CIRCADIAN RHYTHMICITY

Proceedings of the International Symposium on Circadian Rhythmicity, Wageningen, the Netherlands, 26 - 29 April 1971

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Preface

Once every three years the Agricultural University of Wageningen organizes a symposium on a fundamental aspect of natural science. In 1971 the topic was Circadian Rhythmicity. Its influence in biology ranges from the whole organism to the subcellular level. The symposium attempted to cover all these aspects.

Diurnal rhythms were first observed long ago. In plants, for instance, diurnal movements of leguminous plants were noticed in the time of Alexander the Great. A century ago such rhythms were observed in animals, and interest in rhythmicity in man is now rapidly increasing with the advent of jets and space flight.

Circadian rhythms are very important in ecology. Many organisms adapt themselves to cyclic external conditions such as day and night, and seasonal trends. Even tidal movements and moon phases seem important in terrestrial life. There is also a close interrelation between various species in a community. Production of pollen and nectar, and opening and closing of flowers are often diurnal in rhythm. Flight of bees is synchronized with such plant rhythms. Some circadian rhythms may have great influence on survival, since they may synchronize activities with the most favourable moment of the day or season.

A circadian rhythm is endogenous when rhythms continue for at least a few days under constant environmental conditions. Research has advanced rapidly since it became possible to control environmental conditions. Temperature changes of 1 °C and radiation for only a few seconds are now known to modify results.

Besides such close control of the environment, research has been promoted by the precision, with which rhythms can be measured, by the sophistication of mathematical models, and by use of the computer.

This book records the proceedings of a symposium on Circadian Rhythmicity held in Wageningen, the Netherlands, 26–29 April 1971 during which many new aspects were discussed of circadian rhythms in plants and animals at the cellular and subcellular level, kinetic models, biochemical processes and the effect of external factors on rhythms.

We thank the Board of the University for financial support, our contributors for their excellent reviews, the International Agricultural Centre for technical assistance and Pudoc for publishing the proceedings.

J. F. Bierhuizen

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Symptoms, problems, and common features of circadian rhythms in plants and animals

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Introduction

In my Opening Address at the 1960 Cold Spring Harbor Symposium on 'Biological Clocks', I summarized the history of research on endogenous *diurnal* rhythms. I will not therefore repeat myself, except to mention one piece of history. That is the work in the Netherlands of Anthonia Kleinhoonte. Kleinhoonte published her thesis in 1928 in Dutch and published work in German a year later about the time I became interested in these problems. She worked in the Laboratory for Technical Botany in Delft, using the same dark room that Brouwer had used a few years earlier (1926) and the same species of plant. Brouwer believed he had shown the control of the diurnal leaf movements by some unknown external factor. He was not the only biologist considering biological rhythms to be synchronized by a factor X, ascribed variously to electric charges in the air, cosmic rays and radioactive radiations.

History credits Kleinhoonte for showing that it is only necessary to improve control of the well known external factors such as temperature and light for the endogenous nature of the circadian rhythms to become clear. Kleinhoonte showed that even pulses of weak light, not more than a minute long, effectively cause delays or advances of circadian cycles and synchronize them. These were also the first steps in studying what is now known as phase-response curves of circadian rhythms.

Unfortunately, even in later work, authors came to wrong conclusions by ignoring the synchronizing effects of weak control lights and other factors. When, under so-called constant conditions, we find an accumulation of physiological peaks around certain hours of the day or an exact 24-h periodicity, we should ask ourselves: where is the mistake in the experimental conditions? (Fig. 1) I raised this question when I began work together with Kurt Stern in 1928. We found that the mistake underlying previous research was the overlooking of the synchronizing effect of very weak control light. Recent evidence against control of rhythms by unknown external factors comes from Enright's (1965) careful analysis of several published experiments. His analysis gave no support for a role of subtle geophysical factors in synchronization.

It was also left to Kleinhoonte to draw attention to earlier papers by Pfeffer. His publications from 1907–15 were neglected in papers on diurnal periodicity in plants

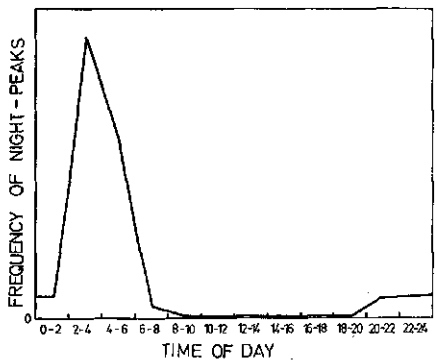

Fig. 1. Example of experiments believed to show the synchronizing effect of 'factor X'. Diurnal leaf movements of *Phaseolus* in a darkroom. Night-peaks (maximum of dawnward movement) mostly occur about 3 h after midnight in spite of 'constant conditions'. After experiments of Stoppel (1916). The 'factor X' was later shown to be weak red control light, used in the preceding morning hours to start the experiment, and to water the plants.

and animals published before Kleinhoonte's work. Actually Pfeffer clearly demonstrated an endogenous component in circadian leaf movements. His only mistake was to publish his mass of results, filling about 500 pages, in *Abhandlungen der mathematisch-physischen Klasse der Königlich Sächsischen Gesellschaft der Wissenschaften.* This sounds very distinguished and aristocratic, but it is, as we say in Germany, 'ein Staatsbegräbnis erster Klasse' (a first class state funeral).

Terminology and some basic phenomena

Sometimes, any type of 24-h cycle is called a circadian cycle. However, this term should be restricted to cycles, which continue in constant temperature and in the absence of diurnal light-dark cycles. Under these conditions, circadian cycles are no longer exactly 24-h periods but vary between about 22 and 28 h and usually between 23 and 25 h. This period varies not only from species to species, but also from one individual plant or animal to the other. Only a few cases are known, where it becomes difficult to find significant deviations from the exact 24-h period when diurnal temperature and light-dark cycles are excluded. Two examples are the fiddler crab *Uca* and the *Canavalia* studied by Kleinhoonte.

However many strictly exogenous diurnal physiological cycles do occur. These should be called 'daily rhythms' or '24-h rhythms', but not 'circadian rhythms' (Wurtman, 1966). It is not always easy to detect the participation of a circadian rhythmicity in a daily rhythm. Continuous light or darkness are often so strongly disturbing or damaging to the organism, that the overt rhythmicity disappears immediately. If, for example, bean plants are brought from the greenhouse into continuous darkness or continuous incandescent light, the circadian leaf movements will no longer continue. This is due to the far-red component of that light. Therefore the earlier experiments with beans were always carried out with plants grown in dark rooms, and thus adapted to this condition. Nowadays this is no longer a problem: greenhouse plants continue with their circadian leaf movements for their whole life time in the light of fluorescent tubes which lacks far-red.

A similar problem may occur with animals. Sometimes the circadian rhythmicity disappears quickly after bringing the animals into continuous darkness or continuous light. Then the application of continuous dim light can be advantageous.

Diversity of circadian rhythms

It is tempting to assume an identical cellular basis for all circadian rhythms, from unicellular organisms up to higher plants and animals. However, this hypothesis is not substantiated by the facts. We know about many developmental cycles showing periods of about 24 h which apparently are self sustaining cycles in the sense, that each of the several morphological steps is comparable with part of the mechanism of a clock. Mitotic cycles can also have features of developmental cycles. Contrary to this, in the 'classical' cases of circadian rhythms, the cycles are maintained without overt morphogenetical steps.

Microscopic or submicroscopic changes can be associated with the running of the circadian clock. For instance, circadian changes in the volume of the nuclei or in the shape of chloroplasts are known. But apparently, they are again just consequences, not components of the unknown clockwork. The reason for this is that in other species or with other experimental conditions, these overt microscopic or submicroscopic changes may be missing, although the clock continues to run. Sweeney and co-workers studied the unicellular alga *Gonyaulax* which shows circadian rhythms in bioluminescence, in photosynthetic capacity, and in several other physiological processes. Cells of *Gonyaulax* were fixed at different times of day in light-dark cycles and in constant light. 'Examination of thin sections under the electron microscope ... failed to show any differences in structure which could be correlated with the time in the cycle when the cells were fixed, although nucleus, nuclear membrane, Golgi bodies, mitochondria, plastids and cell membranes were well preserved' (Sweeney, 1969), Thus, in this respect typical circadian rhythms differ from developmental cycles.

Many recent studies on synchronized cells kept in cultures have shown that in developmental cycles, generation time may vary considerably. Usually these cycles in division have adapted to the 24-h cycle (Bruce, 1965).

During earlier research work on circadian rhythms, all these rhythms were interpreted as developmental cycles or as being analogous to them. That means, gross morphological or biochemical events were assumed to be the cause or consequence of one another, each requiring a certain time, about 24 h in total. Simple feedback explanations are also characteristic of these earlier attempts: running activity was thought to cause fatigue and a period of rest to cause reactivation. A strong upward directed position was believed to cause downward leaf movement in plants, with an overshoot leading to a new upward movement.

Both these simple feedback explanations were shown to be wrong by appropriate experiments. Richter (1965) made rats totally inactive for ten days with electro-shocks. But the clock continued to run during this period because, at the end of the treatment, activity occurred exactly at the predicted time as if nothing had been done to the rat. Pfeffer (1911) reported on an analogous experiment on leaf movements. He prevented

movement mechanically and when the leaves were released they resumed movement without any phase shift.

These are just two examples from a wide range of circadian processes. In contrast to what I called developmental cycles, the 'typical' circadian rhythms behave like technical control systems: the overt circadian rhythms in growth, respiration, photosynthetic capacity, synthesis and hydrolyzation of starch or glycogen, movements, running, hatching from pupea and bioluminescence are not comparable with the mechanism but merely with the hands of the clock. We can inhibit all these processes and capacities to 1% or nearly zero without any significant effect on the rhythm. For example, the circadian rhythm of glycogen deposit in the liver persists even under conditions of starvation. Experiments with reduced temperature or with metabolic poisons show that the circadian clock, like technical control systems, operates on a very low amount of energy requirement which is negligible as compared with the amount necessary for the controlled processes. Even the organism's sensitivity to poisons and light are governed by the circadian clock.

Multifarious processes are controlled by the circadian clock. The organism's ability to measure day length is governed by quantitative and qualitative oscillations in responsiveness to light. Such typical circadian cycles cannot always be distinguished from developmental cycles. Developmental cycles may even be coupled to the circadian cycle. This may happen for instance with respect to mitotic cycles. Quite often, mitosis is restricted to certain phases of the circadian clock, so that its maxima come at night.

In searching for the biochemical or biophysical basis of circadian rhythmicity we should try to study systems without such complications. For instance, studies based on tissues with mitotic activity may lead to wrong conclusions. The well known biochemical characteristics of the several mitotic phases may be wrongly interpreted as components of the underlying circadian clock. This holds for studies on the circadian rhythmicity of nucleic acid metabolism.

We are not sure whether the 'typical' circadian clock operates on the same basis from unicellular algae up to the cells of vertebrates. But many common features are a good argument for such an assumption.

Common features of circadian clocks

Summarizing these common features:

1. Under conditions of constant temperature and continuous light or darkness, the circadian rhythms continue with their free-running periods for several weeks, months, or longer. This is possible even through different developmental stages, for instance from the egg through the pupal stage to the imago in insects (Minis and Pittendrigh, 1968; Bünning, 1967). In mammals, the clock can also continue to function during hibernation (Saint Girons, 1965). The length of the period, as determined by genetic constitution and specific type of constant conditions, is maintained from day to day with slight variations of only a few minutes. Individual differences within a species are significant, but mostly not more than about 10–15 min.
2. The rhythm may stop because of damping out in certain constant conditions.

Otherwise as in a plant or an insect, developing in constant conditions from seed or egg, the arhythmicity is due to the lack of initiating stimulus. Here a single stimulus is sufficient to initiate rhythmicity. Such a stimulus may be a single light-pulse in continuous darkness, a transition from continuous darkness to continuous light, or vice versa, transition from one constant temperature to a higher or lower constant temperature or a single dark-period in continuous light.

Sometimes the absence of overt rhythmicity can be interpreted as resulting from desynchronization between the several cells of a tissue. But this explanation is not always applicable. The possibility of a real arhythmic status becomes clear from observations on unicellular organisms, and on tissues from multicellular organisms (Bünning, 1967). Circadian changes in the volume of nuclei can be seen in many tissues. When the rhythm stops the extreme values of the nuclear volumes are no longer reached (Wassermann, 1959). Of course, one might object that there is also a desynchronization within the individual cells. But very strong evidence is against such an assumption. In the unicellular algae *Gonyaulax*, different circadian processes can be observed in the individual cell. But it has never been possible to change the normal phase angle difference between them. This should be possible if desynchronization inside the individual cell does indeed occur.

True arhythmicity has been substantiated by rhythm inducing and synchronizing studies on the fruitfly (*Drosophila*) (Zimmerman, 1969; Winfree, 1970) and on the churchyard beetle (*Blaps mucronata*) (Thomas and Finlayson, 1970).

3. As already mentioned, little energy is necessary for the running of the clock. This becomes evident from experiments at low temperatures. The temperature may be lowered to a degree that no longer allows growth, running, spore discharge and respiration. On re-establishing normal temperature, the physiological processes may start again without a phase shift in their rhythmicity. The clock is only stopped when the temperature is a few degrees below the minima for the overt processes. With organisms adapted to low temperatures (especially aquatic plants and animals), temperatures below zero may be necessary to inhibit the mechanism of the clock.

4. Very characteristic is the low dependency of the speed of the clock on temperature. The Q_{10}-values for the periods are usually between 1.0 and 1.1, seldom reaching values of 1.2 or more. These extreme values, resulting in periods of about 20 h at constant temperatures of about 15 °C or lower, are known for tropical plants which normally are never exposed to such low temperatures (Mayer, 1966).

Although temperature has a rather limited influence on the length of periods, slight diurnal temperature changes are sufficient to synchronize circadian rhythmicity. Sometimes diurnal fluctuations of less than one degree, in constant light or continuous darkness, are sufficient for synchronization. This synchronizing action is not in contradiction to the almost negligible influence on the length of the periods. The explanation is that certain phases of the circadian rhythms are advanced, others delayed by an increase or decrease of the temperature. The typical phase response curves show this (Fig. 2).

5. Equally important for the usefulness of circadian rhythms in chronometry is the low influence of light intensity on the length of periods. The periods are different in continu-

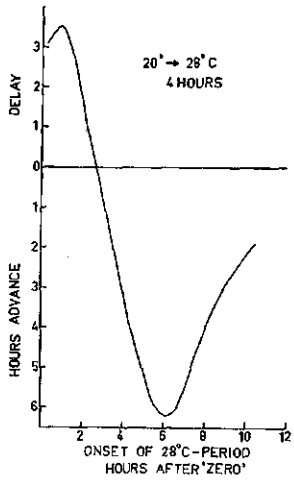

Fig. 2. *Phaseolus multiflorus*, circadian leaf movement in continuous light. The curve shows phase shifts occuring after the constant temperature (20°) has been raised to 28° for 4 h. Abscissa: onset of 28°-period, hours after the maximum night-position of the leaves. After Moser (1962).

ous darkness and in continuous light, and they depend on the intensity and quality of continuous light. But these influences are not much greater than those of the temperature.

On the other hand, diurnal cycles of light and darkness, even very short light pulses of one minute or less, are quite sufficient for a synchronization of the cycles. Light is a much more effective synchronizer than temperature. Again, similar to the temperature effects, typical phase response curves can explain these synchronizations (Fig. 3).

6. The entrainment of the circadian rhythms to cycles differing from the 24-h cycle is possible, but only within narrow limits. Usually these limits are between about 18 and 30 h. With light-dark cycles or cycles of higher and lower temperature that deviate

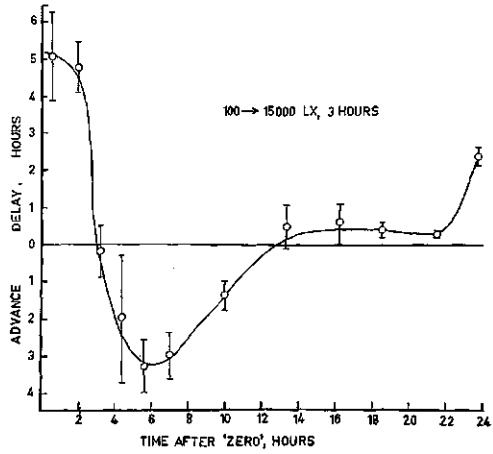

Fig. 3. *Phaseolus multiflorus*, circadian leaf movements in continuous light of 100 lx. The curve shows phase shifts occuring after the light-intensity has been increased to 15000 lx for a period of 3 hours. Abscissa: onset of period with higher intensity, hours after the maximum night-position of the leaves. After Moser (1962).

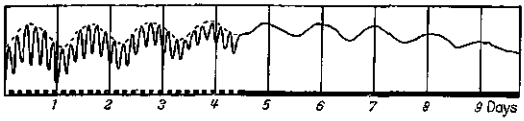

Fig. 4. *Euglena gracilis*. Rhythm in mobility. Superposition of an endogenous circadian and an exogenous 4 h rhythm in 2:2 h light-dark cycles, and circadian rhythm in continuous darkness. Broad black lines indicate darkness. From Bünning (1967).

significantly from these limits the organisms tend to oscillate with free running periodicity. However, external cycles of, for example, 6:6, 3:3, or 2:2 h can synchronize the circadian rhythm to exactly 24-h cycles ('frequency demultiplication' or 'frequency division').

There are some reports in the literature about entrainment to much shorter or longer cycles than 18 and 30 h respectively. Here we are obviously dealing with a combination of a free-running circadian periodicity and an exogenous periodicity (Fig. 4).

7. Several attempts have been made to find 'the site' of 'the clock' in multicellular organisms. Certain organs in vertebrates and invertebrates were believed to shelter or even to be themselves the 'master clock'. Many scientists showed a certain vital control and regulation of circadian periodicity by the brain, by parts of the brain, by certain ganglia in insects, by the pituitary, the adrenal and the pineal gland. But none of these organs can be compared with a 'master clock'. Certain diurnal functions may be the consequence of circadian hormone production. But other circadian processes may continue after removing the respective endocrine systems. Circadian activity is known for instance for the pituitary, the hypothalamus, the adrenal and the pineal gland. However circadian rhythms of activity in mammals are able to continue without activity of the adrenal, the pituitary or the pineal. (Richter, 1967; Roberts, 1965).

This is also true for lower animals. Contraction and expansion of chromotophores belong to the well known circadian processes. Hormonal or nervous influences were believed to be fully responsible for this rhythmicity. Experiments with the polychaete *Platynereis dumerilii* confirmed the existence of nervous and humoral influences. But experiments with isolated pieces of skin suggested that an endogenous component of this rhythmicity was located in every chromatophore (Röseler, 1970).

Plant or animal tissues and even the individual cells in tissue cultures from quite different organs display circadian rhythms independent of any of these controlling organs. The control function of certain glands is especially one of a mutual synchronization in the organism's body. The adrenal, for example, seems to be necessary for coupling a number of diverse functions in the body of mammals, to care for mutual entrainment and appropriate phase relations. The pituitary is necessary for mediating the synchronizing light effect. A mutual synchronization of various circadian processes within the individual organism already occurs in plants. It is more important in animals, and its disturbance leads to rhythm dissociations in the organism often resulting in diseases.

I do not intend to report on the immense biological and medical work on controlling processes. For a general review of common features it is more important to stress that individual cells, from unicellular algae up to isolated cells of higher plants and animals, possess circadian oscillations.

If there is a feature in rhythm entrainment common to all organisms from lower plants up to mammals it is perhaps the means and pathways through which information is relayed. Information has been said to be transmitted primarily via electrical rather than chemical pathways. (Brady, 1969; Roberts, 1965; Bennett, 1970; Azarjan and Tyshchenko, 1970). Many experiments to induce phase shifts by chemical agents were unsuccessful and therefore an additional argument for such a hypothesis.

Effect of light

Light quality and sites of photoreception. Diurnal light-dark cycles are the most important synchronizer of circadian rhythms in plants and animals. Apparently, light never affects the circadian clock directly, but through complicated pathways. In vertebrates it affects the clock via light-reception in the eye. However, even in vertebrates an extraretinal reception of light by the brain, or parts of the brain, has quite often been observed to be effective in synchronizing the circadian clock (Menaker, 1968; Adler, 1969). There are experimental data strengthening 'the hypothesis that all vertebrates may rely on cues perceived extraretinally to regulate biological clocks' (Underwood and Menaker, 1970). In insects, both direct action on the brain as well as action via the eye occur. In plants, light absorption can be effective in green as well as in yellow pigments. Thus, comparing different species of plants and animals, we can recognize a great variety of action spectra for phase shifts and synchronization.

