overt rhythm play a direct role in temperature compensation in this organism. The possibility remains however, that temperature compensation is a property of a more fundamental basic oscillator.

Both radiant flux density and ambient temperature influence the transient of the Bryophyllum rhythm. The reduction of the transient by radiant energy was found to be approximately proportional to the modification of the period. An exception to this was the lowest of the quantum flux densities of monochromatic (660 nm) radiation tested, which reduced the period but not the transient. This finding differs from that of Jones and Mansfield (1972) who reported that the transient of the carbon dioxide compensation rhythm varied considerably more than did the period of the free-running rhythm, with the light intensity.

In some organisms a greater dependence on temperature has been noted for the transients than for the period of the steady-state oscillation. This has been reported for the rhythm of carbon dioxide metabolism in Kalanchoë blossfeldiana (Schmitz, 1951). The time from the onset of darkness to the first peak of the rhythm of stomatal opening ability in Xanthium pennsylvanicum however, is virtually independent of temperature between 15°C and 30°C (Mansfield and Heath, 1964). The greater temperature dependence in darkness, of the transient than of the free-running period

of the Bryophyllum rhythm, previously reported by Wilkins (1962b), was confirmed in the present investigation. Thus, although radiant energy and high temperatures often appear similar in their effects on the rhythm (Wilkins, 1969), their influence on the transient clearly differs.

This investigation of the effects of radiant energy on entrainment, initiation, phase and period of the rhythm included not only the effects of white light, but also the relative effectiveness of selected spectral bands of radiant energy. Only for the leaf movement rhythm of Phaseolus has a similar range of responses been investigated in detail. A number of differences are apparent between the rhythms in Bryophyllum and Phaseolus. There are also differences between the photoresponses of the Bryophyllum rhythm and all the other rhythms which have been studied in rather less detail.

Wilkins (1973) published an action spectrum for the relative effectiveness of equal quantum flux densities of different wavelengths of visible radiation in inducing a phase shift. Activity was strictly confined to wavelengths above 560 nm. The maximum effectiveness was at 660-660 nm and a sharp cut-off occurred at 700 nm. This action spectrum confirmed a previous observation that red, but not blue, light acted like white light in shifting the phase of the rhythm (Wilkins, 1960a). A similar conclusion has been reached by Jones (1973) for the circadian rhythm of carbon dioxide compensation in Bryophyllum. The major feature of the action spectrum, the effectiveness of the red but not the blue or far-red regions of the spectrum in inducing phase shifts, is confirmed in the present investigation.

Only for the Kalanchoë blossfeldiana petal movement rhythm has an action spectrum for the induction of phase shifts been obtained (Schrempf,

1975) which resembles that found for Bryophyllum. In Kalanchoë, as in Bryophyllum, a peak of activity was found in the 600-700 nm region. However, a second peak occurred in the near UV (300-380 nm). This latter region of the spectrum has not been investigated in Bryophyllum.

The study of the effectiveness of different spectral bands in inducing phase shifts of the Bryophyllum rhythm has been extended in this investigation to examine the phase shifts induced at different times in the cycle. The effectiveness of the red but not the blue or far-red regions of the spectrum was confirmed at several times in the cycle. It thus seems highly probable that the same photoreceptor is responsible for mediating both phase advances and phase delays. The same conclusion cannot be drawn unequivocally for all organisms, since in both Phaseolus (Bünning and Moser, 1966) and Coleus (Halaban, 1969) different wavelengths are effective in inducing phase shifts at different times during the circadian cycle.

The greatest modification of the period of the Bryophyllum rhythm was obtained with radiant energy from the red region of the spectrum, which had previously been reported to inhibit the rhythm at high radiant flux densities (Wilkins, 1960a). The rhythm in Bryophyllum differs from rhythms in other plants in which the spectral composition of radiant energy determines the period length, in that none of the wavelengths tested lengthened the period. The period of the leaf movement rhythm of Phaseolus is lengthened by radiant energy from fluorescent lamps and shortened by that from tungsten lamps (Lörcher, 1958). Radiant energy from either of these sources reduces the period of the rhythm in Bryophyllum. This difference can be explained by the fact that tungsten lamps emit a high level of radiant energy in the far-red region of the spectrum, while fluorescent lamps emit in the red region of the spectrum but are deficient

in far-red radiation. The period of the leaf movement rhythm of Phaseolus is shortened by far-red and lengthened by red radiation (Lörcher, 1958), whereas in Bryophyllum both red and certain wavelengths of far-red radiation shortened the period and no wavelengths lengthened it. The period of the leaf movement rhythm of Coleus, like that of the carbon dioxide output rhythm of Bryophyllum, is shortened by radiant energy in the red region of the spectrum (Halaban, 1969). However, in contrast to the Bryophyllum rhythm, the period of the Coleus rhythm is lengthened by blue and is insensitive to far-red radiation.