Therefore, studies of these action spectra do not give any information about the chemical nature of substances which might be wheels in the clock mechanism.

Light intensity. Circadian rhythms are actually used for time measurement by plants and animals. Therefore a reliable accuracy in the running of the clocks is necessary. Two features are required for this accuracy: synchronization to exactly 24-h cycles and the stable phase relation between circadian phases and local time which does not vary from day to day. A constant phase angle difference between sunrise or sunset and the several phases of the circadian clock is required. This can not be obtained by taking the light intensities at sunset or sunrise as discrete reference points. Light intensities at these moments of the solar day fluctuate too much from day to day, depending especially on cloudiness (Fig. 5). On one day a value of 100 lx may be reached at sunrise. The next day, without clouds, the same value may be reached 20 min earlier. Most suitable as reference points are values between about 1 and 10 lx, occuring at dawn and dusk. The rate of change in intensity is greatest, and variations with weather in the moment a certain intensity between 1 and 10 lx is reached seldom exceed 10 min in our regions, and 5 min in tropical regions (Bünning, 1969). Plants and animals actually make use of these dim light regions as reference points. Intensities of about 1 lx have a synchronizing and phase-shifting effect (Fig. 6) and in several animals even

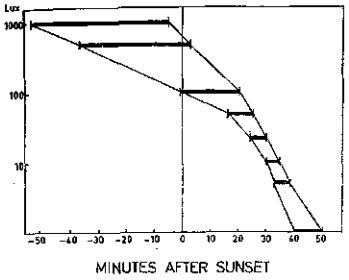

Fig. 5. Intensities of zenith-light, reaching a horizontal plane. Measured in Tübingen, March 1–12, 1969. Note that the rate of change of light-intensities is greatest when the intensity has reached about 10 lx after sunset. Arbitrary variations due to cloudiness have their minimum in these regions of twilight. From Bünning (1969).

intensities of about 0.1 lx have an effect (Menaker, 1968; Chandrashekaran and Loher, 1969; Engelmann, 1969). With intensities of about 10 lx or a little more, the phase angle difference between the solar cycle and the circadian rhythm remains nearly independent of light intensity (Fig. 6 and 7), Thus, the very short span between 1 and 10 lx (or a little more) is mostly used as reference point. This has the advantage of being almost independent of weather. In addition, an abrupt increase (or decrease) in intensity (Fig. 8) controls a physiological process more effectively than a gradual one. Plants and animals sometimes increase this steepness further by their own activity. Several plants quickly bring the blades of their leaves with the help of the circadian movements during the time span between about 1 and 10 lx, from the night to day-position and vice versa. This means a very drastic increase of steepness as far as the

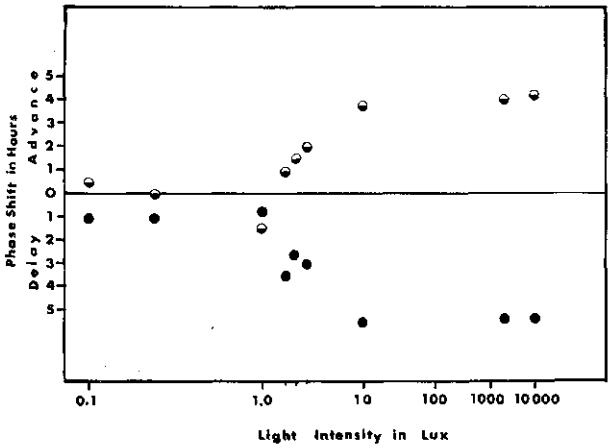

Fig. 6. The extent of advancing and delaying phase shifts effected in the circadian system of *Drosophila* by light pulses of 15-minute and varying intensities.
The circles denote the calculated median hours for the eclosion peaks of experimental populations on day 4 and day 5 after exposure to light pulses. The line separating advances from delays indicates the position of the eclosion peak medians of the control populations which did not receive any light signal. From Chandrashekaran and Loher (1969).

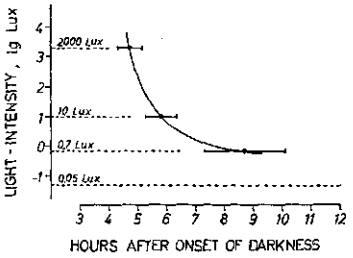

Fig. 7. *Glycine max* (soya bean). Circadian leaf movements in 12:12h light-dark cycles. Abscissa: appearance of night-peaks, hrs after the beginning of the daily dark-period. There was no synchronizing effect in case the light-intensity was only 0.05 lx. With this extremely low intensity, the light-dark cycle acted as continuous darkness, resulting in a free-running period of more than 26 h (this is not shown in the figure). Intensities of 0.7 lx or more synchronized the circadian cycle to 24-h cycles. The figure shows that already with intensities of 10 lx a phase angle difference between the light-dark cycle and the circadian rhythm is nearly reached as it is for light-dark cycles with much higher light intensities. Data from Bünning (1969).

intensity actually reaching the blades is concerned (Fig. 9). Animals may show an analogous behaviour, such as starting morning activity at about 1 lx and thereby exposing themselves to higher light intensities than exists in their shelters or while their eyes remain closed (Fig. 10). In a different way, Wolfson (1970) has stressed this point especially with respect to birds. 'The duration of the light period in a 24-h or circadian period appears to be the most important information for the decision regarding the length of the day, since birds respond spontaneously to light by becoming active.'

Certain animals have been found to start measuring the day with intensities of 0.1 lx. Plants usually start at a level of about 1 lx. This may have an adaptive significance. Moonlight can reach intensities between 0.5 and 1.0 lx. Thus, it may cause phase shifts disturbing the accurate running of the clock. Day-active animals can avoid this disturbance, since they rest in dark places at night. Relatively few species of plants can escape these disturbing influences by the complicated sleep movements of their leaves. This strongly reduces the intensity of moonlight, reaching the surfaces of the leaves (Bünning and Moser, 1969). Other plants, that cannot actively regulate the intensity of light reaching them, must have values of about 1 lx as threshold of response.

One more aspect of optimum light intensities is that the termination of diapause in

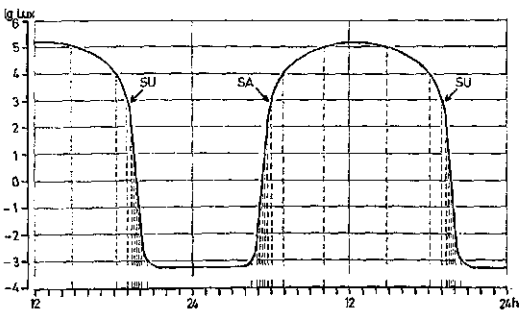

Fig. 8. Diurnal changes of light-intensity at Tübingen on April 2, 1965. Abscissa: time of day. Ordinate: light-intensity, lg lx. SU: sunset, SA: sunrise. The curve shows (just as Fig. 6 does) that intensities between about 1 and 10 lx, reached about $\frac{1}{2}$ h after sunset and about $\frac{1}{2}$ h before sunrise are the best reference values for a physiological process. From Erkert (1969).

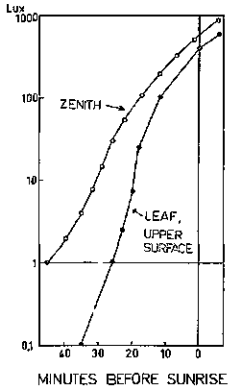

Fig. 9. Comparison of light-intensities reaching a horizontal plane and reaching the upper surface of the leaf of *Parochetus communis* (Papilionaceae). Because of the circadian leaf-movement the rate of change of light-intensity becomes greater than it is during the respective part of twilight. After Bünning (1969).

the pupae of certain insects is controlled by day length measurement. A similar photoperiodic control terminates the rest periods of buds in certain species of plants. The cuticle of the pupae reduces the light intensity considerably, as do the bud scales in plants. How do the organisms overcome this handicap? The pupae of the oak silkworm *Antheraea pernyi* have a window-like transparent zone exactly in the region of the brain, which is the receptor of the photoperiodic stimulus. This transparency is especially for the effective fraction of light, i.e. for blue light (Williams et al., 1965). The analogous trick of a plant: the bud scales of the beech tree have a transparent basal portion that allows sufficient light to reach the leaf-primordia (Wareing, 1953).

Plants and animals may have difficulties with the exact running of the clock, in arctic regions where during the summer months the light intensity never reaches low values (Erkinaro, 1969). However, experimental data from both plants and animals show that

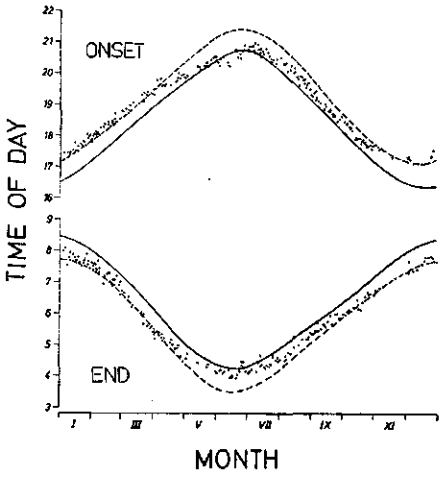

Fig. 10. Onset and end of flying activity in the daw *Coloeus monedula* L., measured at two places (latitude 49° 20′ N). Broken line: the sun is 6° below horizon. Solid line: sunrise and sunset. Comparison with Fig. 7 shows that this bird starts and ends measuring the day with about the same light-intensity as plants do (compare also with Fig. 8). After Aschoff and v. Holst (1960).

even at latitudes of about 70° the diurnal changes in light intensities, co-operating with the rather strong diurnal changes in the temperature, are sufficient at least to co-ordinate the circadian subjective day time with the period of higher light-intensity and temperature (Bünning, 1967; Swade and Pittendrigh, 1967). But plants and animals living in arctic regions cannot use their circadian clocks for such a precise time measurement as is possible and necessary for certain developmental processes in other latitudes. Certain species of animals may even shift to a free-running periodicity under arctic summer conditions (Müller, 1968).

Kinetic analysis approch

There are quite different types of oscillations in plant and animal tissues. Circadian oscillations have certain features in common with short period oscillations of nerves and other tissues in animals and plants which are based on the principle of all-or-none excitations and reactions. Exitation may be accompanied by action potentials, the reaction for example, by contractions.

This type of oscillation has the following features:

1. The oscillation starts spontaneously or is initiated by a single external stimulus.
2. Each excitation and reaction in the oscillation is of the all-or-none type. Reaction is accompanied by a refractory period first absolute and then relative.
3. Certain types of external stimuli, offered to the already running periodicity, can lengthen or shorten the affected cycle (Fig. 11). The effect depends on the phase the cycle is undergoing during this stimulus. The stimulus will have no effect during the absolute refractory period but cause an additional peak during relative refraction. The amplitude of the next peak of this shorter cycle will be less than the normal ones. For example: the well known phenomenon of an extra systole in heart beat (Fig. 12).
4. After such a disturbance, transient cycles follow, before the normal periods are re-established.

All these characteristics seem inherent to circadian rhythms. This does not become evident if only certain phases of the circadian cycle can be recorded clearly. When circadian oscillations are recorded continuously over a few cycles similarities appear with the short-period oscillations. The similarities can be easier seen in leaf movements than in eclosion rhythms of insects. The response curves show absolute refractory

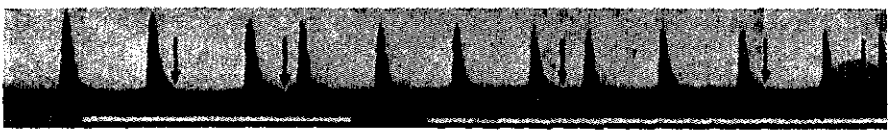

Fig. 11. Example for phase-shifts (advances or delays) in short period oscillations. Electric oscillations in the alga *Chara*, influence of electric stimulations (indicated by arrows), applied at different phases of the refractory periods. Black interruption of white line on bottom indicates 1 min. From Auger (1936).

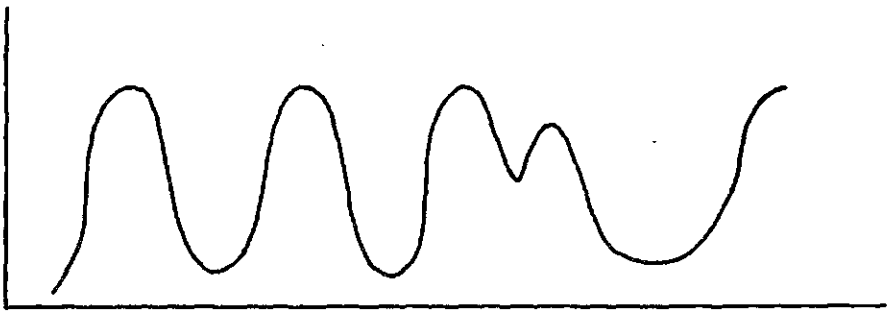

Fig. 12. The well known phenomenon of an extra systole in heart beat, due to electric stimulation in the relative refractory period.

periods, where a light stimulus has little effect (Fig. 3). In certain species the response curves show no absolute refractory period but only a relative refractory period. During this period, a light or temperature pulse induces a reaction only of lower amplitude. Certain reaction curves resemble an extra systole, as does also the gradual decrease in light intensity, required to induce this extra response during the course of relative refractory period. (Fig. 13, 14, 15 and 16).

The kinetic model suggest that a reaction starts at dawn and reaches a maximum about 18 h later. The reaction is accompanied by a refractory period, beginning soon after the reaction starts and gradually diminishing during about 24 h.

The reaction itself requires energy until the maximum and is prevented if energy supply is blocked by poisons or by low temperature. After the 18-h peak, the reaction behaves like a relaxation process, continuing even when the energy supply is blocked.

About 24 after the reaction begins, the next one is initiated either by the next dawn or by the complete disappearance of the refractory period. The new reaction may superimpose on the preceding one (Fig. 15): hence the variation in shape appearance of one of the peaks (Fig. 17).

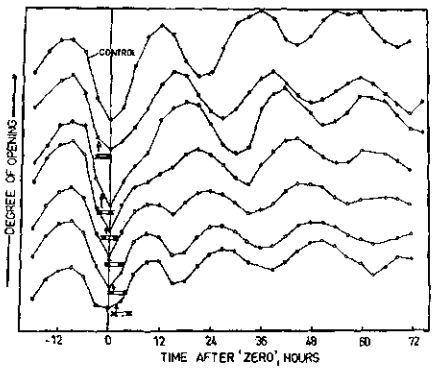

Fig. 13. *Kalanchoë blossfeldiana*. Petal movements in continuous darkness. Time O is the time of complete closure of the flower. The curves show phase shifts after a single exposure to 2 h of light. Time of these signals indicated by symbols in which the arrows mark the beginning. Curve 1: control without light-break. Delays are connected with increased amplitudes. Advances with lower amplitudes, comparable with 'extra-systoles'. Note also transients, especially during advances (lower 3 curves). See also Fig. 14. After Zimmer (1962).

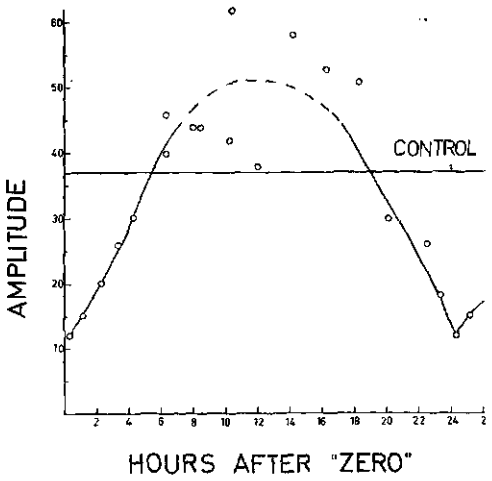

Fig. 14. Result of analyzing a greater number of experiments with *Kalanchoë*, of the type shown in Fig. 13. Advances (after light-breaks from about hour O up to nearly hour 6) are like 'extra systoles' characterized by lower peaks. Delays (after light breaks later than about hour 6) are characterized by higher peaks. After Bünning and Blume (1963).

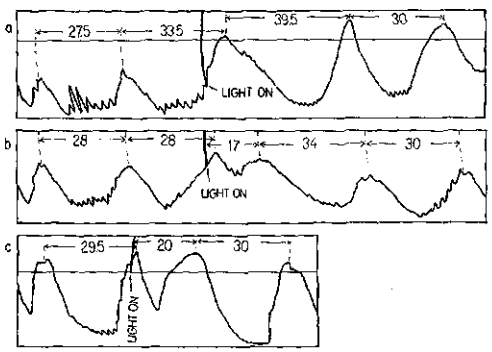

Fig. 15. *Phaseolus multiflorus*. Circadian leaf movements in continuous darkness followed by continuous light at the indicated times. The curves show that, depending on the circadian phase during which the shifting to continuous light started, either a lengthening of the affected cycle appears, or the induction of a new reaction. The new reaction (similar to an 'extra systole' as in Fig. 12 and 13) overlaps with the preceding one. Length of periods in hours. After Bünning & Moser (1967).

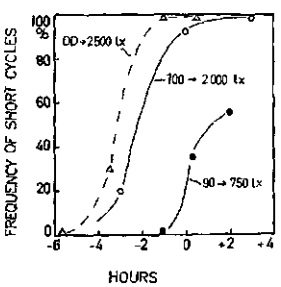

Fig. 16. Experiments as those shown in Fig. 15. The curves show how often (percentage) the replacement of continuous darkness by continuous light or by switching over to a higher intensity of continuous light induced new reactions (as in Fig. 15b and c). Abscissa: time of beginning continuous light (or shift to the higher intensity), hours before (–) and after (+) the peak of the already occurring reaction. The data show that this new induction is the easier the later the light (respectively the higher light-intensity) was switched on. It is also the easier the higher the intensity is. These are typical features of a relative refractory period. After Bünning and Moser (1967).

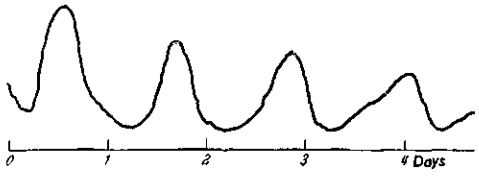

Fig. 17. *Phaseolus multiflorus*, circadian leaf movements in continuous light. Note very different positions of minima (physiological day-time phase) within the 4 cycles. Only the distances between the physiological night-time peaks are constant. From Bünning (1967).

A light or temperature pulse during the refractory period has either no effect (absolute refractory period), or induces an additional reaction which prolongs the refractory period. The amplitude of the new reaction is less. The second reaction is additive if the stimulus precedes the maximum of the first. Such a repeated stimulus lengthens the cycle. The next period ('transient cycle') is also lengthened by the increased duration of the refractory period. Thus the clock is slow.

If a second stimulus comes after the maximum of reaction, there is an 'extra systole' and therefore a shortening of the cycle, i.e. the clock is fast. Such a chain of events explains why delay and advance due to light-pulses are equally dependent on intensity and quality of light (Chandrashekaran and Loher, 1969; Frank and Zimmerman, 1969; Engelmann, 1969).

About 18 h after dawn (i.e. at the mentioned reactions maximum), a light perturbation of appropriate strength can stop the clock, i.e. it can lead to an arhythmic status. Such has been shown for both plants and animals (Winfree, 1970; Takimoto and Hamner, 1965; Bollig and Engelmann, pers. com.).

This model, though based on experimental facts, will certainly require refinement. Similarity with brief physiological cycles (as in nerves) breaks down in that a light pulse during a circadian cycle results in all-or-none reactions only above a saturation value (though the saturation values are very low).

Attempts at a mathematical analysis of circadian rhythms on analogy with oscillations in physics and engineering does not give a satisfactory model. Usually accurate measurement of the period depends on events during one of the extreme phases (peak and trough) of the circadian cycle (Fig. 17).

Approaches towards a biochemical analysis

Many botanical and zoological papers are published on circadian biochemical changes. I cannot summarize or list changes common to all these phenomena, hormone concentration, enzyme activity, respiration rate and photosynthetic capacity. Despite their importance, none of these biochemical changes have been proved to form part of the mechanism of the circadian clock and give no clue to its biochemical or biophysical working, since the clock ticks on when the metabolic rate is extremely low.