The transient of the Bryophyllum rhythm is, like the period, modified by radiant energy in spectral bands centred on 600 and 660 nm but not 450 nm. However, the spectral band centred on 730 nm which was effective in shortening the period did not modify the transient. In both Phaseolus (Lörcher, 1958) and Coleus (Halaban, 1969), the period and the time between the initiation of the rhythm and the first phase reference point, which in these cases is the first minimum leaf position, differ in their responses to radiant energy. In Phaseolus, the latter parameter is virtually unaffected by wavelengths which either lengthen or shorten the period. In Coleus, the first minimum occurs earlier with all wavelengths tested, irrespective of their effect on the period.

Entrainment of the rhythm in Bryophyllum to 0.25 h of white light every 24 h can be attributed to wavelengths principally from the red region of the spectrum. Activity was also found at 730 nm in the far-red region of the spectrum but not at 450 nm. The results for the effectiveness of periodic exposure to monochromatic radiation in entraining the rhythm correspond closely to those for the effectiveness of continuous radiation in reducing the period. In two other plants entrainment can be achieved by periodic exposure to red and far-red radiation. 0.25-h

exposures to red or far-red radiation every day are effective in entraining the carbon dioxide output rhythm of Lemna (Hillman, 1971). The leaf movement rhythm of Phaseolus (Lörcher, 1958) is entrained to an abnormal cycle length by 10-h photoperiods of red or far-red, but not blue or green, radiation alternating with 12 h of darkness.

The relative effectiveness of different wavelengths of radiant energy in experiments where the rhythms ~~was~~ were initiated by a 'light-off' signal is similar to that for entrainment and period length determination. Bryophyllum thus differs from the few other plants where defined spectral bands have been employed in initiating the rhythm by either 'light-on' or 'light-off' signals, in that blue light is quite ineffective.

Certain generalisations can therefore be stated concerning the wavelengths of radiant energy responsible for photocontrol of the Bryophyllum rhythm. The responses obtained with white light result principally from the effects of wavelengths in the red region of the spectrum. Although only a single 25 nm wide spectral band was tested in the 400-500 nm region of the visible spectrum, in view of previous investigations (Wilkins, 1960a, 1973) it may be concluded that this region is ineffective in photocontrol of the circadian rhythm. The various responses tested do however show some differences in their sensitivity to different wavelengths and may be divided into two groups. While modification of the period, reinitiation of the rhythm and entrainment can be achieved with both the red and, to a lesser extent, the far-red radiation employed, the latter appears to be ineffective in either modifying the transient or in inducing phase shifts when applied as a single 4-h perturbation.

The characteristics of the action spectra for phase shifting the rhythm had previously indicated that phytochrome might be the pigment involved in this response (Wilkins, 1973). The main features of the

action spectra are very similar to those of the absorption spectrum of phytochrome with the exception of the absence of a minor peak in the blue region. The absence of significant activity in the blue region does not however rule out the involvement of phytochrome and has been reported for other phytochrome mediated responses such as the induction of bean hypocotyl opening (Withrow et. al., 1957). Several kinds of experimental approach using sequential or simultaneous exposures to monochromatic red light and a higher radiant flux density of far-red radiation in a broad spectral band showed that these interact with each other in their effect on the rhythm. With the phase shift studies this interaction quite frequently did not take the form of a clear partial or total reversal of the effect of red by far-red radiation, but rather an abolition of the rhythm when far-red radiation was applied. This abolition of the rhythm may arise in a number of ways. For example, the far-red radiation may interact with the red light to abolish oscillation of the basic circadian system in each of the cells of the leaf. On the other hand, total reversal of the phase shift induced by red light may occur in the basic circadian system in some cells and not in others. This would result in abolition of the overt rhythm of carbon dioxide output from the leaves because the rhythms in some cells would be approximately 12 h out of phase with those in other cells. The occurrence of small peaks at 12-h intervals in some phase shift experiments may result from such cellular desynchronisation.

The entrainment studies have provided a clear demonstration of complete red/far-red reversibility in the Bryophyllum rhythm. Red/far-red reversibility was achieved in experiments in which the leaves were exposed to radiant energy once or twice in every 24-h cycle. In experiments which involved exposing the leaves to a single red light pulse each day, a subsequent exposure to far-red radiation prevented the clear entrain-

ment of the rhythm obtained with red light alone. Far-red radiation applied daily, immediately before a single red pulse, was without effect. Far-red reversal of the effects of red radiation also occurred in schedules in which 0.25-h exposures were separated by 11.75 h of darkness. Such experiments provided the clearest indication of the involvement of phytochrome, since reversal of one of the red light pulses led to entrainment of the rhythm by the other. The resulting rhythm differed in phase and persistence from a rhythm in leaves exposed only to two pulses of red light each day.

It is thus probable that phytochrome is the photoreceptor pigment which mediates each of the aspects of photocontrol studied. Although the spectral band centred on 730 nm is effective in entraining the rhythm but not in inducing phase shifts, some degree of reversibility or evidence for an interaction of red and far-red radiation was obtained for each of these responses. This, together with the likelihood that both responses involve the same basic, phase shifting mechanism, suggests that an explanation for the different sensitivity of the responses to the 730 nm spectral band should be sought in terms of phytochrome action rather than in the possible involvement of alternative or additional pigments.

The effectiveness of monochromatic (730 nm) far-red radiation can be explained as a phytochrome response, since radiant energy of this spectral composition will, because of the long wavelength absorption of Pr, result in a photostationary state in which a small proportion of the phytochrome is present in the active Pfr form (Mohr, 1969). This phenomenon has also been cited to account for the action of both red and far-red radiation in entraining the rhythm of carbon dioxide output of Lemna (Hillman, 1971). With Lemna however, blue light is effective,

and the results are consistent with the proposal that blue light establishes a level of Pfr intermediate between those established by red and far-red radiation. The reason for an absence of a blue light induced response in Bryophyllum is not clear. It is possible that pigments not involved in photocontrol of the rhythm, including the large amounts of anthocyanin present in Bryophyllum leaves, selectively remove, or reduce to an ineffective level, radiant energy from the blue region of the spectrum. The lack of an effect of this region of the spectrum even at the very high radiant flux densities used by Wilkins (1960a) does, however, cast some doubt on this suggestion.

The greater effectiveness of radiant energy at 730 nm in shortening the period than in shifting the phase of the rhythm might be attributable to the generally lower activity at 730 nm than at 600-660 nm. Thus a small effect would be more easily detected with continuous irradiation than with a single exposure of a few hours duration between the first and second peaks of the rhythm. This suggestion may also offer an explanation for the different sensitivities of the phase shifting and entrainment responses to monochromatic far-red (730 nm) radiation. This spectral band might induce only very small phase shifts when given at the times selected for the single exposures, and these shifts might be below the level of statistical significance. When an irradiation treatment is repeated in each cycle however, these small phase shifts would in effect be added with the result that a gradual entrainment of the rhythm would occur. This could also explain why entrainment to far-red radiation is slower than to red light.

A change in the sensitivity of the leaves during the experiments may offer an alternative explanation of the greater effect of the 730 nm

spectral band in the entrainment than in the phase shifting responses. Although virtually no entrainment was apparent during the first three cycles, it was subsequently quite rapid. The concept of increasing sensitivity may also be invoked to explain why continuous exposure to monochromatic far-red (730 nm) radiation or to the lowest of the quantum flux densities of monochromatic red radiation tested, reduced the free-running period of the rhythm but not the transient. However, no evidence for an increasing sensitivity was found in studies of the period, this being constant in leaves continuously irradiated with each of the spectral bands. Further evidence suggests that the different effectiveness of spectral bands in reducing the period and the transient does not result simply from increasing sensitivity. Continuous irradiation with a spectral band centred on 530 nm was much less effective than with one centred on 730 nm in reducing the period of the rhythm. In contrast, whereas irradiation at 530 nm resulted in a reduction of the transient directly proportional to the reduction of the period, irradiation at 730 nm had no significant effect on the transient. Since phytochrome is almost certainly the photoreceptor responsible for mediating these responses, the length of the transient may be determined in part by the Pfr level present at the time of transfer of the leaves to darkness or continuous irradiation, and the subsequent changes in this level during the first few hours of the experiment. The changes in the level of Pfr would presumably depend upon the wavelengths employed. The effects of radiant energy on the period are, in contrast, assessed by comparing the free-running rhythms of irradiated and unirradiated leaves after a sufficiently long time for the Pfr level of the ~~latter~~ former to have reached a photoequilibrium. It is not clear whether the additional factor of initial changes in Pfr levels contribute to the different spectral sensitivities of the rhythm during the transient and free-running states. No firm conclusion can be drawn in the absence of data concerning either

the photostationary state of phytochrome in each of the experiments or the length of the transient in darkness after experimental manipulation of the initial Pfr level.

Photocontrol of the Bryophyllum rhythm appears to be a rather atypical phytochrome response. The spectral dependence of phase shift induction and the occurrence of red/far-red reversibility are consistent with this being a low energy phytochrome response. The fact that the phase shift response is saturated at very low radiant flux densities (Wilkins, 1960a) also supports this conclusion. Typically, the magnitude of a phytochrome mediated response is directly proportional to the logarithm of quantum ~~flux densities~~ doses which do not saturate the phytochrome system (Smith, 1975). In Bryophyllum, this relationship can be seen to hold for the effect of quantum flux density on the free-running period and transient of the rhythms.