Experiments on the effect of actinomycin D indicate DNA-dependent synthesis of RNA in the clock mechanism. There is some evidence for the role of the nucleus, but other evidence for the continuation of the rhythmicity in enucleate cells. There is evidence for and against participation of protein synthesis. I refer here particularly to a

recent paper by Vanden Driessche, Bonotto and Brachet (1970). A study of the biochemical basis of circadian rhythms is published by Hastings (1970), who says: 'What is it that oscillates? Is it enzyme quantity, enzyme localization, or enzyme activity? Does it relate to the synthesis of inhibitors and/or substrates, or perhaps to some kind of compartmentalization?' ... 'It is highly likely that even if the clock function does involve circadian production of RNA, there exists a large background of noncircadian RNA production as well. It is also to be hoped that more studies in more laboratories will be devoted to biochemical characterizations of circadian systems, especially those in single-celled organisms. A great improvement in our perspectives would result from improved biochemical knowledge.' Others who have studied this topic are Vanden Driessche and Bonotto (1969); Feldman (1968).

There is some speculation on the possible role of short-period biochemical oscillations building up the circadian rhythm. We know something of such rhythms with periods of a few minutes or about half an hour in the feedback between production and activity of enzymes, and in membranes (Katchalsky and Spangler, 1968).

However, there is no convincing evidence that oscillations with shorter periods take part in building up circadian rhythms. We know that the organism's circadian rhythmicity can split up, but this obviously is a desynchronization between individual cells or tissues. In individual cells showing 2, 3 or more different circadian processes, no experimental treatment has ever changed the phase angle difference between these several processes. Also frequency demultiplication, referred to earlier, does not support this hypothesis. For example, a 6:6 hour light-dark cycle may result in overt rhythmicity with 12-h periods. But, where this is not because of desynchronization inside the individual, it is merely an imposed exogenous periodicity, not continuing in constant conditions. Again, in individual cells it was never possible to modify the circadian periodicity to a 12, 6, 3 or 2 h periodicity that continues in constant conditions.

Frequency demultiplication is by no means an amazing phenomenon. One has only to think of the different responsiveness of the several phases in the circadian rhythm. Let us consider what happens if there are short light-pulses at 3-h intervals. One of these light-pulses within each 24-h period will be the nearest to the maximum responsiveness of the circadian cycle, i.e. the nearest one to a peak of the phase response curve. This light-pulse, in co-operation with the one offered 24 h later will have the greatest phase shifting effect (delay or advance), whereas comparatively the other pulses will only have a small effect. Phase shifting will continue until the most effective pulses coincide with a phase that does not respond to the light.

Thus, it is an interesting hypothesis that populations of short biochemical oscillations are the basis for the circadian clock (Pavlidis, 1969). But there is no convincing experimental evidence for this hypothesis. Moreover, the kinetic behaviour, previously described, is evidence against this hypothesis.

A biophysical approach may be more succesful than the biochemical approach. A more detailed knowledge of membrane biology might be helpful. The influence of heavy water (D_2O) and alcohol may support this suggestion as both are very effective in causing phase shifts as well as drastic increases in length of periods. (Suter and

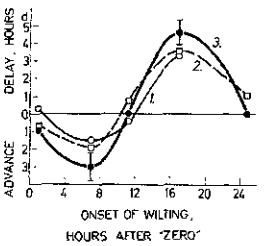

Fig. 18. *Phaseolus multiflorus*, circadian leaf movements. Influence of a 6 h period of wilting. Abscissa: onset of the wilting period, hrs after the preceding maximum night position. Ordinate: Delay or advance in the occurance of the 1st, 2nd, and 3rd night-peak after wilting. (After Bünning and Moser, 1968).

Rawson, 1968; Bünning and Moser, 1968). The phase shifting effect can be as strong as the effect of light pulses. In *Phaseolus* the free-running period of plants supplied with D_2O can increase up to 34 h. A continuous wilting also has a strong influence, resulting in remarkable increases of the free-running periods. A transient wilting for a few hours induces delays or advances, depending on the affected phases. This results in phase response curves being very similar to those for light or temperature pulses (Fig. 18).

The effect of alcohol, D_2O and water on the clock strongly support a more biophysical approach, especially as the very slight effect of many other substances is checked. This biophysical approach also has the advantage of bypassing the small influence of temperature on length of period. Circadian biochemical events such as amino acid incorporation (Feldman), and RNA synthesis (Vanden Driessche and Bonotto) seem to be secondary processes. With other physiological processes such as certain hormone actions and certain responses to light, there was strong evidence that genes were activated primarily, but later these effects were proved to be secondary, following changes in membranes. Photoperiodic induction of flowering is usually mediated via light absorption in phytochrome. This photoreception in phytochrome activates certain genes. But, according to convincing experiments, membranes are primarely affected. There is a striking analogy between the effect of light and water on circadian rhythms and on flowering. Halaban and Hillman (1970a), working with the duckweed *Lemna perpusilla* found that a daily transfer from the nutrient solution to distilled water for short periods of time had an effect on flowering similar to that produced by light pulses offered during the respective phases of the circadian cycle.

Dual relation between light and circadian clock

Results of experiments on the dual relation between light and the circadian clock support the suggestion that we should study membrane processes when searching for the mechanism.

Light sets the phases of circadian rhythms. There is no example of a circadian rhythm not responsive to light. On the other hand, the clock can cause a circadian rhythm in the quantity and quality of the organism's responsiveness to light, especially in developmental processes.

In general, photoreceptor pigments of plants and animals are in membranes or lamellae or at least near to such structures: the pigments involved in setting the

phases of the clock, such as visual pigments in animals, and flavins, chlorophyll and phytochrome or other photoperiodic photoreceptors in plants.

Light can shift the phases of the clock very quickly perhaps because the clock is near the photoreceptor. If light acted through metabolic products moving from the photoreceptor to a clock elsewhere, we would expect substances applied experimentally to disturb the rhythm.

Thus, structural changes in membranes or lamellae, or the orientation of pigments in those structures might be the basic phenomena in circadian rhythmicity, i.e. the basis of the clockwork itself and for the influence of the clock on quantity and quality of developmental responsiveness to light.

There is another problem concerning the dual relation between the clock and light. Sometimes, both in plants and animals, the action spectra for phase shifts and synchronization are indentical with the action spectra for photoperiodic responsiveness. That means, the same pigments seem to be used in both processes. Are the organisms nevertheless able to distinguish the meaning of the several light signals? To a certain extent they have this ability and the problem is solved on a structural basis, sometimes at the macroscopic level.

In plants, phytochrome is usually the decisive photoreceptor for photoperiodic reactions. Phytochrome is also very important in the phasing of the circadian rhythm (Bünning and Moser, 1966). Nevertheless, the plant can distinguish: the main site of photoperiodic reception is the blade of the leaf, but the main site of light absorption which leads to phase shifts of the rhythm is the leaf joint (Bünning and Moser, 1966).

Now a zoological equivalent. From experiments with the house sparrow *Passer domesticus* Menaker et. al. found that the photoperiodic control of testis growth is mediated entirely by extraretinal photoreceptors in the brain. The eyes do not participate in photoperiodically significant photoreception, but are involved in synchronizing the circadian rhythm. (Moreover, the threshold intensities for these two responses are different).

Sometimes the organisms distinguish between the 'meaning' of the several light signals by using different pigments. At least in certain species of plants, the action spectrum for phase shifts differs from the action spectrum for the developmental effects of light breaks offered during the dark period. (Bünning, 1967; Halaban and Hillman, 1970b).

More research is needed at microscopic and submicroscopic levels to discover the site of photoreception for the synchronization and phasing of the clock and for the photoperiodic responsiveness.

Thus, at present there is no satisfactory hypothesis on the biochemical or biophysical basis of the circadian clock. I myself would hazard the guess that more research on periodicities in membranes would be more fruitful than classical biochemical research.

Acknowledgments

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Discussion

Participants: Bünning (Author), Bink, Cumming, Lindenmayer, Truman, Wassink

A few questions were asked in connection with the sometimes assumed effect of subtle geophysical factors in synchronizing circadian rhythms. It was stated again that there is no convincing experimental evidence for such an assumption. There is, for example, no influence of geographical longitude.

Other questions were about the relation between circadian rhythmicity and photoperiodism. Circadian oscillations are certainly not the only timing system involved in photoperiodism. Other processes are well known as components. Sometimes only non-oscillating timing processes are decisive. There was the more special question about qualitative changes in flowering response to red and far-red light during the light and the dark period respectively of 24-h cycles. The following statements were made: the experimental results may be quite different depending on the species or variety of plant, and on special experimental conditions. Moreover it was stated that often no relation of these well known changes to circadian rhythmicity becomes evident.

Another part of the discussion was concerned with the suggestion to look for processes in membranes when searching for the 'master clock'. I did not intend to make a precise hypothesis but only to encourage scientists, especially biochemists and biophysicsts, to extend themselves in looking for biophysical oscillations. In connection with this problem, the question was raised whether mutants which lack the clock, are known. Unfortunately it is not easy to decide whether certain known mutants without overt circadian rhythms do lack the clock, or whether just the coupling of the special processes to the clock is missing. In this context Truman reported on interesting unpublished work by Benzer about clock mutants in *Drosophila*: strains with quite different periods or without any overt rhythmicity were isolated. These mutants are apparently due to single genes.

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The role of circadian rhythmicity in photoperiodic induction in plants

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A complete coverage of general concepts and possible experimental approaches seems unnecessary, as well as being impossible, in view of the extensive literature that is relevant to this subject[1]. I will, instead, after outlining historical events that seem to have been particularly significant, refer to recent work that may provide a theoretical and experimental basis for future work. The question posed by the title of this paper involves many ambiguities because of gaps in our fundamental knowledge. As I will explain in more detail later, I am questioning whether the term photoperiodic 'induction' should be restricted just to developmental responses such as flowering, dormancy, bulbing, and so forth, or whether it should be applied to circadian rhythmic phenomena in plants that can be induced or controlled by photoperiod. To avoid becoming involved in unnecessary semantics I will now consider my subject under the broader title of 'The role of circadian rhythmicity in photoperiodic responses in plants'.

Evolutionary context

'I saw Eternity the other night,
Like a great ring of pure and endless light,
All calm, as it was bright;
And round beneath it, Time in hours, days, years,
Driv'n by the spheres,
Like a vast shadow mov'd; in which the world
And all her train were hurl'd.'

Silex Scintillans. In: The world
Henry Vaughan, 1655

In his 'First principles', published in 1862, the prophet of evolution, Herbert Spencer, referred to evolution as 'a change from an indefinite incoherent homogeneity, to a definite coherent heterogeneity'. A question, relevant to our context, is 'Did photo-

[1] See, for example, Bünning (1967), Cumming and Wagner (1968), Sollberger (1965), Sweeney (1969).

periodism and circadian rhythmicity originate sequentially or as interdependent manifestations of an essentially unitary mechanism?' This question seems to belong to the which-came-first-the-chicken-or-the-egg category – which suggests part of the root of our present problem: if the one phenomenon exists, must the other? The subject can be highly circuitous.

Let us consider two possible views. The one might state that circadian rhythmicity and photoperiodically controlled processes have evolved in many different ways at different times as a result of the ubiquitous 24-hour solar days prevailing on planet earth, and the potential capacity and need for different organisms to develop both (self-sustained?) oscillations of various periodicities and (enforced?) hourglass processes. The other view is partly an answer to the former one, and focuses our attention on the possibility of a common origin of circadian rhythmicity, and photoperiodic responses in different organisms. It seems reasonable to take cognisance of both of these views, which we might categorize as the convergent versus monophyletic concepts (cf. Pittendrigh, 1966).

A very useful criterion for classifying a rhythmic phenomenon as being circadian has been the display of at least two or three approximately 24-hour free-running oscillations in continuous light or darkness. However, under natural conditions suitable for the active metabolism and growth of plants, the organism has been continually subjected to a daily alternation of light and darkness – (except near the Earth's poles) – in which the daily dark period does not normally exceed about 16 hours, if that. It is these natural cycles which have provided the main cues for photoperiodic timing by the organism and it is also under these conditions that circadian rhythmicity has evolved. The simplest conception of the photoperiodic timing mechanism would be the operation of one or more non-cyclic processes having time-dependent optima or hourglass principles. However, the demonstration of the remarkable property of circadian rhythmicity in a phenomenon that can be photoperiodically controlled, and the common properties of circadian timing that are displayed by widely different organisms, are in themselves strong arguments, although not proof, of a timekeeping role of circadian rhythmicity in photoperiodically controlled responses.

Historical context

Reference to the historical background is helpful, since it allows us to see that there have been some coherent trends in this field; also, we are forced to admit that the progressive history of this subject shows that antecendent work has tended to receive less attention than it merited.

Retrospective references to the study of leaf movements through the centuries provide us with a perspective that has remained to a remarkable degree unchanged until the present time. Many of the characteristics of circadian rhythms can be summarized by tracing the history of work on leaf movements, although other rhythmic phenomena were also studied during the same period. In this respect I would refer you particularly to the historical coverage provided in some books and reviews (e.g. Pfeffer, 1906; Bünning, 1958, 1967; Kayser and Heusner, 1967; Cumming and Wagner, 1968).

Functionally, as well as historically, the movements of leaves or floral parts are in some ways ideal for the study of circadian rhythms, because they are readily discernible 'hands' of a clock that can be continuously recorded mechanically and which may provide indications of some basic or master oscillation(s).

Androsthenes, while exploring an island near Bahrein in Arabia, noticed that the leaves of some legumes opened at sunrise, then folded up at sunset; the inhabitants of the island said that during the night the plants 'sleep' (Theophraste, 372–287 B.C.; Bretzl, 1903). Thus, an analogy (albeit not very deliberate) was drawn between a rhythmic process that occurs in plants and the sleeping rhythm that occurs in man. This analogy may seem just as apt today if we consider some of the metabolic events that are associated with these two phenomena: for example, there are changes in potassium flux and water movement associated with rhythmic leaf movements in *Albizzia julibrissin* (Satter et al., 1970; Satter and Galston, 1971) and with the circadian metabolism of man (Lobban, 1960). The sleep movements of certain plants were also noted in Pliny's 'The natural history' which appeared in AD 77 (cited in Pfeffer, 1875).

We can trace the definite study of rhythmic leaf movements from the time of de Mairan's experiments, published in 1729, in which he described how daily leaf movements continued in darkness away from sunlight and open air – experiments that were later confirmed by Hill in 1757 using *Glycine abrus*. Carl von Linné (Linnaeus), in 1755, published a lengthy list of plants that 'sleep'. Linnaeus also proposed using flowers to build a living clock; different flowers would open and close at regular intervals to indicate the time of day. Zinn, in 1759, reported that *Mimosa virgata* leaf movements persisted in constant darkness in a cellar, and that they were not affected by fluctuation in temperature or relative humidity. In 1832 de Candolle reported inverting a rhythm of leaf movements by reversing the alternation of light and darkness; his work also indicated that the leaf movements of *Mimosa pudica* showed a period of 22-23 hours in constant darkness. The subsequent papers of Julius Sachs provided clear evidence that rhythmic leaf movements are inherited. Sachs' 1857 publication on the photoreversible control of leaf movements by different wavelengths of the visible spectrum is a monument to our common failure to notice relevant literature. It was only in the 1950's that the photoreversibility of many photoperiodically controlled processes, that is mediated by the photoreversible pigment phytochrome, became recognized as an important physiological phenomenon in plants (Borthwick et al., 1952 a and b). The phytochrome system may in fact provide a very sophisticated basis for an hourglass timing component in leaf movements (Fondeville et al., 1966, 1967) and in other phenomena such as flowering response in different photoperiodic cycles (see review, Cumming and Wagner, 1968).

The extensive studies of Wilhelm Pfeffer from 1875 until well into the present century, laid a solid experimental foundation for the generation of concepts regarding endogenous rhythmicity and control by cycles of light and darkness. He showed that 12h L:D cycles resulted in a corresponding daily rhythm of leaf movements that in some plants was still sustained in continuous light or darkness. Pfeffer (1875, 1909, 1915) projected the idea that the approximately daily rhythm which occurred in continuous darkness ensured – under the normal conditions of daylight – that the plant

would be in the right disposition when the new light supply became available. Pfeffer thus projected a basic idea of the possible role of circadian rhythmicity in responses to natural daylengths. His 1909 paper reported that, in a bean plant, *Phaseolus multiflorus*, the rhythm of leaf movements corresponded to L:D changes of 18:18h, but that the internal tendency to produce an approximately 12:12h rhythm was shown in subsequent continuous darkness. He reasoned that this reaction depended on the arrangement of the inner conditions of the system and compared this with a pendulum: when there was no light the pendulum was free, but if there was light the pendulum was forced. It is noteworthy that near the fourth quarter of the 20th century we are still thinking in rather similar terms. Later studies of Pfeffer (1915) showed that leaf movements could be forced to adopt cycles of much higher frequency e.g. oscillations with a 6-hour period, in synchrony with photoperiodic cycles of a similar duration.

The book of Charles and Francis Darwin, published in 1898, entitled 'The power of movement in plants', is also receiving more attention these days, partly because of the interesting observations of rapid movements of leaves with a periodicity of as little as a minute. The Darwins emphasized the inherent nature of rhythmic phenomena but wondered about the adaptive value of such things as leaf sleep movements. Clearly, if leaves move their surfaces away from the sky at night, they may need to reorient them towards the sky during daylight hours to receive sufficient radiation. But one can still ask why they close at night. It is noteworthy that Bünning and Moser (1969a) have recently obtained evidence that may partially answer the question posed by Darwin. The adaptive value of leaf movements in some plants may be not so much to reduce radiation from their surfaces into the sky but, rather, to reduce radiation from the sky (i.e. from moonlight) which could otherwise interfere with photoperiodic timing. Bünning and Moser (1969b) have also found that if the leaf movements of *Perilla ocymoides* or *Chenopodim amaranticolor* are prevented from occurring, by fixing them with wires (*Perilla* leaves in a horizontal position, *Chenopodium* upright) during short-day inductive treatment, flowering is strongly inhibited.

In 1932, Kleinhoonte showed that in *Canavalia ensiformis*, light breaks of 1 minute duration could, if given at certain times, delay or advance the phase of the rhythm in leaf movements.

Bünning's (1936) hypothesis of the physiological clock was based to a considerable extent on his own and previous observations of rhythmic leaf movements. Thus, Bünning and Stern reported, in 1930, that the average period length of *Phaseolus multiflorus* leaf movements was 25-26 hours. Although previous workers, such as de Candolle and Pfeffer, had shown that there were rhythms deviating from 24 hours, it was Bünning who, in 1936, postulated certain essential features of such rhythms. First, that the periodicity is in fact 'circadian' (I am jumping ahead to a term originated by Halberg et al. in 1959), rather than being exactly 24 hours. Second, that the rhythms can be regulated to an exact 24-hour period by the natural daily light-dark alternations – which also determine the periodic order of the individual photo- and scotophil phases. A central issue of Bünning's 1936 hypothesis was, to quote his own words (Bünning, 1960) 'that the time-measuring processes in photoperiodic reactions are not carried out by the hourglass principle, but, rather, by means of endodiurnal osclillations'.

With the foregoing as a background we now need to turn our historical clock back a little because I have so far deliberately omitted any reference to studies that led directly to the formulation of the theory of photoperiodism. This is because it seems important to highlight one of the reasons why misconceptions have arisen in this field of work.

A. Henfrey, in 1852, had theorized in his book 'The vegetation of Europe' that the length of day is a factor in the natural distribution of plants and that they may be circumscribed within certain latitudes (cited by Allard, in 1944). The invention of a commercially available incandescent lamp by Edison in 1879, led to experiments in 'electrohorticulture' by Bailey (1891), Gaston Bonnier (1895) and others (cited in Garner and Allard, 1920), which showed that the flowering of several horticultural plants could be accelerated by extending natural daylengths with incandescent light – presumably these were what we would now call long-day plants. No significant theory arose from this work at that time. Tournois, however, in 1912, reported experimentally inducing earlier flowering in *Humulus japonicus* (hop) and *Cannabis sativa* (hemp) by covering plants with boxes to exclude daylight for all but 6 h daily. He clearly attributed precocious flowering to the 'shorter time of the intensity of light in winter than in the normal growing season'. In his 1914 paper Tournois also suggested that precocious flowering was caused not so much by the shortening of the days as by lengthening of the nights. This was the first explicit report of the experimental induction of flowering in what we now call short-day plants. Tournois was killed at the front shortly after the publication of his 1914 paper. Klebs, in 1913, reported inducing flowering of *Sempervivum funkii* by exposing plants to continuous light: a species that we would now classify as a long-day plant. He concluded that, in nature, the time of flowering is probably determined by the increase of daylength after the spring equinox.