The lack of ~~reciprocity~~ reciprocity reported for phase shifting the Bryophy rhythm (Wilkins, 1973) and the observation that far-red radiation centred on 730 nm is effective in modifying other aspects of the rhythm applied continuously or periodically are, however, more suggestive of the involvement of a 'prolonged-light' or 'high-energy' type of phytochrome response. Action spectra for the high-energy response vary markedly between species, even for the induction of a similar response (Smith, 1975). This makes it impossible to deduce the nature of the photoresponse simply from the spectral sensitivity of the Bryophyllum rhythm. It is clear however that the additional activity in the blue region of the spectrum, typical of a high-energy response is absent in the photocontrol of the Bryophyllum rhythm.

The quantum flux density of radiant energy at 730 nm, effective

in this investigation, is similar to the lowest quantum flux density which induces a significant inhibition of lettuce hypocotyl extension (Hartmann, 1966). A significant reduction of the period of the Bryophyllum rhythm was however recorded under continuous irradiation at 660 nm with less than 1 pE cm^{-2} s^{-1}, approximately 2% of the quantum flux density used in the 730 nm spectral band. This flux density may well be too low to implicate a high irradiance response. A comparison of the quantum flux densities required to induce different responses in different organisms, rather than their respective dependence on the irradiance is however of doubtful value and further investigation would be required to ascribe phytochrome control of the Bryophyllum rhythm to the involvement of low-energy or high-energy responses or indeed possibly both.

Phytochrome is held to be involved in entrainment of the rhythm of carbon dioxide output in Lemna perpusilla (Hillman, 1971) and in initiation of the leaf movement rhythm in Phaseolus multiflorus (Lörcher, 1958). These conclusions are based on the reversible effect of red and far-red radiation, no detailed action spectra having been determined for these plants. The effects of different wavelengths of radiant energy in modifying the period with continuous irradiation or shifting the phase with a single irradiation have not been investigated in Lemna. It is unlikely that results which would permit further comparison with the Bryophyllum rhythm could be obtained for these aspects of the photocontrol of the Lemna rhythm. This is because the Lemna rhythm persists for only one or two cycles in continuous conditions, and its amplitude is very small.

Additional aspects have been studied for the Phaseolus rhythm and have revealed a more complex situation. In this organism, far-red radiation possibly exerts an effect which is essentially different from

that of red light. Red and far-red radiation give different phase-response curves and, more significantly, are effective only when absorbed by different parts of the plant (Bünning and Moser, 1966).

Phytochrome has also been implicated in the control of the circadian rhythm of sensitivity to flower induction in Xanthium (Salisbury, 1965; Denny and Salisbury, 1970). The rhythm in sensitivity to germination promotion in Sphearocarpus donelli may also be controlled by phytochrome (Steiner, 1969). Schrempf (1975) considers that phytochrome may be involved in the phase shift response of the Kalanchoë petal movement rhythm. Phytochrome is clearly not involved in the photocontrol of the circadian rhythms in Oedogonium cardiacum or Neurospora crassa which are affected by wavelengths of radiant energy from the blue and near UV regions of the spectrum. Muñoz and Butler (1975) conclude that a flavin is the photoreceptor involved in Neurospora and also in the insects Drosophila and Pectinophora. Neither is phytochrome always the pigment involved in circadian rhythms in higher plants. Karvé et. al. (1961) conclude that the induction of rhythmic petal movement of Kalanchoë flowers by 'light-on' or 'light-off' signals probably depends upon radiant energy absorbed by chlorophyll. The photosynthetic pigments are probably also responsible for photocontrol of several circadian rhythms in Gonyaulax polyedra (Sweeney, 1969).

The involvement of different photoreceptors in controlling the circadian rhythms in different organisms may suggest that the individual rhythms studied are derived from different basic mechanisms, affected more or less directly by radiant energy. Alternatively, if a single universal oscillator is responsible, it is clear that organisms have evolved with different pigments responsible for mediating the effects of radiant energy on that circadian system.

Although it is apparent that phytochrome-absorbed radiant energy influences the circadian rhythm in Bryophyllum, the biochemical mechanism of this effect remains undetermined. Little is known of the events which occur between the absopption of light by phytochrome and the enormous variety of developmental and non-developmental responses which follow. The biochemistry of the basic oscillator is equally unknown. It is possible to consider the role of phytochrome in terms of the various, general hypotheses for oscillating systems. Clearly if any of these is to apply to the rhythm in Bryophyllum, it must be able to accommodate a specific role for phytochrome. Alternatively, consideration may also be given to the possible involvement of phytochrome in the control of the mechanisms underlying the overt rhythm in Bryophyllum, which may or may not represent the basic oscillator.