The 1920 paper of Garner and Allard, entitled 'Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants' was a cornerstone in the development of our subject. I quote here from a book published in 1969 on the induction of flowering, in which Evans refers to Garner and Allard's work: 'Their first paper is a classic not only in the range of species, of kinds of experiments, and of plant responses used to establish that daylength influences plant behaviour, but also in the breadth of the discussion which includes consideration of the effects of daylength on plant distribution, on crop yields, and on the behaviour of algae and the migration of birds. They also introduced the terms photoperiod for daylength and photoperiodism for the response of organisms to the relative length of day and night. Yet, despite the power and the sweep of this paper, it was almost rejected by the editorial panel, to which it was submitted, as being insufficiently novel.'

It should be noted that Garner and Allard suggested the term 'photoperiod' to designate the favorable length of day for each organism. They also referred to precocious flowering as being 'induced' and projected the idea that 'the appropriate length of day acts, not merely as an accelerative, but rather as an initiative influence in bringing into expression the plants potential capacity for sexual reproduction.' They also introduced such terms as 'short-day plant', 'long-day plant', and 'critical length of day for flowering'. These authors showed remarkable perception of the role that day-

length control of flowering plays in the natural distribution of plants and in their vegetative and flowering response during different seasons. It is however noteworthy that of the twenty six references included in Garner and Allard's 1920 paper, none referred to any of the work that had been previously published on rhythmic phenomena such as leaf movements. Retrospectively, we can see that this is one of the reasons why misconceptions arose regarding the role of circadian rythmicity in photoperiodically controlled processes. The relationship between photoperiodism and circadian rhythmicity had not been realized, and it remained for Bünning to trace the connection in his hypothesis of the physiological clock, sixteen years later. Yet it was still appropriate for Bünning to state in 1960 that 'In botany, above all, grave errors have arisen in that the problem of photoperiodism has often simply been equated with the problem of flowering.'

Nevertheless, it seems to be fair to say that from the time of Garner and Allard's formulations early in the 1920's, and Bünning's postulations in the 1930's, there was a steadily increasing awareness of the scope of photoperiodically controlled phenomena and of rhythmic processes, and their interconnection. Although for a considerable time progress seemed to be along parallel tracks, that did not converge, collisions were perhaps inevitable when the two tracks met in the 1950's. A statement made by Melchers in 1952 during a series of lectures at Imperial College, University of London, perhaps sums up the situation at that time: 'The theory of photoperiodism developed since 1937 by Bünning has been completely overlooked outside Germany. This can hardly be explained merely by the inability of physiologists in other countries to read German. I have noticed that the lack of willingness to take this theory seriously or even to discuss it is far more a matter of personal feeling or irrational refusal to accept its premises.'

One cannot help wondering what the future will decide about the extrinsic (or exogenous) rhythm hypothesis, that is primarily attributable to Brown (see, for example, 1969 reference) but is supported for various organisms by work from diverse sources. This concept refers to the influence of changes in subtle uncontrolled geophysical factors which may be continually imparted to an organism, even under so-called constant conditions. A recent representative statement (Brown, 1965) is that 'the organism under natural conditions possesses no intrinsic daily rhythmicity but is, instead, rhythmic as a consequence of responding simultaneously and continuously to such rhythmical geophysical factors as light and temperature on the one hand, and to subtle geophysical factors on the other.' Brown has conceded that both intrinsic and extrinsic factors may be involved in the timing of biological periodisms. The organism may act as a 'variable frequency transformer' deriving different periods from a specific period input through autophasing. Autophasing involves the assumption that a circadian rhythm may bear any phase-angle relationship to the environmentally imposed 24-h periodic input – even locking to it in some manner. While it must be conceded that considerable scepticism is attached to some of Brown's postulations, and it is at present difficult to prove or disprove some of his concepts, we should be wary of too hasty negative conclusions in the present state of our ignorance of the basis of rhythmic phenomena. Nevertheless, this particular aspect of our subject will

not be considered directly in the following discussion, because essentially the same basic question still remains: what do we know about the endogenous mechanism that provides both circadian timing and photoperiodic response?

Present context

Various preliminary questions can be signified that may help us towards answering the primary one that is confronting us. Further aspects of these will be considered in greater detail when I later mention some current approaches to the question.

Can we define and ascribe a general significance to circadian rhythmicity and photoperiodic induction?

Arising from Bünning's hypothesis, Pittendrigh (1966) has stressed that the fundamental properties of the endogenous free-running rhythm endow the subjective organism with two distinctive but essentially characteristic responses: first, the photoperiodic induction of a particular phenomenon, and, second, the ability of the rhythmic response to be rephased or entrained by light. The corollary to this is of course the dual action of the light cycle: in other words, as pointed out most clearly by Bünsow (1960) and by Pittendrigh and Minis (1964), firstly, light effects the photoperiodic induction of a particular phenomenon by extending, or not extending, into the scotophil phase of the subjective endogenous cycle, and, secondly, light phases the oscillation. These are crucial points, and, although I will cloud the issue to some extent in the following discussion by questioning whether we can define the term 'induction' very explicitly, this will serve to highlight the specific instances where we can be explicit. I quote here an apt comment of Pittendrigh and Minis (1964). 'It is a remarkable fact that so much effort has been put into testing the Bünning hypothesis without any fundamental inquiry being made into the mechanism of how light entrains (and hence phase controls) the rhythm. Indeed, it is not clear that the problem has been recognized as especially pertinent.'

Considering 'induction' to mean the 'act or process of causing, initiating or bringing about', there seems to be no very clear reason (except one of historical precedence) as to why we should consider photoperiodic induction in a restrictive sense as applying only to phenomena such as seed germination, vegetative development, succulency, tuber and bulk formation, flowering, and the induction and breaking of dormancy – to which one can clearly attribute a developmental change.

It is not really clear where one might draw the line, for example, between dormancy and some quantitative changes in growth rate that are controlled by photoperiod. Furthermore, such phenomena may represent only one result of a series of changes in processes that are coupled at different levels of organization: various enzymes, for example, are also inducible or affected by photoperiod: Queiroz (1969) has shown in young leaves of *Kalanchoë blossfeldiana* that short days induce a rise in PEP carboxylase and malic acid enzyme activities, together with the establishment of a circadian rhythm of activity. At what level of organization does photoperiodic induction belong in such an instance?

Rhythmic leaf movements of some species can be induced in light and in darkness,

ehty can be affected by light quality, and can be forced to adopt different periods according to the length of the photoperiodic cycle. Furthermore, some of the changes induced by photoperiod may be sustained for a time after the Zeitgeber has been suspended. This was shown (Fig. 1) for the circadian leaf-movement rhythm of the bean seedling (*Phaseolus multiflorus*, Syn. *P. coccineus*) by Bünning (1956). A brief exposure to bright light caused the leaves to change prematurely from a sleeping (lowering) to a waking (rising) motion, but the sleep movement was again resumed when the light was turned off. The significant point was that the 're-awakening' response was repeated at approximately the same time on successive days, although no further bright light pulse(s) were imposed after the first day of treatment. The induction of such rhythmicity may involve changes that are 'encoded' in some way. Brown (1970) uses the analogy of entering a recording on a tape; the process also seems to be somewhat analogous to the memory system of animals. Recordings of such effects in plants are not common, so it is worth mentioning that Pirson et al. (1954), and Schön (1955), working with the Alga *Hydrodictyon reticulatum*, found that rhythms of growth and oxygen metabolism were entrained by 6:$\overline{6}$, 10.5:$\overline{7}$, and 12:$\overline{12}$ cycles of light and darkness and that the rhythms persisted for three days with periods of 12, 17.5 and 24 hours, respectively.

One can consider photoperiodism as a process evolved by organisms for timing their activities on a daily and seasonal basis, in which circadian rhythmicity acts as a basic timing mechanism. Ecologically, the main feature of photoperiodism is the timing, coordination, and economy of primary processes within the organism so that it is adapted as perfectly as possible to its environment. The spectrum of circadian rhythmic responses that are shown by various types of organisms, and the differences in the sensitivity of time measurement that exist among closely related genotypes, underline the difficulty of applying broad generalizations from specific cases, but one is encouraged by the intimations of the universality of rhythmicity in living systems.

There is an inherent difficulty in any experimental approach to determining the role of circadian rhythmicity in photoperiodic induction in systems which show dual sensitivity to light. This arises from the fact that we cannot study a free-running (circadian) system and also study how it is forced (photoperiodically) at one and the same

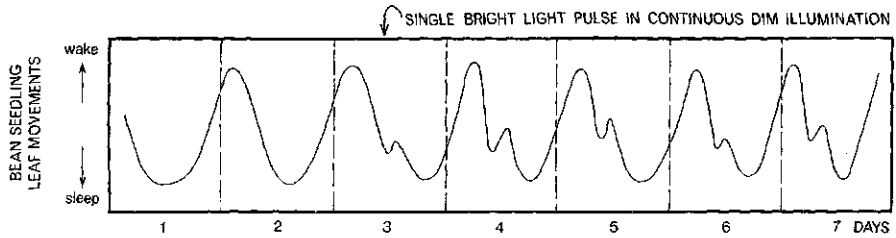

Fig. 1. Leaf movements of *Phaseolus multiflorus*. Plant maintained in constant temperature and constant dim illumination except for brief exposure to bright light on day 3. (After Bünning, 1956).

time without making some assumptions about the basic mechanism involved – which is of course begging part of the present question.

What is the range of rhythmic phenomena shown by different organisms?

Our knowledge of the range of circadian rhythmic phenomena has increased markedly in the last decade. However, it is still difficult to consolidate the scattered information into a clear picture to show either the generalizations that can be applied to different organisms, or the extent to which many of the phenomena are controlled by photoperiod.

In multicellular plants (including fungi) circadian rhythmicity has been shown in at least the following categories of plant functions: enzyme activity, cellular metabolism, changes in organelle size or shape (which may also indicate possible changes in metabolism), gaseous exchange, photosynthesis, luminescence, pigment formation, translocation, the movements of various organs, cell division, growth, germination, reproduction (see review, Cumming and Wagner, 1968). The example of the dinoflagellate *Gonyaulax polyedra* (that is discussed by Sweeney in this symposium) is sufficient to emphasize that a multiplicity of circadian rhythmic phenomena can be exhibited by a single-celled organism, and this fact raises interesting questions regarding the cellular basis of circadian rhythmicity in multicellular organisms.

Are we being realistic in our choice of test objects and in our experimental approaches?

It may be salutary to realize that our choice of organisms with which to work is often quite arbitrary and may be restricted either by our lack of knowledge of potentially suitable material, or by the unavailability of suitable technical resources. This situation is compounded by our progressive investment of time and energy in using particular organisms and specialized technical paraphenalia that may become quite unsatisfactory or obsolescent as our study progresses. Hopefully, the scientific method lessens the problem by fostering contact between scientists and allowing better utilization of our resources. Certain ideas can be mentioned:

1. The plant or parts of it should be easy to grow and maintain in a viable condition, and small in size during part of its life cycle so that it can be replicated and grown on a large scale under carefully controlled environmental conditions. Conversely, at some stage in its life cycle the plant should be capable of producing large amounts of vegetative material for specific extraction and assay purposes.
2. The plant should be suitable for sterile (axenic) culture, for propagation of its tissues as cell suspensions in a liquid medium, as callus on a solid medium, in the presence or absence of light over prolonged periods. It should also be capable of various means of vegetative as well as sexual propagation and reproduction. Further, genetically, the population represented by the organism should comprise a number of closely related but distinct genotypes, ecotypes, races, clones or species, that exhibit wide differences in circadian rhythmicity and photoperiodic response.
3. The organism should exhibit some overt circadian rhythmic phenomenon that can be recorded continuously and automatically. Present examples of this include leaf movements and gaseous exchange, but this is presumably only a fraction of the future possibilities. Such overt rhythms may provide a starting point in tracing the

coupling of various rhythmic processes at different levels of organization and, hopefully, enable us to distinguish between the hands of the clock and the clock mechanism or basic oscillator. A further advantage of overt rhythms is that the immediate effects of electromagnetic factors, chemicals, or surgical treatments can be more directly assayed with less likelihood that we will be confused by artifacts.

4. There should be sufficient sensitivity of the rhythmic response to photoperiod and other factors. In studies of rhythmic flower induction in a short-day plant, for example, there are many experimental advantages if flowering can be readily induced by a single dark period, or if a single light pulse can stimulate or inhibit flowering and/or rephase the rhythmic response.

5. It would not be untoward if the plant has some recognizable ecological significance or practical importance, since we may then hope to put our knowledge to some quite direct social use. This might also stimulate administrators to cough up funds on a rhythmic basis that is sufficient to prevent feedback repression (a well known phenomenon in biological systems that oscillate).

Some of the foregoing ideals will be illustrated in the remainder of my paper.

What types of answers can different organisms provide?

The multicellular fungi can serve as a starting point of simplicity. They may be particularly valuable because of their simplicity of growth and reproduction and their greater dependence on nutritional factors in the growth medium, since they are heterotrophs, lacking chloroplasts. Furthermore, fungi may provide a physiological link between organisms which do and those which do not exhibit classic circadian rhythmic responses. The latter would include such organisms as the bacteria, yeasts, and blue-green algae. Different strains or mutants of the fungus *Neurospora crassa* illustrate this possible link. Sussman et al. (1962) have shown that in some strains of this species, the rhythm of zonation in growth and sporulation is endogenous but not truly circadian: the rhythmicity may depend strongly on temperature and composition of the growth medium with periods ranging from 15 to 90 hours, but these strains do not respond to photoperiodic cycles (e.g. Neurath and Berliner, 1964). In contrast, Sargent et al. (1966) have shown that in *Neurospora crassa* 'Timex' the rhythm of conidiation is truly circadian, since there is (i) a periodicity of 22.7 h at 25 °C in constant darkness (DD); (ii) a Q_{10} of 0.95 to 1.21 in a temperature range of 18 to 35 °C; (iii) the rhythm persists in DD at constant temperature for at least 14 days without damping out; and (iv) the rhythm can be shifted in phase by a single brief exposure to light. Photoperiodic control of this rhythm is shown by its synchronization to L:D cycles of 24 h, while in continuous light sporulation is continuous rather than rhythmic.

The fungus *Leptosphaeria michotii* seems to incorporate mimic- and truly-circadian rhythmic features. Lacking zonation in continuous darkness, a single light stimulus can initiate a 24-h rhythm which, however, damps out rapidly. But on glucose agar in DD, a persistent rhythm with a period of 5 days at 22 ° and 3 days at 24 °C has been observed (Jerebzoff and Lacoste, 1963a). The rhythm appeared to be entrained to L:D cycles of 12:12 at 24 °C because a 24-h rhythm was shown along with the 3-day rhythm (Jerebzoff, 1965). The periodicity of zonation could be influenced by asparagine concentration; with higher concentrations of asparagine the rhythm became persistent

and stable with a period of ca. 28 h (Jerebzoff and Lacoste, 1963b).

In higher plants, as already intimated, the induction of leaf or petal movements, and flowering, are the phenomena which have provided us with some of the clearest answers regarding the role of circadian rhythmicity in photoperiodic induction. Nevertheless many other phenomena are also informative and current approaches on some of these will also be considered in the following section.

Current approaches to the question

Current approaches, though more critical and analytical, are still primarily descriptive and empirical. This stems from the fact that we are still searching for definite indications of what constitutes the basic oscillatory mechanism that is involved in overt timekeeping by different organisms. It seems fair, however, to say that recent work is at least providing us with some clearer indications of where we may look to answer this question.

Correlated phenomena and circadian timekeeping

My historical review dealt largely with studies of leaf movements, and some recent studies of this phenomenon by Alford and Tibbitts (1970, 1971) are noteworthy because of the care which they took to obtain constant environmental conditions. The circadian and higher frequency movements of the primary (unifoliate) leaves of the adzuki bean (*Phaseolus angularis*) were studied in an environment with controlled levels of carbon dioxide, relative humidity, temperature, light, nutrient concentration and water tension (Fig. 2). Rhythmic circadian leaf movements with a period of 27.3 hours were evident as the leaves unfolded and these persisted during development of the leaves. The measurements of the leaf movements, that were recorded in angular degrees (Fig. 2), showed that there were irregular short period upward and downward movements of the leaves, but these were small compared with the main circadian upward and downward movements (Figs. 2b, 3b). Apart from the foregoing irregular high frequency movements, there were also rhythmic rotational leaf movements (Fig. 3a, 4, 'blade rotation' measured in millimetres), which were only pronounced when the leaves were in an upward (horizontal) 'awake' position, and faded out when the leaves were downward in a 'sleep' position (Fig. 3b). This blade rotation was persistent and self sustaining in the uniform environmental conditions and, as pointed out by Alford and Tibbitts (1971), therefore fit Sweeney's (1969) definition of a true biological rhythm. The mean period of the blade rotation was 53.2 $\pm$ 4.3 minutes.

Although the circadian leaf movements for the two primary leaves on the same plant were usually closely in phase they could be several hours out of phase (cf. also Hoshizaki and Hamner, 1964). In contrast, however, the short period blade rotation of the two leaves on the same plant were closely synchronized and in phase (Fig. 4). However, neither the circadian nor the short period rotation movements were synchronized on different plants (Fig. 2, 3) – which suggested that these rhythms were not entrained by any concurrent environmental fluctuation.

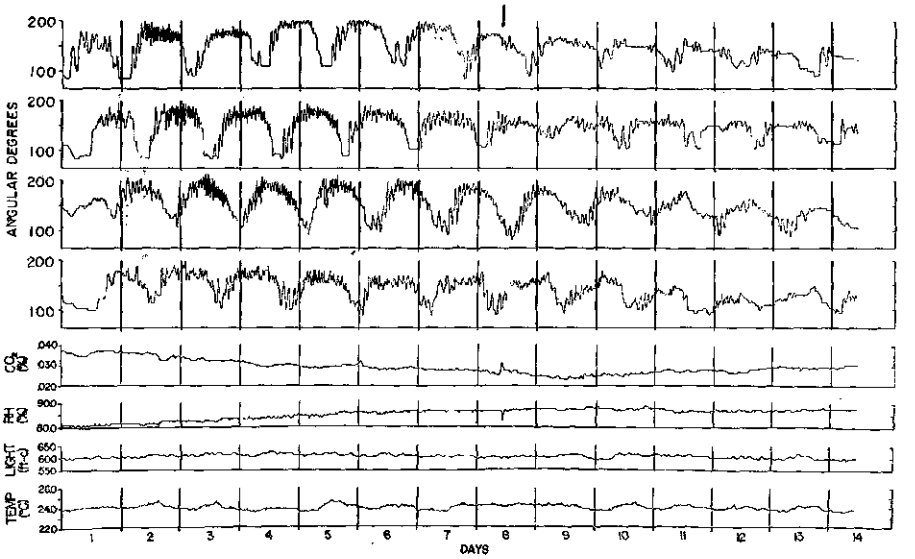

Fig. 2. Movements of individual primary leaves from four plants of *Phaseolus angularis* recorded from the time of leaf unfolding, together with environmental data recorded simultaneously. The arrow indicates time of removal of apical meristem and trifoliate leaves. (Alford and Tibbitts, 1970).

The high frequency rhythm in blade rotation draws further attention to the question (cf. Cumming, 1969a; Cumming and Wagner, 1968) of whether circadian rhythmicity might be derived (by frequency multiplication, modulation, etc.) from higher frequency oscillations. The problem, however, is to determine whether such high frequency oscillations are an integral part of the circadian clock mechanism or whether they are merely overt indicators (clock hands) of the basic oscillator involved in circadian timing. The experiments of Alford and Tibbitts were conducted at only one constant

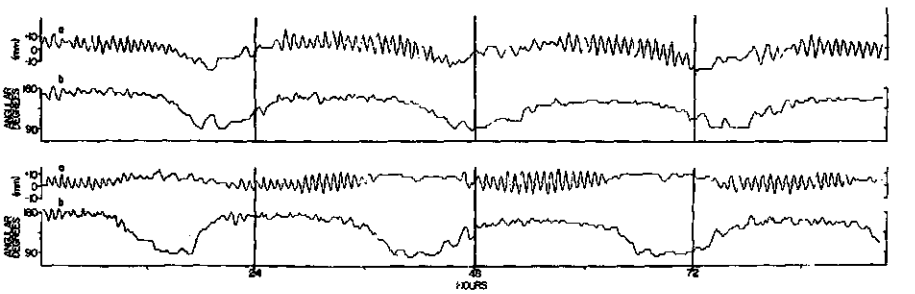

Fig. 3. Movements of primary leaves for two separate *Phaseolus angularis* plants grown simultaneously: (a) blade rotation in mms, as recorded by measuring the distance between the lateral margins of the leaf as it appeared on the projected image; (b) upward and downward move ment in angular degrees. (Alford and Tibbits, 1971.)