Two fundamental modes of action have been proposed to account for the action of phytochrome. Mohr (1966) proposed the hypothesis that phytochrome regulates development by controlling gene expression at the genome level. This proposal implied that Pfr directly, or possibly indirectly (Mohr, 1972), influences the genome by selectively inducing or repressing transcription. The occurrence of a number of responses to radiant energy absorbed by phytochrome, which are too rapid to be explained by an effect on transcription, led to the alternative hypothesis that phytochrome acts by modifying membrane permeability (Hendricks and Borthwick, 1967). Evidence that phytochrome mediates effects on membranes comes from the observations of rapid red/far-red reversible changes of the electric potential of etiolated oat coleoptiles (Newman and Briggs, 1972) and mung bean root sections (Jaffe, 1968). Further evidence for an initial effect of phytochrome on membranes comes from observations of the nyctinastic leaflet closure of Albizzia julibrissin which is promoted by red light and inhibited by far-red radiation (Hillman and

Koukkari, 1967). The two general hypotheses of phytochrome action are not however mutually exclusive, as long term effects on gene expression may result indirectly from an initial effect on membrane systems.

The hypotheses for the mechanism of the basic circadian oscillator can be divided into two general types which correspond rather closely to the proposals for the mode of action of phytochrome. There are thus proposals which account for rhythmicity in terms of either cycling, sequential transcription or in terms of rhythmic changes in the properties of membranes. The 'chronon concept' of Ehret and Trucco (1967) implies an ordered, sequential transcription of RNA from DNA. The cyclic nature of such a system is thought to result from the positive feedback of the product of translation of a terminator cistron on transcription at a cistron which initiates the next transcription sequence. If the action of phytochrome were to induce or repress transcription of genes involved in this cycle, it can easily be envisaged that the normal transcription sequence could be perturbed.

The alternative theory, of overt rhythmicity resulting from cyclic changes in the properties of membranes, is most cogently expressed in the membrane-ion gradient hypothesis of Njus et. al. (1974). This theory accounts for the effects of radiant energy on circadian rhythms by suggesting that it acts by perturbing ion concentration gradients. A number of rhythmic processes can readily be attributed to the movement of ions. There is a good correlation between the movement of leaflets of Albizzia julibrissin, controlled by an endogenous rhythm, and the movement of potassium ions between the ventral and dorsal motor cells of the pulvinule (Satter and Galston, 1971a, 1971b). Furthermore, the movement of potassium ions in this system is influenced by red and far-red radiation acting through phytochrome (Satter and Galston, 1971b). The

supposition of the membrane-ion gradient hypothesis, that light acts by modifying the changes in ion gradients which are responsible for endogenous rhythmicity, is thus borne out in Albizzia. The role of phytochrome in control of circadian rhythms can therefore be envisaged as altering the permeability of membranes either directly, if membrane bound phytochrome were to act as a 'photosensitive ion gate', or indirectly, possibly through the rapid production of a membrane active hormonal substance.

The rhythm of carbon dioxide output in Bryophyllum leaves in carbon-dioxide-free air results from the rhythmic fixation of respired carbon dioxide by periodic activity of the enzymes responsible for CAM. Phytochrome might thus mediate the effects of radiant energy on the rhythm either by inducing or repressing enzyme synthesis, or by activating or inhibiting enzymes already present in the leaves. Queiroz (1965, 1966, 1967, 1968a, 1968b, 1969, 1970, 1972a, 1972b), Queiroz et. al (1971, 1972) have investigated the CAM pathway of Kalanchoë blossfeldiana in detail. The induction of rhythmic variation in the activity of enzymes of this pathway is a short-day photoperiodic response. Red/far-red reversibility of 'night-breaks' revealed that phytochrome is involved in this response. The induction of rhythmicity in Kalanchoë by short days is accompanied by an overall increase in the activity of malic enzyme and PEP carboxylase in leaf extracts. Queiroz (1972a) concludes that this increase occurs as a result of both an increase in enzyme synthesis and a decrease in the level of an inhibitor. There is thus some evidence to suggest that phytochrome, acting in a long term photoperiodic response, may increase the level of activity of the enzymes responsible for CAM in Kalanchoë.

In Bryophyllum, large amounts of PEP carboxylase can be extracted from the leaves even at phases in the cycle at which no fixation occurs

(Wilkins, 1969). This has been interpreted as indicating that circadian variation in the amount of PEP carboxylase does not control the rhythm of carbon dioxide fixation (Wilkins, 1969). A number of investigators have suggested that CAM involves feedback control of the enzyme responsible for carbon dioxide fixation, by the products of this reaction, in particular malate (Kluge and Osmond, 1972; Queiroz et. al., 1972). In Kalanchoë, there is a close correlation between the malate content of leaves and the activity of enzymes responsible for carbon dioxide fixation and deacidification. It is thus likely that both the circadian rhythm of carbon dioxide fixation in Bryophyllum, and the diurnal variation in the activity of the enzymes of the CAM pathway in crude extracts of Kalanchoë leaves are the result of periodic inhibition or activation rather than synthesis of the enzymes.