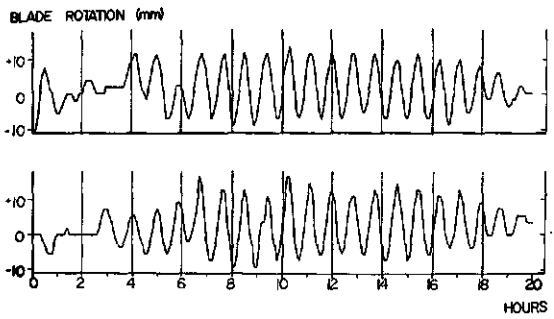

Fig. 4. Synchrony in blade rotation of the two unifoliate leaves on a plant of *Phaseolus angularis*. (Alford and Tibbitts, 1971.)

temperature, so that it is not known whether the period of the leaf rotation is relatively temperature independent or not. If such rhythms are temperature-dependent (as was found for some high frequency leaf movements that were studied by the Darwins (1898)), the likelihood of these being overt expressions of interval timers that are involved in circadian rhythmicity is less, although it is not ruled out, since various possibilities of temperature-compensated interacting processes still exist. Alford and Tibbitts (1971) have pointed out that stomatal apertures may regulate water levels within the pulvinus and thus might exert some control on blade rotation. Stomatal opening rhythms with periods between 40 and 50 minutes, superimposed on a circadian rhythmicity, have recently been observed in another bean species, *Phaseolus vulgaris* (Hopmans, 1971).

The possibility that such different rhythmic phenomena are indicative of a common oscillatory mechanism deserves consideration. The high frequency stomatal rhythms observed by Hopmans (1971) were temperature-dependent: the period decreased with increase in temperature.

Some organisms show synchronous timing of different circadian rhythmic phenomena and thus provide indirect evidence that the same basic oscillatory mechanism may be involved in different photoperiodically controlled processes. An excellent example of this has been given by Bünning (1969). Soybean plants were maintained in light: dark cycles of 14:$\bar{1}0$h that did not induce flowering. They were later transferred to an inductive light program in which 8 hours of light was given only once within 72 hours, instead of every day (Fig. 5). Under such conditions, a circadian rhythm was evident in the flowering response to a short light break that was given once at different times during each long dark period, in that flowering was successively inhibited then promoted above the uninterrupted long dark treatment (control: Fig. 5). Particularly significant is the fact that the leaves moved, that is, were raised ('waking' or 'subjective day' response) and lowered ('sleeping' or 'subjective night' response) rhythmically, during each uninterrupted dark period, in excellent synchrony with the time at which flowering was inhibited by a light interruption (subjective night or scotophilic phase), or promoted by it (subjective day or photophilic phase). As Bünning has pointed out, the leaf movement rhythm can be looked upon as a good 'hand' of the clock – that is indicative of the endogenous rhythm. The photoperiodic control of flowering and the synchrony of this response with the free-running leaf movements is strong indirect

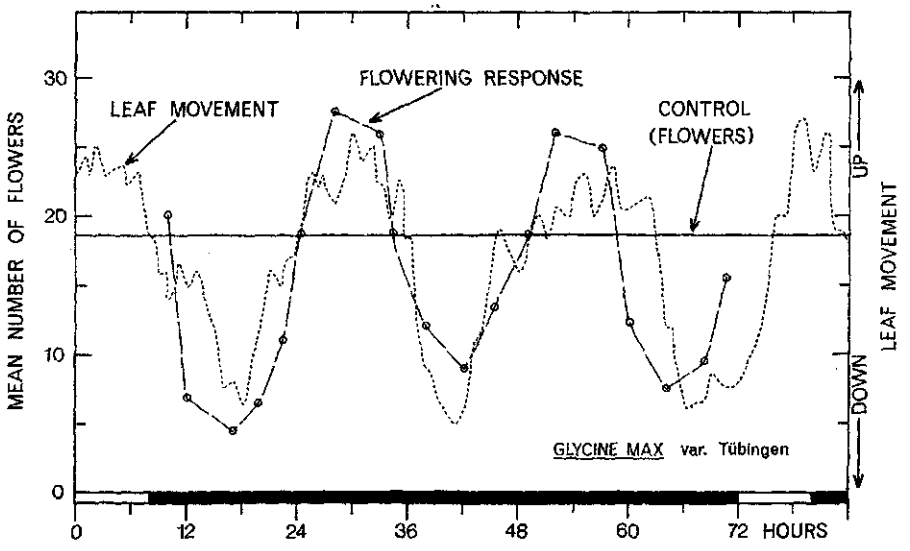

Fig. 5. *Glycine max*, variety 'Tubingen'. Synchronous circadian changes in photoperiodic responsiveness to half-hour light breaks during a long dark period and in circadian leaf movements. The leaf movements also refer to the 8:64 h light:dark cycle, but without light break. The plants were maintained in light: dark cycles of 14:10 h before starting the special treatments hight was white seed germination. (Bünning, 1969).

evidence that, in this instance, circadian rhythmicity was involved in photoperiodic induction.

Quite similar evidence has been provided by Halaban (1968b) who has shown that, when plants are subjected to different daily photoperiodic cycles, the inductive phase for flowering of the short-day plant *Coleus frederici* has a fixed phase relation to the phase of rhythmic leaf movements (Fig. 6). The experiments of Halaban were designed to locate the inductive phase, that is, the clock time when illumination inhibited flowering in daily dark periods of either 20, 16 or 12 hours, given with the corresponding daily photoperiod of 4, 8 and 12 hours, respectively. While inhibition of floral induction by light started 10 hours after the onset of darkness when the daylength was 4 or 8 hours, it started 8 h after the onset of darkness with a 12-h daylength. The onset of maximum floral inhibition by a light break therefore always occurred 5 hours after the time of the minimum leaf position in each respective photoperiodic cycle. Thus, the inductive phase apparently maintained a fixed phase relation to the phase of the circadian rhythm of the plant (indicated by the rhythm of leaf movements: a circadian rhythm of leaf movements has been shown by Halaban (1968a) in *C. blumei* × *C. frederici*). This evidence, although limited in scope, supports the theory that a circadian clock participates in the time measurement process of photoperiodic floral induction in *C. frederici*. Halaban (1968b) has suggested that if an hourglass mechanism were operating here as the time-measuring process, the inductive phase would be timed from the onset of darkness and should thus show a fixed relation to it in different photoperiods.

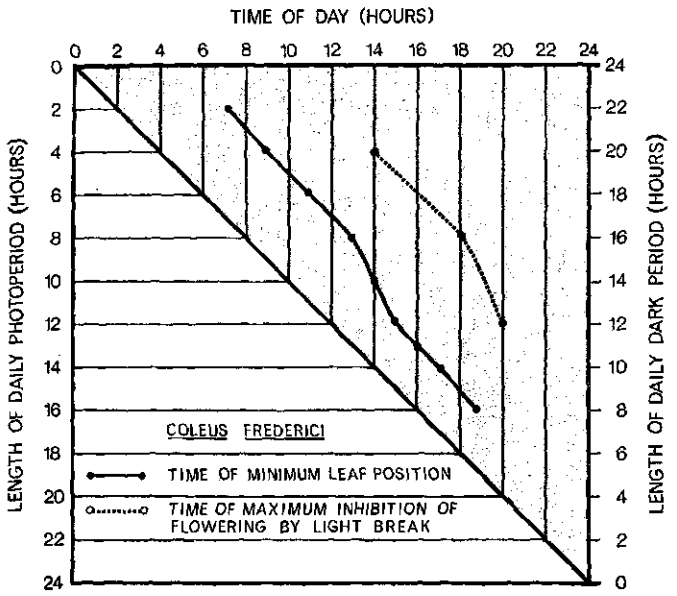

Fig. 6. *Coleus frederici.* Plot points with solid line: time of mean minimum leaf position in different daily light: dark cycles. Diagonal line indicates the onset of darkness. Plot points with dashed line: time of onset of maximum inhibition of flowering by a light pulse given at different times of a daily dark period. Light pulse was 60, 30 and 30 min, respectively, in a 20-, 16- and 12-h daily dark period. (Modified from Halaban, 1968b.)

Evidence from the use of skeleton photoperiods

Evidence for a significant role of endogenous circadian rhythms in photoperiodic response has been obtained by Hillman (1964, 1969, 1970) using a duckweed, *Lemna perpusilla* 6746. This plant is particularly valuable for studies of flowering because it thrives on a liquid medium and can be grown in non-photosynthetic conditions by culturing the fronds axenically on media with sucrose. With sucrose present to supply carbohydrates, flowering can be maximal with as little as 15 minutes of light per day (and 23 3/4 hours of darkness). These attributes led Hillman to use this plant to study the effects of skeleton photoperiods, a term used by Pittendrigh and Minis (1964) in studies of the *Drosophila pseudoobscura* eclosion rhythm. These authors showed that the action of a complete photoperiod – for example, eight hours of continuous light – could be almost fully simulated by two short (15-minute) pulses eight hours apart. The first observations of Hillman (1963) were that skeleton cycles such as 1 (9 3/4) 1/4 (13) permitted much less flowering than the corresponding full cycles, 11 (13). (The numbers in brackets refer to a dark period, the others to a light period.) These results were interpreted simply as an inhibition due to the long dark interruption of the complete 11-h photoperiod (Hillman, 1963). The 'inhibition' was reversible by brief light interruptions

of the dark 'interruption' – that is, the schedule 1/4 (2½) 1/4 (2½) 1/4 (2½) 1/4 (2½) 1/4 (13) permitted high levels of flowering – indicating that the amount of light was not the factor involved, but rather its distribution (Hillman, 1963). Hillman (1964) revised his conclusion that a skeleton schedule such as 1/4 (10½) 1/4 (13) was always inhibitory compared to the full photoperiodic cycle, when he found that if plants were transferred from continuous light into the reverse schedule, with the longer dark period coming first – that is, 1/4(13)1/4(10½) – then they flowered very rapidly. Thus, it became clear that the effect of such skeleton schedules depended on which of the dark periods the plants experienced first after being transferred from continuous light. Further experiments then showed that the effects of skeleton schedules were changed according to the length of a single dark period given between the end of continuous light and the start of the skeleton, and a circadian rhythm in flowering response was obtained (Fig. 7, lower section). For example, seven cycles of the schedule 1/4(13)1/4(10½) – referred to as 13:10.5 skeleton photoperiod in Fig. 7 – gave a higher percentage of flowering if preceded by 0, 24, or 48 hours of darkness (after continuous light) than if preceded by 8 or 32 hours of darkness. Conversely, six cycles of 1/4 (10½)/1/4(13) gave a higher percentage flowering when preceded by 15 or 39 hours, than by 0, 24 or 48 hours of darkness. These results are explicable if a rhythm that influences flowering starts from the light-off signal created by the transition from continuous light to darkness (the stock plants were previously in continuous light for years). Then, the amount of flowering would depend on the length of the initial dark period so that there is always maximal flowering if there is 13–15 hours of darkness (+ 24-h increments) before the first 15-minute light pulse. The persistence of the effect of a single variable dark period, after seven daily skeleton photoperiodic cycles, is explicable and possible if the 15-minute light pulses are not long enough to reset the rhythm in sensitivity to light that is started by the continuous-light-to-dark transition (Hillman, 1969). In contrast, seven repetitions of a full schedule, such as 10(14) are not modified by a single preceding dark period of varied length because the full photoperiod apparently resets the initial rhythm (Hillman, 1969). Some of the skeleton photoperiodic effects obtained by Hillman in *Lemna* have been interpreted in terms of the *Drosophila* circadian oscillation by Pittendrigh (1966).

Although this work on flowering suggests that photoperiodism in *L. perpusilla* 6746 involves an endogenous circadian rhythm, Hillman (1970) has emphasized that such evidence should be related if possible to an overt rhythm that occurs in the same plant. He has recently studied the carbon dioxide output rate of axenic cultures supplied with sucrose. In continuous darkness, following either dim red light or a 12(12) L:D schedule, Hillman (1970) found that the rate of CO_2 output oscillated through successive maxima and minima for two days, with a circadian periodicity, before apparently damping. In red light the rate was linear. When plants were transferred from continuous light to six or seven days of a skeleton photoperiodic schedule similar to the one used for flowering (Fig. 7, upper section) there was a more clearly sustained rhythm when the skeleton schedule 1/4(13)1/4(10½) rather than 1/4(10½)1/4(13) was imposed. Thus, the two skeleton schedules that had markedly different effects on flowering also differed in their action on the rhythm of carbon dioxide output and in both instances

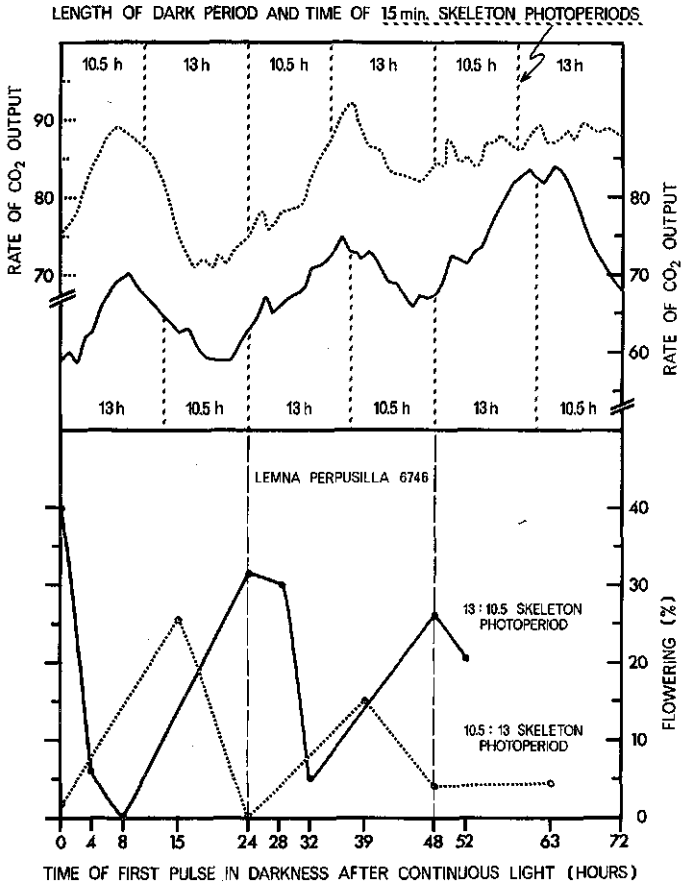

Fig. 7. *Lemna perpusilla* 6746. Lower figure: flowering response after various durations of darkness followed by seven (13:10½) or six (10½:13) repetitions of the skeleton schedule. (Modified from Hillman, 1964.) Upper figure: CO_2 output of two sets of cultures; curves drawn between points represent hourly rates in arbitrary units. Vertical lines represent 15-min light breaks; numbers between light breaks indicate length of dark period. (Modified from Hillman, 1970).

the skeleton schedule 1/4(13)1/4(10½) was more favorable for the 'induction' of a response. Such induction appears to be obtained when the photoperiodic cycles are appropriately timed in relation to an endogenous circadian rhythm.

Multiplicity of rhythmic responses in Chenopodium rubrum

Chenopodium rubrum shows extremely sensitive morphogenic responses to differences in the relative duration of light: dark cyclus, or to a single period of darkness that

interrupts continuous light. This is shown by the fact that a number of different rhythmic processes can be shown in this plant when seeds are germinated in continuous light that is interrupted by a single dark period, of varied duration, a day or two after the cotyledons have emerged from the testa. Some of these different rhythmic processes will be considered after providing details of our approaches to studies of flowering. Certain observations of rhythmic flowering responses have encouraged us to search for its basis at the metabolic level.

Considering the role of circadian rhythmicity in photoperiodic induction, any generalization should ideally take account of the different dimensions of timekeeping that are involved in the individual system. This is illustrated by the induction of flowering. In *C. rubrum*, imposition of a single dark period interrupting continuous light can lead to the visible initiation of flowering within a week. The amount of flowering that is obtained will depend upon the length of the dark period because there is a circadian rhythm in sensitivity to light within the organism and this controls flower induction. This rhythm is set by the transition from light to darkness and it measures the length of the dark period through oscillations in sensitivity to light (which provide alternate phases in which flower induction is inhibited, then promoted by light). Under this type of single cycle regime the response is primarily qualitative because any one plant either does or does not flower within quite a short period of time. However, the percentage of plants in the population that flower in response to different dark period lengths does provide a quantitative basis for the measurement of circadian rhythmicity. Using the same population of plants, it can be shown that the effects of non-optimal daily photoperiodic cycles may be progressively summated over a much longer period of time (weeks to many months) because the time to onset of flowering can depend markedly on the relative length of the daily light:dark cycles. In this instance the quantitative control over a developmental transition is much more evident and is clearly indicative of the adaptation of photoperiodic response to natural conditions: vegetative growth on the one hand and flowering and seed set on the other is partitioned to provide responses that are timed according to the season. In the foregoing experiments, the influence of circadian rhythmicity is quite clear in the single cycle experiment, but it is less clear in the photoperiodic cycle experiments, because the free-running system will be affected to some extent by the repeated dawn and dusk signals. From a philosophical viewpoint the display of circadian control in the one case does strongly suggest its controlling influence in photoperiodic induction, particularly in view of the close correspondence that can be shown between peaks of the circadian free-running oscillation and the photoperiodic response curve optima. More critical experimental approaches involving suspending or resetting of the circadian rhythm do support this view.

The experimental procedure that we generally use is to germinate seeds and maintain the seedlings either in alternating light:dark cycles, or in constant light that is interrupted by a single period of darkness. Seedlings that produce a visible floral primordium are classified as flowering, the remainder are considered to be vegetative (Fig. 8). Experiments on flowering can be completed within 2–3 weeks after sowing seeds, thus economizing on both space and time (Cumming, 1959, 1967a and 1969b). As many as 400 6-cm Petri dishes may be used in one experiment, each containing about 120 seeds

sown on filter paper or agar that incorporates a chemically defined medium; chemicals can be added to the medium for prescribed periods, in either light or darkness and then washed off after appropriate test period(s). Flowering may be induced by a single dark interruption of constant light as follows: after an initial period of 3 or more days in low intensity white fluorescent light (600 ft cd), during which the temperature is varied to stimulate germination, seedlings are left in the same light intensity or are transferred to 3000 ft cd fluorescent or 1000 ft cd incandescent light (Cumming, 1967b, 1969a). To induce flowering, 4½-day-old vegetative seedlings are placed in darkness and the length of the dark period is varied by returning replicated dishes to light after successively longer periods. Alternatively, a dark period of a duration known to induce subsequent flowering can be interrupted by light of a particular quality and intensity, and the effect of this light on flowering is compared with the control flowering response (Cumming et al., 1965). After removal from the dark, seedlings are left in continuous light for about 1 week and the percentage of seedlings showing a flower primordium is determined using a binocular dissecting microscope. The percentage of flowering can be plotted against the time at which light terminated or interrupted the dark period, and a sine curve with circadian periodicity may be obtained. For example, a rhythmic flowering response was evident when the duration of darkness interrupting continuous light was varied between zero and 5 days (Fig. 8) but the amplitude of the last one of the four oscillations was very small (i.e., 'damped' out) and there was no flowering after a dark period of more than 5 days duration. It was found that feeding glucose or sucrose either in the light period before darkness (Cumming, 1967b), or during the whole of the dark period, can enhance and sustain the rhythmic flowering response very markedly (Fig. 9). Although the rhythmic display of flowering in control plants (without sugar) was much less pronounced with low intensity (600 ft cd) than high intensity (3000 ft cd) light preceding darkness (Cumming 1967b), the degree of enhancement of flowering by sugars was more apparent when the light intensity before the dark period was 600 rather than 3000 ft cd – that is, when the energy supplied by photosynthesis was more limiting. There was also progressively greater stimulation of flowering the higher the concentration of glucose (between 0.2 and 0.6 *mM*) supplied throughout the dark period, whether preceded by low or high intensity light (Cumming, 1967b). Both inhibitory and stimulatory effects on flower induction have been obtained from glucose application, depending on the time when it was applied during darkness (Cumming, 1967b). For example, glucose caused some inhibition of subsequent rhythmic flowering response when applied for only the initial scotophil phase of darkness (0 to 9 hours), but it was stimulatory when applied during the time approximating the first photophil phase of darkness (9th to 20th hour – making allowance for uptake through the roots). Corroborative evidence of such alternately inhibitory and stimulatory effects was evident from the repeated inhibition or lack of stimulation of floral induction that was obtained when glucose was applied in successive scotophil phases of a long inductive (72 h) dark period; also, it was indicated by the considerable stimulation of induction that resulted from glucose application during one of the photophil phases (Cumming, 1967b). These results, although requiring further elaboration, do provide an obvious parallel with those resulting from the light interruption of

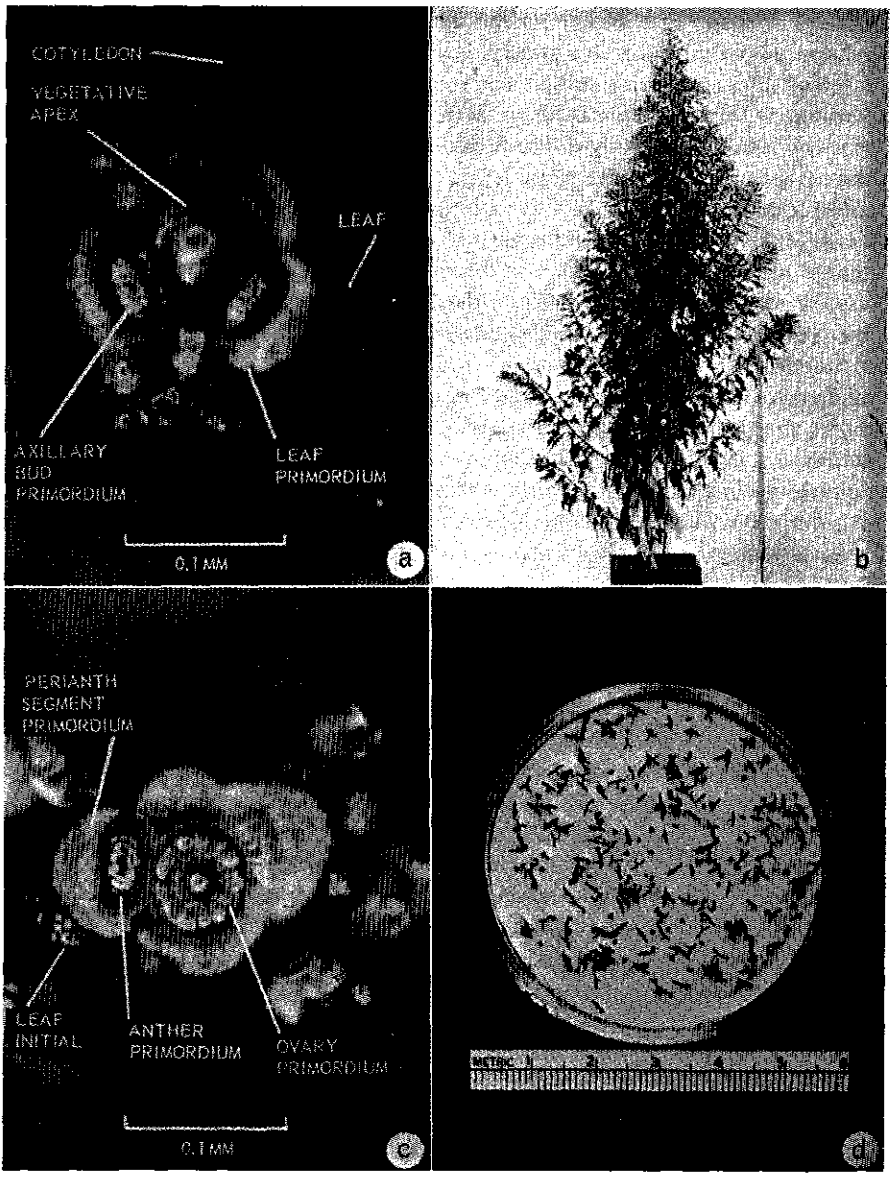

Fig. 8. Transition of apical primordium of *Chenopodium rubrum* from (a) vegetative to (c) floral state; (b) vegetative plant of *C. rubrum*, ecotype 49°58′N, grown in continuous light for 250 days; (d) five-day-old seedlings of *C. rubrum*, ecotype 60°47′N; seedlings at this stage are readily induced to flower by a single dark period interrupting continuous light. (After Cumming, 1959 and 1963; Cumming et al., 1965.)

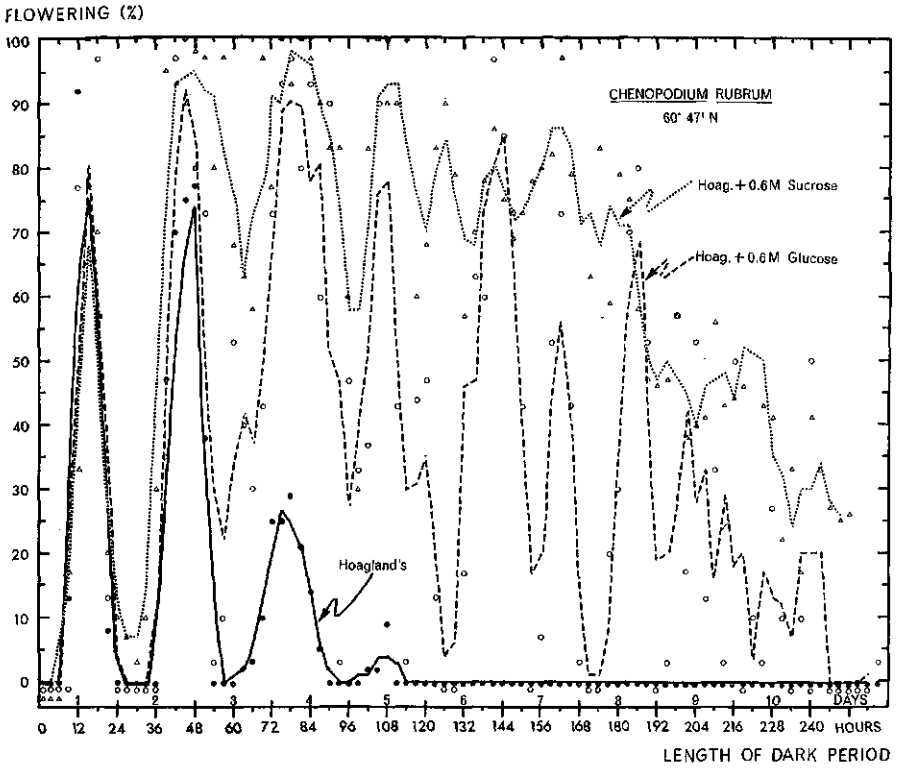

Fig. 9. Percentage of flowering of *Chenopodium rubrum*, ecotype 60°47′N. Germination for 3 days in fluctuating temperature and light intensity. Thereafter, 20 °C and transference to 3000 ft cd white fluorescent light for 36 h preceding single dark period of 0 to 260 h. Plants supplied throughout darkness with either Hoagland's solution or 0.6 *M* glucose or sucrose in Hoagland's solution. All plants in 600 ft cd after dark period. Plot pionts are raw data, curves are first 3-point moving mean. (Cumming, 1967b.)

darkness – in which light is alternately inhibitory and stimulatory to subsequent flowering if applied during a scotophil or photophil phase, respectively, of the free-running circadian rhythm (Cumming et al., 1965). However, rhythmic changes in sensitivity to glucose pulses applied during continuous light after darkness have also been found (King, 1971). The latter results suggest that the response to glucose might involve events occurring at the shoot apex that determine the capacity to flower, rather than directly influencing the induction of flowering.

Before discussing further evidence of the role of circadian rhythmicity in the photoperiodic control of flowering in *C. rubrum*, observations of other processes that occur in this species merit attention. They provide clear indications that a multiplicity of rhythmic phenomena with different periodicities and phase relationships may occur within a single organism. These observations lead one to question whether such periodic phenomena are involved in the generation of circadian rhythmicity or are

themselves derivatives of a more basic oscillatory system. In this connection we should bear in mind that both higher and lower frequencies could be derived from single or multiple oscillatory system(s) through differential coupling, specific phase relationships, frequency modulation and amplification, frequency multiplication or demultiplication.

In *C. rubrum* ecotype 50°10′N, betacyanin synthesis is light dependent (completely dark grown seedlings contain no betacyanin). In continuous light, accumulation of betacyanin is linear with time. However, when a single dark period interrupts continuous light, both the amount of betacyanin and chlorophyll synthesized during a given period of time in light after the dark interruption show a rhythm. This rhythmicity reflects differences in the rate of, and(or) the capacity for pigment accumulation that is dependent on the duration of the dark period (Wagner and Cumming, 1970). The rhythm in chlorophyll content was higher in frequency than circadian, with a period of about 15 h, while rhythmicity in the rate of betacyanin synthesis was circadian. When glucose was supplied throughout darkness interrupting continuous light, the phasing of the rhythm was positively correlated with the rhythm controlling flower initiation; but, without glucose, under relatively low intensity light, there was no clearly defined rhythm in betacyanin synthesis and there was no flowering (Fig. 10). Considered by themselves, the foregoing results suggest a simpler situation than appears to be the case. When phenylalanine, instead of glucose, was supplied during darkness interrupting continuous light, the rhythms of betacyanin synthesis and flower initiation were not correlated because the phasing of the betacyanin rhythm differed according to light intensity (Wagner and Cumming, 1970). Wagner and Frosch (1971) have concluded that a circadian rhythm which controls the capacity for betacyanin accumulation can be initiated or synchronized by the cyclic temperature conditions and changes in light intensity that are imposed during germination – although betacyanin accumulation during that period is essentially linear (Wagner and Cumming, 1970). This conclusion is based on the fact that rhythmic fluctuations in the time course of betacyanin content during darkness show correlations with the previous alternating germination conditions, rather than with the onset of darkness. This suggests that the underlying oscillation controlling the capacity for rhythmic betacyanin synthesis in darkness, is established or synchronized by the early fluctuating conditions and is not rephased by the transition from prolonged light to darkness (Fig. 11) – a situation different from flowering, in which such a dusk signal is normally decisive in setting the phase of the rhythm (Cumming et al., 1965). In light following darkness, the respective level of betacyanin accumulation is increased, but the phasing of the rhythm that occurs in darkness is not altered (Fig. 10).

Since endogenous rhythmicity is an adaptation of the organism to cyclic environmental conditions of energy supply, it is of particular interest to determine the enzyme activities concerned with energy transformation in different biochemical sequences and in different compartments of the cell. Working in the author's laboratory, Frosch (1971) has studied the activity of three enzymes in *C. rubrum* – 50°10′N (Fig. 12): nicotinamide adenine dinucleotide phosphate-dependent glyceraldehyde-phosphate dehydrogenase (NADP-GPD) – that is located only in the chloroplasts and is involved

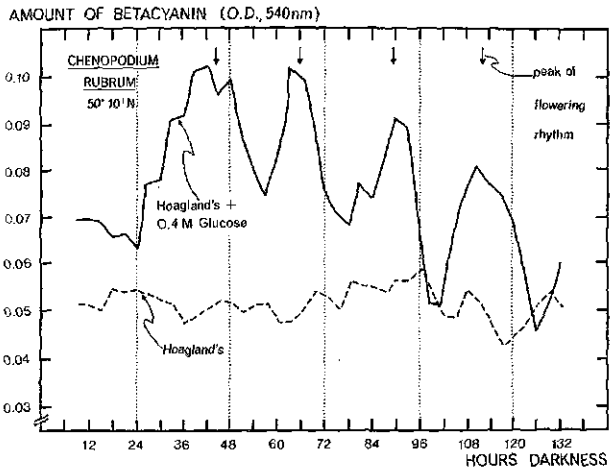

Fig. 10. *Chenopodium rubrum*, ecotype 50°10'N. Germination for four days in fluctuating temperature and light intensity. Thereafter 28 °C, 1 day in 600 ft cd fluorescent light with terminal 30 min of 220 ft cd incandescent light before darkness (3-h increments). Seedlings supplied with 0 (dashed line) or 0.4 *M* glucose (solid line) in Hoagland's solution during darkness. 600 ft cd fluorescent light for 1 day after darkness, then betacyanin extracted. The arrows above the curve indicate the peaks of the rhythm in flower initation when 0.4 *M* glucose was supplied throughout darkness; there was no flower initiation without glucose.

in the carbon reduction cycle of photosynthesis (Calvin cycle) – this has a rhythm in activity with a period of 15 hours; NAD-GPD – that is mostly located in the cytoplasm and is involved in glycolysis – this also has a rhythm in activity with a period of 15 hours; adenylate kinase (AK) – that is located in the chloroplasts, mitochondria, and cytoplasm, and is involved in the regulation of the adenylate system (ATP, ADP, AMP, and P_i) – this has a 30-h (circadian) periodicity with two sub-peaks in each circadian period and the main minima at the 30th and 60th hours of darkness (i.e. these minima delineated the termination of each circadian period and coincided with the minima of the rhythm controlling flowering). The phasing of the NADP-GPD and the NAD-GPD rhythms was almost opposite, with the peaks occurring at 9, 24, 42, 54 and 69 hours (NADP-GPD) and at 15, 27, 45 and 60 hours (NAD-GPD). However, there was quite close correlation between the peaks of the NADP-GPD activity and the adenylate kinase peaks – the latter occurred at 9, 21, 39, 51, and 69 hours (Fig. 12).

It appears that the activity of the enzymes in light is determined by the phasing of the rhythm at the time when a single dark period is terminated by light. Although light following darkness increases or amplifies the overall metabolic activity it does not alter the phase of the dark rhythm (Frosch, 1971). In other words, the increases in enzyme activity after different dark periods show an essentially parallel relationship – the curves for activity after a given period of light would show higher activity than the dark activity curves in Fig. 12, but the two curves would be essentially parallel to each other.

I suggest that some significant conclusions can be derived from the results shown by

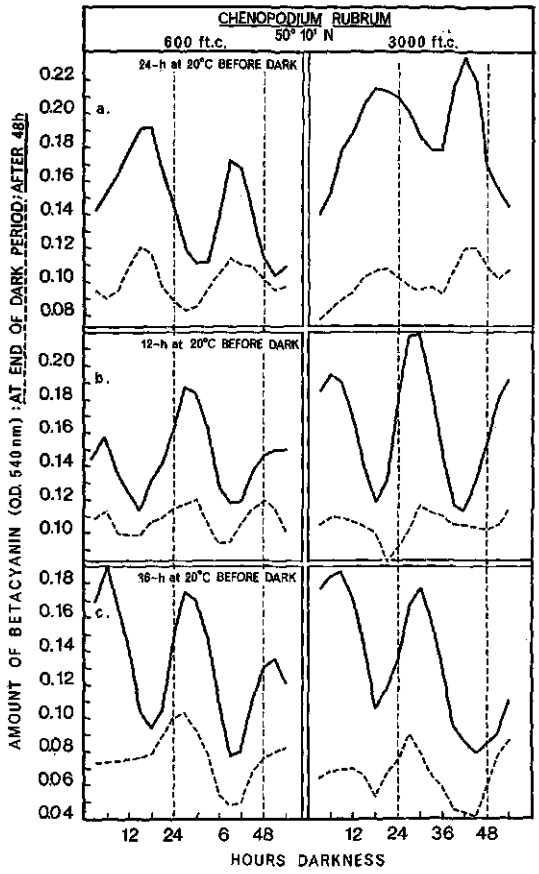

Fig. 11. *Chenopodium rubrum*, 50°10'N. Germination for four days in fluctuating temperature and light intensity. Then constant 20°C; 600 or 3000 ft cd white fluorescent light for (a) 24 h, (b) 12 h, and (c) 36 h, followed by a single dark period of varied duration (3 h increments). Dashed line represents amount of betacyanin extracted at the end of each respective dark period. Solid line represents betacyanin extracted at end of 48 h light period following each respective dark period, with same light intensity as preceded darkness. The dark period medium was 0.4 *M* glucose in Hoagland's solution. (Wagner and Frosch, 1971.)

the betacyanin and enzyme activity results. Although there is no apparent rhythmicity in the continuous light conditions preceding a single dark period, some basic oscillation(s) – which may be established or entrained by the light intensity and (or) temperature fluctuations – apparently exist during that period. These oscillation(s) later affect the capacity for pigment synthesis, and the activities of different enzymes, in such a way that they show various periods and phase relationships. Significance must be attached to the dark period and its duration if this serves to establish the overt rhythms and the relative levels at which the synthetic or metabolic activities will be amplified during the

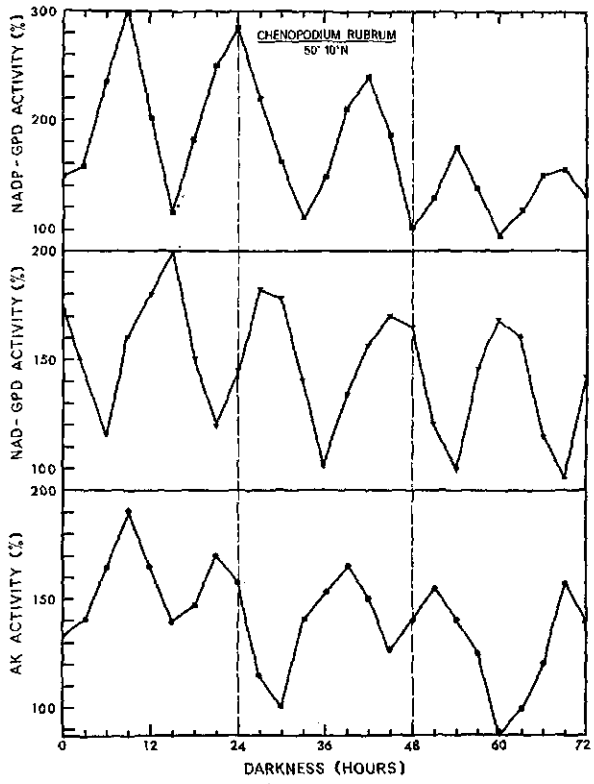

Fig. 12. *Chenopodium rubrum*, 50°10'N. Germination for 4¼ days in fluctuating temperature and light intensity. Thereafter constant 20 °C; 600 ft cd fluorescent light for 1 day, then darkness. Enzyme activities determined at respective time in darkness (3-h increments). (After Frosch, 1971.)

subsequent light period. In other words, for any particular rhythmic phenomenon, the timing and duration of the dark period in relation to light is the means towards the differential amplification of a metabolic process in the subsequent light period. Previously, I have drawn an analogy between darkness acting on the organism in a manner similar to letting in the clutch of an automobile (disengaging the gear). In the latter condition the engine revolutions can be varied via the accelerator but there is no direct progression; however, if the clutch is then let out, so that a particular gear is engaged (analogous in the organism to the transition from darkness to light), then the specific signal, information, and energy that is available can result in different rates of progressive activity or development (Cumming, 1971).

It is also clear in the foregoing examples of metabolic control that light energy input not only acts as a timing component but also as a source of energy whereby the response to a signal can be amplified.

Further indications of rhythmic changes in metabolism have been obtained by Chia-

Table 1. *Chenopodium rubrum.* Comparisons of rhythmicity, at 20 °C, shown in net photosynthesis, dark respiration, chlorophyll content (ecotypes 60°47′N and 50°10′N) dry weight and flowering (ecotype 60°47′N only). Responses measured during a single dark period of varied duration (3-h increments) started 5½ days after wetting seeds (dark respiration, dry weight), or in continuous light (1000 ft cd incandescent) following darkness (net photosynthesis, chlorophyll content and flowering) (Chia-Looi, 1971).