It has been proposed that active and passive transport of malate to and from the vacuole is also involved in the control of CAM. The resulting changes in the size of the cytoplasmic pool of malic acid may lead to inhibition and promotion of the enzymes of the pathway (Kluge and Heininger, 1973; Kluge and Lüttge, 1974). The importance of the transporting properties of membranes in the operation of CAM has also been stressed by Willert (1972) and Willert and Kluge (1973). Luttge and Ball (1974a, 1974b) have drawn attention to the possible rhythmic control of CAM by malate and proton fluxes across the tonoplast in a system analogous to the potassium fluxes which control the leaflet movement of Albizzia. It might thus be possible to account for the Bryophyllum rhythm by the membrane-ion gradient model of Njus et. al. (1974). If this were the case, the carbon dioxide fixation phase of the rhythm would cease only when sufficient malate had accumulated in the vacuole to exert a negative feedback effect on its transport to that site. However, Wilkins (1959) found that the fixation of the rhythm in leaves in both

normal air and carbon-dioxide-free air lasted for approximately the same time. A greater net fixation of carbon dioxide occurred in normal air than in carbon-dioxide-free air (Wilkins, 1959). Carbon dioxide fixation therefore presumably ceases at a time when leaves exposed to different levels of atmospheric carbon dioxide contain different amounts of malate. An analysis of the malate content of leaves exposed to the various constant conditions employed in this investigation might usefully clarify this point.

In Albizzia, the action of phytochrome, in its interaction with the endogenous rhythm, appears to be to alter the movement of ions across membranes. A similar function may be proposed to account for phytochrome control of the Bryophyllum rhythm. If exposure to radiant energy were to modify the intracellular location and transport of malate, it is likely that this alone could alter the rhythm by virtue of the well-documented, inhibitory effect of malate on PEP carboxylase (Kluge and Osmond, 1972) and possible, stimulatory effect on malic enzyme activity (Queiroz, 1972). If this hypothesis is correct it should be possible to observe an effect on CAM, of radiant energy absorbed by phytochrome. The possibility can never be ruled out however, that any such effects are mediated by phytochrome-induced changes in a more basic oscillating mechanism. There is little evidence of a direct effect of phytochrome-absorbed radiant energy on CAM.

Lasher and Bonner (1955) reported that the effectiveness of different spectral bands in deacidification of Bryophyllum leaves did not agree with the general action spectrum of photosynthesis, red light being much more effective than blue. The significance of these results is not clear however, since a rapid rate of photodeacidification has, with the use of photosynthetic inhibitors, been shown to depend on the occurrence

of photosynthesis (Denius and Homann, 1972). Bruinsma (1958) suggested that the anthocyanin present in Bryophyllum leaves might in part explain a strong non-effective absorption of blue light, but he also discussed the possibility of a more direct effect of radiant energy on CAM. He speculated that a change in irradiation or temperature conditions might alter the permeability of intracellular membranes and, by that, the availability to cytoplasmic enzymes, of acids accumulated in the vacuole. The rates of acid breakdown and formation would then change.

There is an increasing understanding of the mechanism of CAM and evidence for phytochrome control of the circadian rhythm in the activity of this pathway. This suggests that further, detailed investigation of the control of the CAM pathway, in experiments in which phytochrome can also be shown to influence the circadian rhythm, presents the best opportunity for elucidating both the basic oscillating mechanism and its photocontrol in Bryophyllum.

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APPENDIX 1

The sequential recycling timer.

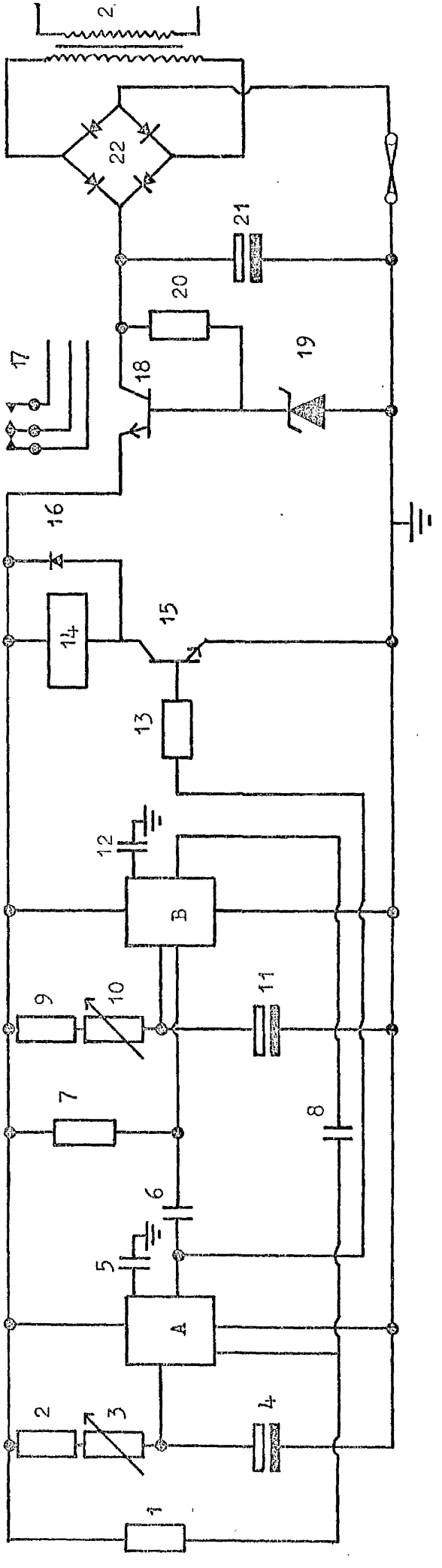
Description and circuit diagram.