Rhythmic phenomena	Ecotype 60°47′N				Ecotype 50°10′N				
	Hours of dark to			Period (hours)	Hours of dark to				Period (hours)
	1st peak	2nd	3rd		1st peak	2nd	3rd	4th	
Net photosynthesis	15	36	57	21	9	24	39	54	15
Chlorophyll content	18	39	60	21	9	24	36	54	15
Dark respiration	12	39	63	27	15	36	60	–	21–24
Dry weight	18	36	54	18					
Flowering	12	42	72	30					

Looi (1971) in my laboratory; she has studied the relationship between net photosynthesis, chlorophyll content, dark respiration, dry weight and flowering in two different ecotypes of *C. rubrum.* These show specific rhythmicities that appear to be genetically based (Table 1). Thus, within both ecotypes, the peaks of the rhythms in net photosynthesis and chlorophyll content were closely correlated; however, the periodicity in 60°47′N was 21 hours and in 50°10′N it was 15 hours, and the successive peaks (maxima) occurred at an earlier time in darkness in 50°10′N than in 60°47′N. In general, the periodicity of the photosynthetic enzyme, NADP-GPD, in ecotype 50°10′N, occurred at 9, 24, 42, 54 and 69 hours (Fig. 12), while the peaks for net photosynthesis were very similar and occurred at 9, 24, 39, and 54 hours (Table 1) – the periodicity being 15 hours in both phenomena. A more detailed discussion of differences in flowering response between these two ecotypes is included in the next section (see also Fig. 14).

It can be concluded from the foregoing results that there are indeed various rhythmic phenomena within a particular ecotype that show differences in frequency and phasing.

Circadian rhythmicity and the photoperiodic induction of flowering

The purpose of this section is to provide a model for the summation of many of the aspects that have just been discussed. Seedlings of *C. rubrum* – ecotype 60°47′N, for example, that have been germinated in constant light – display a circadian rhythm in the initiation of flowering that depends on the duration of a single dark period interrupting continuous light (Fig. 13). The change from continuous light to darkness sets the phase of the rhythm – as shown, first, by timing dusk differently with respect to the start of germination and, second, by the effects of light interruptions of prolonged darkness. A circadian rhythm of flower initiation is also evident when a prolonged inductive dark period of, for example, 72 hours is interrupted once by a brief red irradiation (Fig. 13). The red light is inhibitory to flowering when interpolated in the scotophil

phase, but stimulatory when interpolated in the photophil phase. The stimulatory action of red light indicates that there is an optimal requirement for a high level of the active form of phytochrome-P_{fr} during each photophil phase. Also, since red light can set P_{fr} at a maximum level at the beginning of darkness, yet red radiation is again required later in darkness for maximum flowering, it is evident that some reversion of P_{fr} to P_r, or decay of P_{fr}, has occurred in darkness before that time (Cumming et al., 1965). Furthermore, such partial reversion or loss of P_{fr} occurs during the first scotophil phase of darkness and this can provide an hourglass timing component in the photoperiodic induction of flowering (Cumming, 1963; Cumming et al., 1965; Kasperbauer et al., 1964; King, 1971). Photoreversible control of flowering (Borthwick et al., 1952b) by red and far-red radiation is evident in *C. rubrum* (Fig. 13, and Kasperbauer et al., 1964), but it is much less evident during the scotophil than photophil phase(s) of darkness (Cumming et al., 1965; King, 1971); this provides an example of interactions that may occur between circadian rhythmicity on the one hand, and hourglass timing that is mediated by phytochrome on the other hand.

In daily light:dark cycles, earliest (optimal) flower initiation of *C. rubrum* occurs in a particular range of photoperiodic cycles, depending on genotype and environmental conditions. The time elapsed before flower initiation, when plotted as a dependent variable of the length of the daily dark period (Fig. 13), provides a response curve that can be very similar to the first oscillation of the endogenous circadian rhythm that is elicited by a single dark period (Fig. 13). This similarity is substantial evidence that the endogenous circadian rhythm provides a primary basis for time measurement in the daily photoperiodic control of flower initiation. The more advanced phasing of the photoperiodic response curve, as compared with the free-running circadian rhythm (Fig. 13), can be explained on the basis that the daily dusk and(or) dawn signals entrain the endogenous rhythm of 30 hours; the position of the peak is in effect advanced to an earlier time in darkness, and the rhythm is entrained from its subjective circadian period to a 24-hour (solar) period. King (1971) has shown that the phase of the rhythm in *C. rubrum* – 60°47′N can be advanced or delayed according to the timing and duration of light pulses, and the significance of these results will be discussed subsequently. However, it should be noted that if we postulate that the circadian rhythm is entrained to a solar day then the calculation: 24 (solar day)/30 (circadian period) × 15 (first maximum) = 12 hours, as compared with the first peak of the daily dark response at 10 h (Fig. 13) – even though the experimental conditions for the free-running rhythms and daily photoperiodic response differed in light intensity and temparature. The curve for phasing of the rhythm of flower induction after interruption of prolonged darkness by 4 minutes of red irradiation was more retarded (delayed) than the other rhythmic flowering responses; this is probably indicative of a lack of phase resetting by such a brief light pulse, and corroboration of this suggestion has recently been provided by King (1971).

The endogenous rhythmic flowering responses of different latitudinal ecotypes show specific characteristics that are also expressed in daily photoperiodic cycles. The responses of three ecotypes of *C. rubrum*: 62°46′N, 60°47′N and 50°10′N, that were maintained constantly at 25 °C after germination, illustrate some of these characteristics

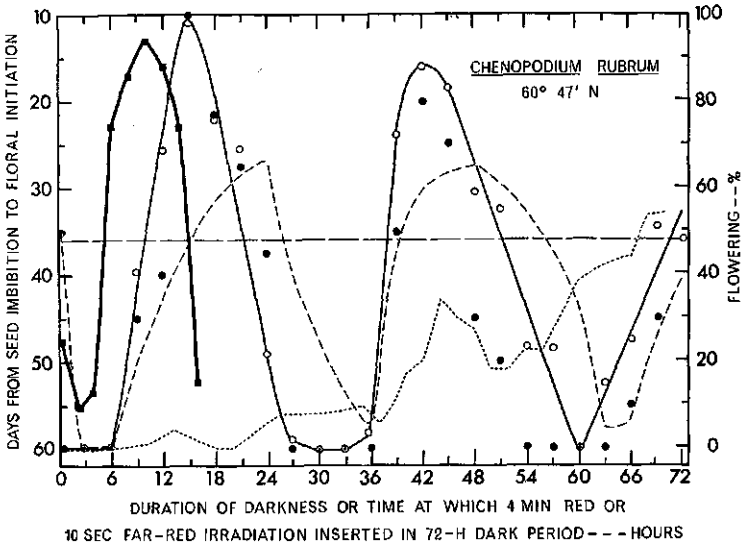

Fig. 13. *Chenopodium rubrum*, 60°47'N. Flower initiation. Daily dark response curve (heavy solid line): days from seed imbibition to flower initiation at 15 °C, photoperiod 250 ft cd CW fluorescent. (Modified from Cumming, 1967a.) Endogenous rhythmic response at 20 °C: (open circle, light solid line) average flowering when single dark period of 3 to 72 h was begun at -6, 0 and +6 h with respect to start of germination period and high-intensity light period; solid circle plot points are experimental values for –6 h (which deviated most, but not significantly, from the average). Single 72-h dark period (control: long dash) interrupted once by 4-min red irradiation (medium dash) or 10-sec far-red irradiation (short dash). (Modified from Cumming et al., 1965)

(Fig. 14). First, there are differences that are correlated with latitude of origin; the most northern ecotypes flower in a wide range of daily dark periods. Earliest flowering at any particular temperature is in shorter daily dark periods in the northern than in the southern ecotypes. Thus, one can generalize that the ability to measure small changes in night length, and the absolute requirement for darkness, is greater in the southern than in the northern ecotypes. This accords with the fact that day-to-day changes in night length are least near the equator and progressively greater towards the north pole, while the night length during the growing season is progressively shorter towards the north pole. In the northern hemisphere therefore, if a plant near the equator is to flower at a certain time between the spring and autumn equinox each year (and the ecotypes considered here do so; see Cumming, 1963), then the qualitative and quantitative control of induction must be over a narrower range of differences in length of dark period – in dark periods of relatively longer absolute length (see Fig. 14 and Cumming, 1963 and 1969a).

Some characteristics of the daily dark response curves appear to be derived from the endogenous free-running oscillations that occur in prolonged darkness. Such similarities are substantial evidence that endogenous oscillatory mechanisms can provide a

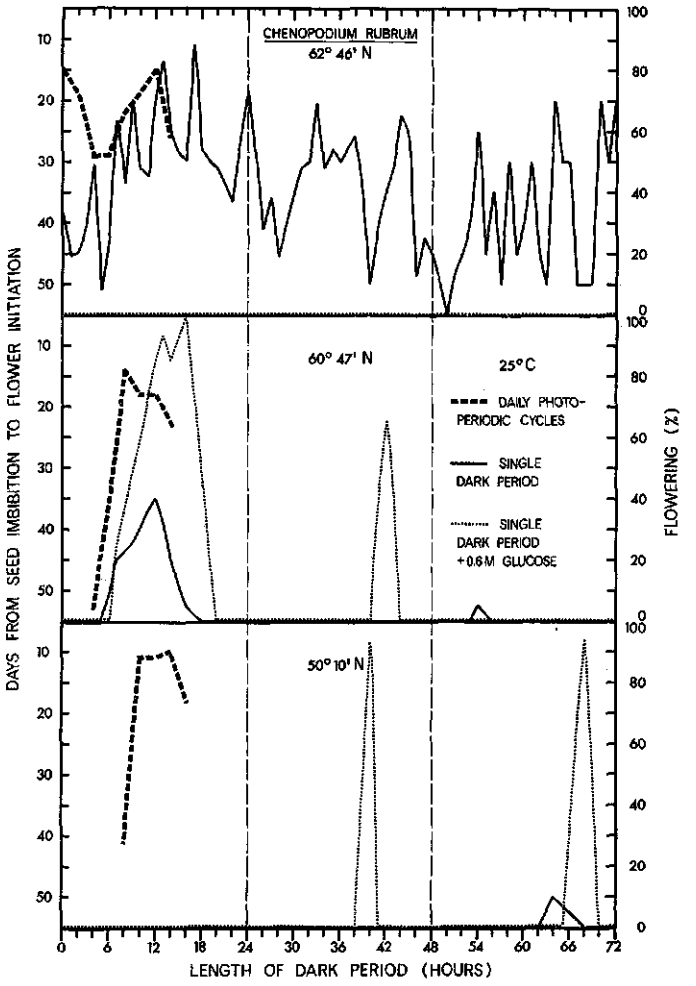

Fig. 14. Flower initiation of *Chenopodium rubrum*, 62°46′N, 60°47′N, and 50°10′N. Daily dark response curves (heavy broken line) with daily photoperiods of 250 ft cd CW fluorescent, 25 °C (Modified from Cumming, 1967.) Endogenous rhythmic response: germination for 3½ days in fluctuating conditions of temperature and light intensity. Thereafter 25 °C and 3000 ft cd CW fluorescent light before and after darkness of 0–72 h (3-h increments). Hoagland's solution (solid line) or 0.6 *M* glucose in Hoagland's throughout darkness (dotted line). (Cumming, unpublished.)

primary basis for time measurement in the daily photoperiodic control of flower induction. The response of ecotype 62°46′N is discussed first because there is a close correlation between its unusual endogenous rhythms and its photoperiodic response (Fig. 15). These responses are different from the circadian rhythms and the unimodal daily photo-

periodic response curves shown more generally in other ecotypes (Fig. 14).

The raw data of induction of this ecotype at 25 °C in response to a single dark period of 0 to 72 h are shown for 3 h and 1 h increments (Fig. 14 and 15, respectively). High frequency oscillations with a period of 3 to 4 h continued throughout darkness (Fig. 15). Also, there was amplitude modulation in these oscillations which may provide a basis for frequency multiplication; moving mean calculations showed that there were maxima with a periodicity of 10.2 h, and the phasing of this rhythmicity was closely correlated with the ambiperiodic optima that occurred in 0 (continuous light) and 12 h daily dark periods at 25 °C (Fig. 15). Furthermore, the pronounced minima at 3, 27, 49 and 67 h (Fig. 15) delineated three circadian oscillations each composed of two subperiods peaking at 13, 22.5 32.5, 43, 52 and 64 h respectively (10.2 h period), – providing evidence that the modulation and multiplication of high frequency rhythms can provide a basis for time measurement in daily photoperiodic cycles. Related to the foregoing, it is of added interest that this same ecotype of *C. rubrum* (62°46′N) shows a rhythm in net photosynthesis having a higher frequency than ecotypes 60°47′N and 50°10′N (Chia-Looi, 1971).

When a comparison is made between the rhythmic flowering response of ecotypes 62°46′N, 60°47′N, and 50°10′N, to the varied length of a single dark period interrupting continuous light (3-h increments), with or without 0.6 *M* glucose supplied throughout darkness, pronounced differences in rhythmic response are apparent. Thus, without glucose, there was some flowering (high frequency rhythm) after all dark period durations in ecotype 62°46′N, but there was only a small first maximum in ecotype 60°47′N, while in ecotype 50°10′N there was initially no flowering after any dark period length. In contrast, with glucose, there was no flowering in ecotype 62°46′N but there was a pronounced first maximum and smaller second maximum in ecotype 60°47′N, while, in ecotype 50°10′N, there was no first maximum but there were a narrow second and third maxima.

Ecotype 34°24′N raises a different problem in assessing the role of endogenous rhythmicity in photoperiodic induction, because flowering has not been obtained in response to a single dark period of any length between 0 and 8 days, even with the addition of sugars and gibberellic acid (Cumming, 1969a). A partial indication of the basis of this lack of response has been shown by the requirement for at least four consecutive daily photoinductive cycles to induce any flowering; furthermore, induction was restricted to daily dark periods of 15 to 18 h, even when seven cycles were given. A possible basis for lack of induction in this ecotype may be rapid dark reversion or loss of the active form of phytochrome P_{fr}. This could provide extreme sensitivity in time measurement if coupled with an endogenous circadian rhythm involving the availability of some substrate(s). Then, flower induction would only be obtained in daily photoperiodic cycles which could provide the necessary cyclical input or availability of metabolites, coupled with the appropriate cyclical photoconversion of P_r to P_{fr}. Preliminary experiments involving cyclical light pulses and the application of sucrose lend some support to this idea (Cumming, 1969a). Chia-Looi (1971) has found that ecotype 34°24′N displays rhythmicity in net photosynthesis and chlorophyll content (15-h periodicity) but there was no clearly defined rhythm in dark respiration.

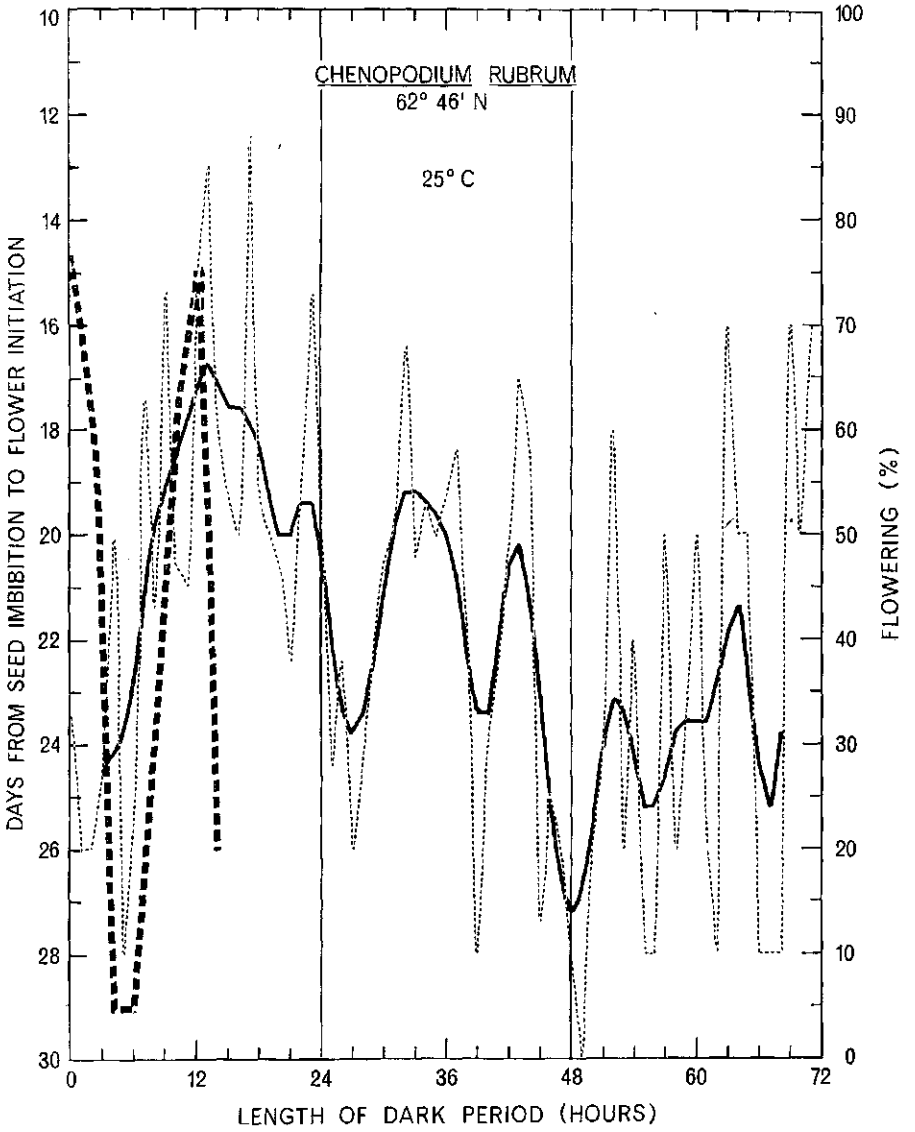

Fig. 15. Flower initiation of *Chenopodium rubrum* ecotype 62°46'N at 25°C. Daily dark response curve (heavy broken line): days from seed inhibition to flower initiation, 250 ft cd CW fluorescent photoperiod. (Modified from Cumming, 1967a.) Endogenous rhythmic response: germination for 3½ days in fluctuating temperature and light intensity. Thereafter 25°C; transfer to 3000 ft cd CW fluorescent for 36h preceding single dark period, with same light after dark. Thin dotted line drawn through raw data; heavy solid line represents third 3-point moving means. (Modified from Cumming, 1967b.)

Control of flower induction by changes in phytochrome

Evidence that the photoperiodic induction of flowering can be changed by altering the endogenous level of phytochrome-P_{fr}, suggests that the rhythmic system can be forced or by-passed so that induction occurs more nearly on a linear or hourglass basis (Cumming, 1963 and 1969c). When *C. rubrum* ecotypes 62°46′N and 60°47′N were illuminated throughout each photoperiod with different red/far-red ratios, it was shown that a low level of P_{fr} maintained by constant light of low R/FR ratio (R/FR-0.07) resulted in earlier floral initiation than did photoperiods of similar R/FR ratio but with a daily dark period (Table 2 and Cumming, 1963). Thus, darkness was not essential for early flowering of these short-day plants and the results suggest a promotive role of P_{fr} throughout a large part of the long photoperiod of low R/FR ratio. This reasoning is based on the assumption that, with photoperiods of any particular R/FR ratio, dark periods of greatest length would result in the most reversion or loss of P_{fr} and therefore the lowest level of P_{fr}, on a time-concentration basis. The reaction of the foregoing ecotypes of moderate sensitivity contrasted with those of greater sensitivity (e.g. ecotype 50°10′N, Table 2). This ecotype required longer dark periods for optimal initiation, but in 16-h photoperiods flowering was much earlier when the R/FR ratio was decreased.

It therefore appears that partial but not complete reversion or loss of P_{fr} provides one of the timing components in flower induction. Brief interruptions of prolonged darkness with different mixtures of red and far-red irradiation have shown that, during the successive photophil phase(s) of prolonged darkness, there is a requirement for a high level of P_{fr} for optimal flower induction – in contrast to the intermediate or low level of P_{fr} that is required during the scotophil phase (Cumming et al., 1965). Such dependence of flower induction on P_{fr} level and endogenous rhythmic processes is in harmony

Table 2. Days from start of 8-, 16- and 24-h photoperiods to floral initiation, with red to far-red ratios of 18, 11 and 0.07, respectively, throughout photoperiod. Constant 15°C. Experiment terminated after 100 days. (Cumming, 1963).