FIGURE 39

The figure shows two NE 555 timers (A and B) connected sequentially, and their associated power supply. The first timer (A) is started by a pulse on pin b when the 24-h time clock micro switch closes. It runs for a predetermined time governed by the 470 µF capacitor (4) and the setting of the 2 MΩ variable resistor (3). At the end of its timing cycle, it triggers the second timer (B), which runs for its preset time. After this time the whole process is repeated. This recycling is effected by feeding the output of B, pin c, back to the input of A, pin b. The sequential recycling of the timers will continue until the 24-h time clock micro switch opens and effectively removes the power supply from the circuit. For clarity, enlarged diagrams of A and B are shown to illustrate the contacts made with the 8 (a-h) pins of the NE 555 integrated circuits.

FIGURE 39

1.	15 KΩ	5.	0.01 µF	9.	10 KΩ	13.	10 KΩ	17.	Relay contacts shown separately.	21.	2500 µF
2.	10 KΩ	6.	0.5 µF	10.	2 MΩ	14.	Relay coil	18.	BDY 62	22.	REC 70
3.	2 MΩ	7.	27 KΩ	11.	470 µF	15.	40348	19.	15.7 VZ	23.	24-h time switch
4.	470 µF	8.	1 µF	12.	0.01 µF	16.	OA 47	20.	820Ω		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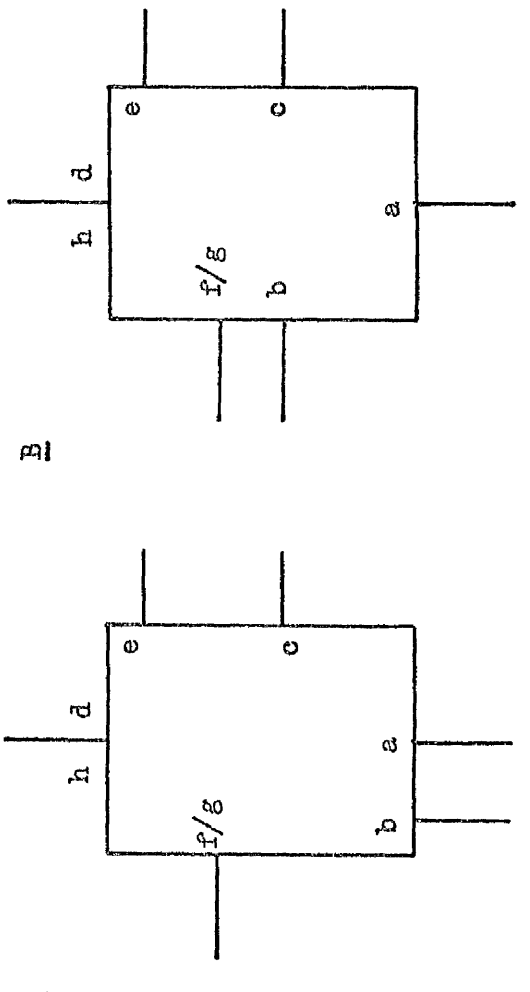
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APPENDIX 2

TABLE 1

The effect, on the period of the rhythm, of continuous darkness or an incident quantum flux density of 47 pE cm^{-2} s^{-1} in 25 nm wide spectral bands.

Irradiation treatment	Number of individuals readings	Period mean ± S.E.	Significance of difference from dark control	
			t value	probability
Darkness	42	24.20 ± 0.17		
450 nm	12	24.42 ± 0.23	0.6413	N.S.
530 nm	12	23.00 ± 0.25	3.4331	<0.01
600 nm	14	19.38 ± 0.36	13.3128	<0.001
660 nm	23	19.32 ± 0.35	14.0722	<0.001
730 nm	18	21.37 ± 0.23	9.2734	<0.001
760 nm	17	24.61 ± 0.29	1.3400	N.S.

TABLE 2

The effect, on the transient of the rhythm, of continuous darkness or an incident quantum flux density of 47 pE cm^{-2} s^{-1} in 25 nm wide spectral bands.

Irradiation treatment	Number of individual readings	Transient mean ± S.E.	Significance of difference from dark control	
			t value	probability
Darkness	16	25.86 ± 0.21		
450 nm	4	25.46 ± 0.46	0.8525	N.S.
530 nm	4	24.68 ± 0.19	2.7338	<0.02
600 nm	6	21.70 ± 0.22	11.3297	<0.001
660 nm	10	21.85 ± 0.32	11.0031	<0.001
730 nm	6	26.09 ± 0.33	0.5782	N.S.
760 nm	6	26.22 ± 0.37	0.8785	N.S.

TABLE 3

The effect, on the period of the rhythm, of continuous darkness or various quantum flux densities of monochromatic radiation in a 25 nm wide spectral band centred on 660 nm.

Irradiance pE cm^{-2} s^{-1}	Number of individual readings	Period mean $\pm$ S.E.	Significance of difference from dark control	
			t value	probability
0.00	31	23.74 $\pm$ 0.20		
0.98	12	22.61 $\pm$ 0.38	2.8800	<0.01
3.30	10	21.93 $\pm$ 0.35	4.5664	<0.001
13.00	10	20.31 $\pm$ 0.19	9.4348	<0.001
22.00	14	19.93 $\pm$ 0.41	9.5400	<0.001
47.00	23	19.32 $\pm$ 0.35	11.7753	<0.001

TABLE 4

The effect, on the transient of the rhythm, of continuous darkness or various quantum flux densities of monochromatic radiation in a 25 nm wide spectral band centred on 660 nm.

Irradiance pE cm^{-2} s^{-1}	Number of individual readings	Transient mean $\pm$ S.E.	Significance of difference from dark control	
			t value	probability
0.00	13	26.24 $\pm$ 0.31		
0.98	4	26.24 $\pm$ 0.17	0.0057	N.S.
3.30	4	24.91 $\pm$ 0.67	1.9923	N.S.
13.00	4	24.11 $\pm$ 0.28	3.6195	<0.01
22.00	6	23.12 $\pm$ 0.28	6.2579	<0.001
47.00	10	21.85 $\pm$ 0.32	9.7330	<0.001

TABLE 5

The effect of temperature on the period of the rhythm in leaves in darkness or irradiated with an incident quantum flux density of 47 pE cm^{-2} s^{-1} at 660 nm.

Temperature °C	Darkness		Irradiated		Significance of difference between dark and irradiated	
	Number of individual readings	Period mean ± S.E.	Number of individual readings	Period mean ± S.E.	t value	Probability
15	29	23.67 ± 0.20	23	19.32 ± 0.35	11.3222	<0.001
20	9	24.21 ± 0.34	16	21.85 ± 0.31	4.8211	<0.001
25	21	24.57 ± 0.35	17	22.75 ± 0.39	3.4800	<0.02
30	15	20.92 ± 0.32	12	20.57 ± 0.51	0.5958	N.S.

TABLE 6

The effect of temperature on the transient of the rhythm in leaves in darkness or irradiated with an incident quantum flux density of 47 pE cm^{-2} s^{-1} at 660 nm.

Temperature °C	Darkness		Irradiated		Significance of difference between dark and irradiated	
	Number of individual readings	Transient mean ± S.E.	Number of individual readings	Transient mean ± S.E.	t value	Probability
15	11	26.02 ± 0.32	10	21.85 ± 0.32	9.1377	<0.001
20	6	21.98 ± 0.49	6	18.80 ± 0.40	5.0410	<0.001
25	8	19.84 ± 0.37	8	18.58 ± 0.32	2.5636	<0.05
30	5	15.68 ± 0.13	4	15.88 ± 0.24	0.7600	N.S.

TABLE 7

The significance of the difference between the periods of the rhythms in leaves at different temperatures.

Temperatures °C	Darkness		Irradiated, 47 pE cm^{-2} s^{-1} at 660 cm	
	t value	Probability level	t value	Probability
15/20	1.1946	N.S.	5.1528	<0.001
15/25	2.3465	<0.05	8.1352	<0.001
15/30	7.5302	<0.001	2.0521	<0.05
20/25	0.6097	N.S.	1.7911	N.S.
20/30	6.6304	<0.001	2.2500	<0.002
25/30	7.3249	<0.001	3.4524	

TABLE 8

The significance of the difference between the transients of the rhythms in leaves at different temperatures.

Temperatures °C	Darkness		Irradiated, 47 pE cm^{-2} s^{-1} at 660 nm	
	t value	Probability level	t value	Probability
15/20	7.0963	<0.001	5.8785	<0.001
15/25	12.5513	<0.001	7.0901	<0.001
15/30	20.8470	<0.001	11.0710	<0.001
20/25	3.5855	<0.01	0.4460	N.S.
20/30	11.4544	<0.001	5.4609	<0.001
25/30	8.5747	<0.001	5.4379	<0.001