C > rubrum ecotype	R/FR ratio	Daily photoperiod		
		8h	16 h	24 h
62°46′N	18	11	12	51
	11	16	13	40
	0.07	D	39	16
60°47′N	18	8	17	78
	11	12	13	NF
	0.07	D	19	17
50°10′N	18	9	62	NF
	11	10	32	84
	0.07	D	8	NF

* NF = no floral initiation; D = dead within 65 days, no floral initiation.

with the externally forced system of light:dark cycles that prevails under naturally inductive conditions. In view of these results and conceptions, I postulated that *C. rubrum* may respond inductively to darkness not necessarily because there is some essential reaction that cannot occur in light, but, rather, because cessation of light terminates external photosynthetic energy input and also permits some decrease in the level of phytochrome-P_{fr}. If this is so, then it should be possible to induce flowering by completely replacing a dark interruption of continuous light with a light interruption, if such light were to maintain phytochrome-P_{fr} and photosynthetic energy utilization at a level that duplicates, or substitutes for the metabolic processes occurring in darkness (on some integrated duration-concentration basis). This has been validated experimentally in one ecotype of *C. rubrum* (60°47′N): light of low R/FR ratio that should maintain some phytochrome continuously in the P_{fr} form, and which has sufficient energy to allow some photosynthesis to occur, can bring about induction of flowering when it completely replaces a single dark period interrupting continuous high intensity white light (Cumming, 1969c, and Fig. 16). Note, however, that a longer

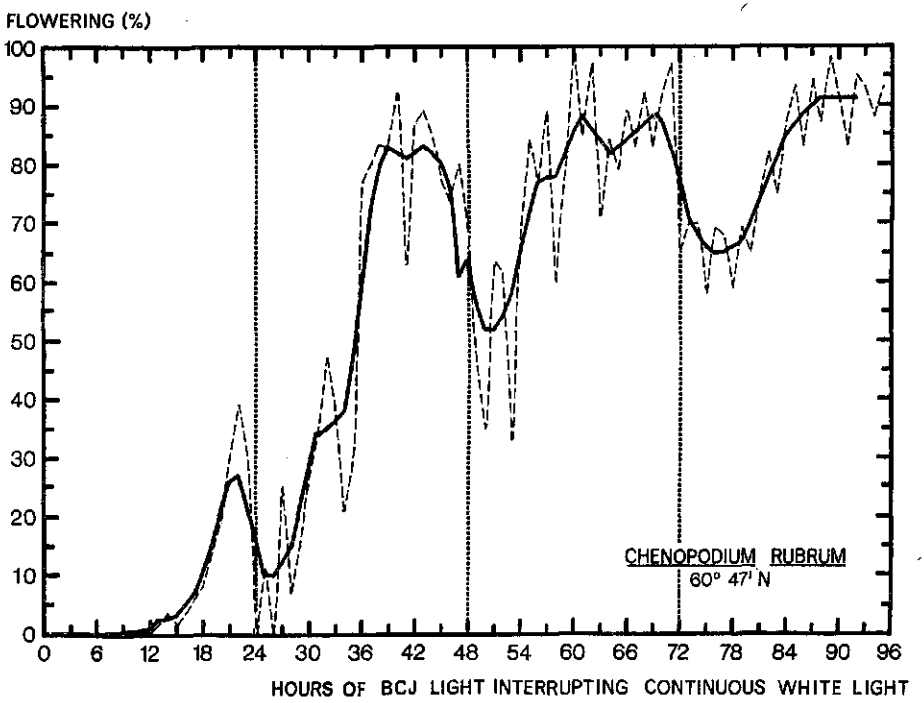

Fig. 16. Percentage of flowering of *Chenopodium rubrum*, 60°47′N. Germination for 3½ days in fluctuating temperature and light intensity. Thereafter 20°C. Transfer for 24 h to 970 ft cd incandescent. Then single period of BCJ light (35 ft cd, 0.97 $mW.cm^{-2}$, 380–750 nm), 1-h increments, followed by continuous 970 ft cd incandescent. Dashed line is raw data, solid line is third 3-point moving mean. (Cumming, unpublished; cf. also, Cumming, 1969c.)

period of inductive light than darkness was required even for minimal initiation, also, that at least 60 h was required for 100% flower initiation. The result of such forcing of the system by inductive light, as compared with the circadian rhythmic induction that occurred in darkness (Fig. 13), was a change to a more linear inductive response. It is also noteworthy that the oscillations of higher frequency (Fig. 16 and Cumming, 1969) are not normally observed when flowering was induced by darkness, and these were superimposed on the circadian periodicity that was less pronounced after inductive light than darkness.

One further important point is that the inductive light conditions were, in themselves, near enough to optimal so that if prolonged to 5 days or more, there was onset of flowering without returning plants to white light (Cumming, 1969c; Sawhney and Cumming, 1971). Many weeks normally elapse before plants of ecotype 60°47'N initiate flowering in continuous white light, furthermore, plants maintained in continuous darkness without returning them to high intensity white light do not flower and the level of flowering is very low even when sucrose is supplied (Cumming, 1969c).

Previous findings that changes in light intensity may be sufficient to influence some rhythmic phenomena are of interest in relation to the foregoing, although they do not bear directly upon the question of phytochrome action because changes in light quality were not involved. Wilkins (1960a and b) observed an approximately linear relationship between the initial intensity of illumination to which the leaves of *Bryophyllum fedtschenkoi* were exposed and the intensity at which the rhythm commenced. Imposing either gradual or sudden changes in light intensity (3000-0 lux), Wilkins found that it was necessary to reduce the light intensity by at least 80% to initiate a rhythm, rather than to reduce it below a critical level (Fig. 17). Engel and Friedrichsen (1951)

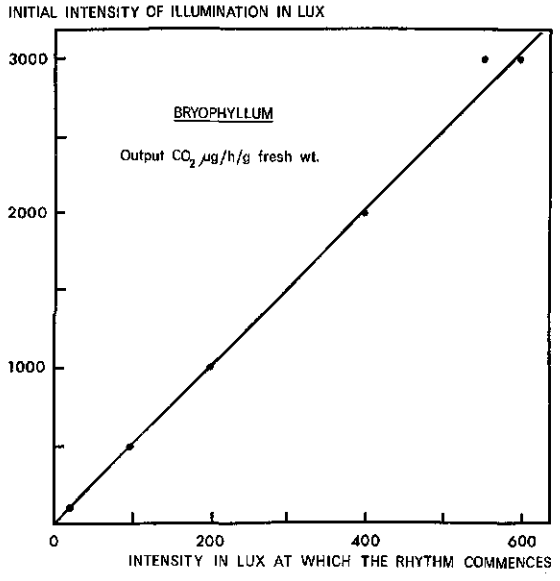

Fig. 17. *Bryophyllum fedtschenkoi*. Approximately linear relationship between the initial intensity of illumination to which the leaves are exposed and the intensity at which the rhythm commences. Relationship based on output of CO_2 in µg per hour, per gram of fresh weight. (After Wilkins, 1960a and b.)

found that the rhythm of guttation in *Avena sativa* seedlings can be induced by increasing the light intensity.

Phase response curves and the role of circadian rhythmicity in photoperiodic induction

In *Chenopodium rubrum* the light-off or dusk signal is of primary significance in controlling the phase of the rhythm of flower induction when continuous light is interrupted by a single period of darkness (Cumming et al., 1965, and Fig. 8 and 13). But even in this instance there may be doubts about the absence of any light-on (dawn) effect, unless we can utilize some indicator rhythm to determine the phase of the endogenous rhythm controlling flower induction. An approach to this question is to impose a short light treatment during prolonged darkness, that does not, in itself, rephase the free-running endogenous rhythm, but which does provide an indication of the sensitivity of the rhythm to light. This approach has been discussed here (Fig. 13) and is elaborated in detail elsewhere (Cumming et al., 1965). It is clear from such experiments that when continuous light is interrupted by a single period of darkness, the effect of the dawn signal on phasing of the rhythm is negligible compared to the light-off signal.

To avoid the difficulties and ambiguities associated with the use of indicator rhythms, King (1971) has made direct measurements of the phasing of the rhythm of flowering under various photoperiodic conditions. To assess rhythm phase control and entrainment, a single light pulse of 18, 12 or 6 h duration was given at different times in darkness, and hence at different phases of the free-running rhythm. In all of the following experiments the dark period was started $5\frac{1}{2}$ days after sowing the seeds. Red light was used so that the photosynthetic effect of the light break would be reduced as far as possible. After the light pulse, an advance or delay in the phasing of the rhythm was assayed by allowing the rhythm to run free in a subsequent dark period of varied duration; then the plants were returned to continuous light until assayed for flowering.

Phase response to a 12- or 18-h light interruption. A single 12-h red irradiation (115 $\mu W/cm^2$) was imposed at different times in darkness. The raw data (Fig. 18) and the derived relationship between rhythm peak times and the timing of the start of the light pulse (Fig. 19) both indicate that the phase of the first rhythm peak maintained a fixed relationship to the timing of the light pulse and hence to the timing of the light-on and the light-off signal. The period of the rephased rhythm was close to 30 h just as in the control, peaks occurring on the average at $13\frac{1}{2}$ and 46 h after the light-off signal. Thus, irrespective of the phasing of the rhythm when the 12-h light interruption was given, there was the same amount of rephasing, so that the next peak of the rhythm of flowering occurred at about 12-15 h after plants were returned to darkness. Essentially the same results were obtained when an 18-h instead of a 12-h red light pulse was used. It is noteworthy that the timing of the rhythm was identical whether low intensity red light, or fluorescent light of 600 ft cd, was given as the 12-h of 18-h light pulses. Thus photosynthetic differences did not affect the phasing response.

Phase response to a 6-h light interruption. Single 6-h red irradiations were imposed

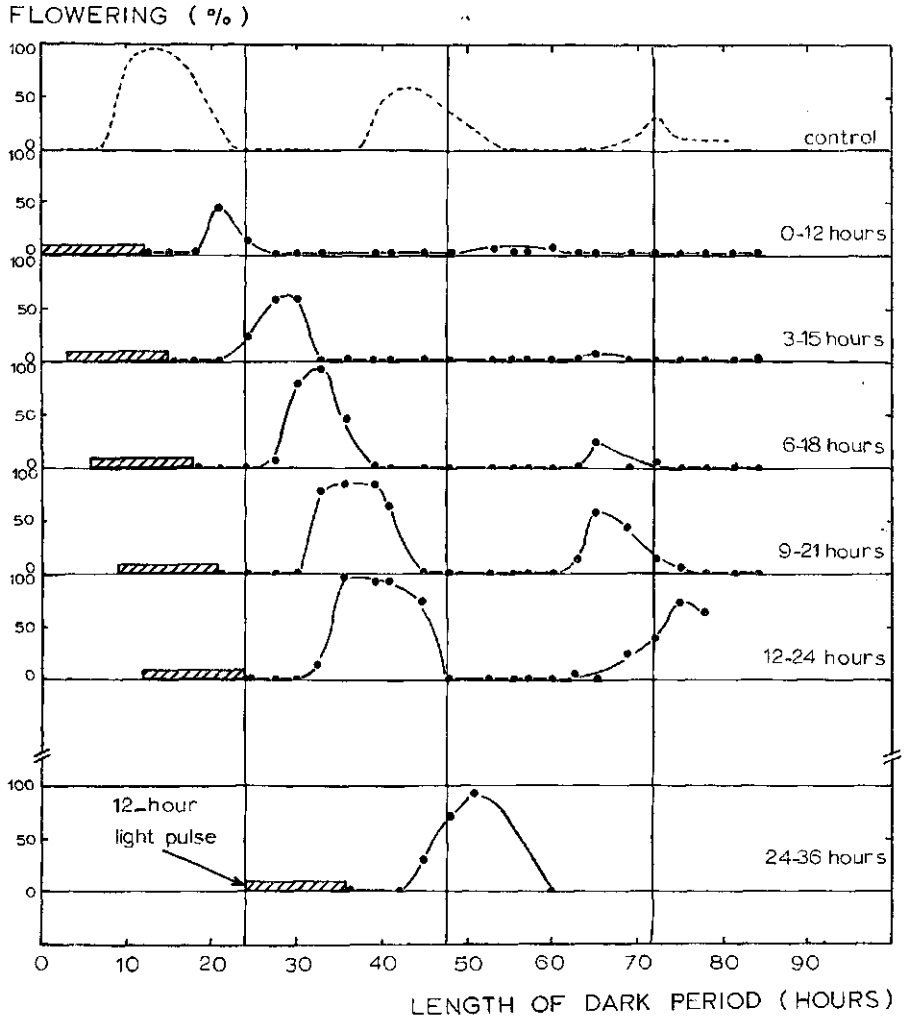

Fig. 18. Flowering response of *Chenopodium rubrum* 60°47'N, to a single dark period of varied duration interrupted by a 12-h period of red light (115 $\mu w.cm^{-2}$) that was given at different times in darkness. Continuous 600 ft cd CW fluorescent light before and after single dark period. Control: no red light interruption. (After King, 1971.)

at different times in darkness. The raw data (Fig. 20) and the derived relationship between the rhythm peak times and the timing of the start of the light pulse (Fig. 21), indicate that there was not just the simple effect on rhythm phasing that was obtained after 12- and 18-h light pulses. By comparing the rhythm of flowering in the control plants lacking a 6-h interruption, with those subjected to a 6-h pulse, it can be seen that the rhythm phase shifting was greatest when light impinged on the positive slope of

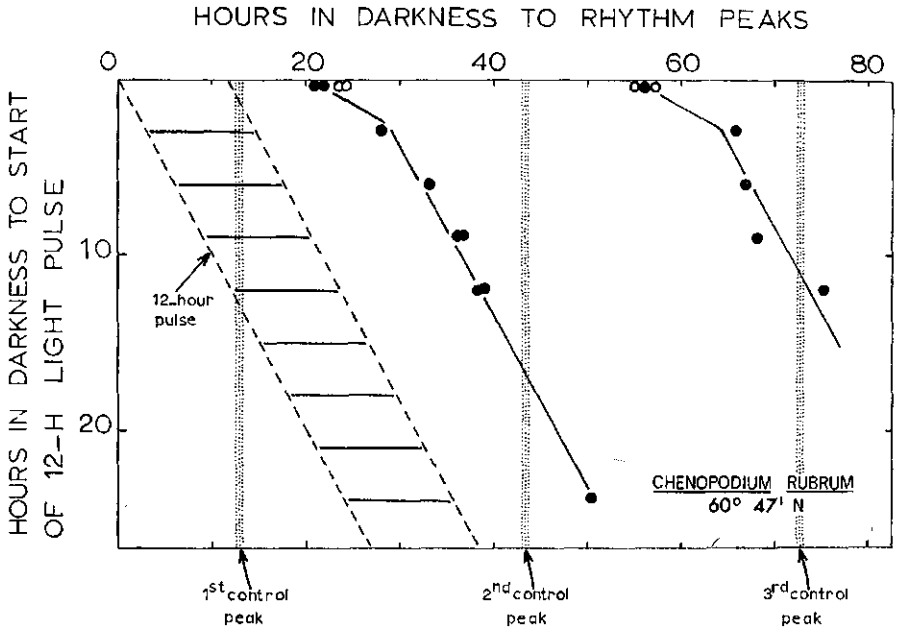

Fig. 19. *Chenopodium rubrum*, 60°47'N. The relationship between the time in the dark period when a 12-h exposure was given and the time of maximum flowering at the first or second peaks of the rhythm of flowering. The dark period was varied to reveal rhythmic response after 12-h break. Experimental details given in Fig. 18. (Modified from King, 1971).

either the first or the second peak of the free-running rhythm (Fig. 20 and 21). If the light came later than the control peak e.g. between 12 and 24 h in darkness, there was little influence on the rhythm phase. Essentially the same phasing effects were obtained when white fluorescent light was used instead of red light. The calculation of the rephasing of the rhythm by the 6-h light pulses relative to the control peaks (Fig. 21) was assessed by dividing the dark period into 30-h intervals (i.e. 0-30, 30–60, 60–90), and then the phasing of the peaks after each light interruption was related to the peak within each successive 30-h interval.

The foregoing conceptions and observations of phase shifting are incorporated into the composite representation shown in Fig. 22. The flowering curve and the phase response curve for phasing of the control rhythm by a 6-h light pulse are based on the data of King (1971). The phase response curve is only included for the first 30 h (one circadian oscillation) in darkness. The results of King (1971) indicate that the extension of the response curve beyond the 30th hour of darkness provides a repetition of the first 30-h phase response curve – as would be expected on theoretical grounds. Since a light period from 0 to 6 hours in darkness provided only a light-off signal, the similar rephasing that resulted from a light period given from the 30th to 36th hour in darkness may have depended on the light-off signal alone. However, since there was no set phase

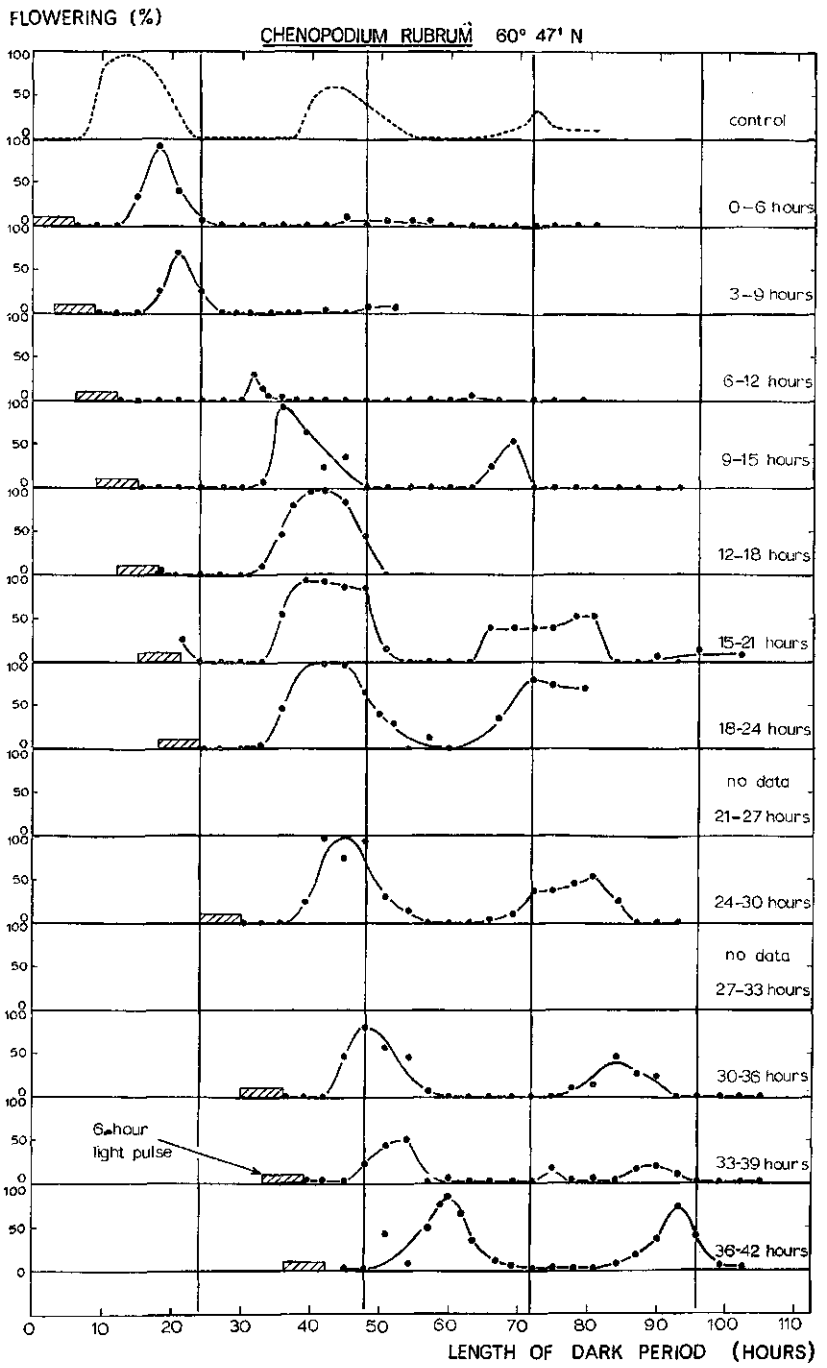
FLOWERING (%)
CHENOPODIUM RUBRUM 60° 47' N
control
0–6 hours
3–9 hours
6–12 hours
9–15 hours
12–18 hours
15–21 hours
18-24 hours
no data
21–27 hours
24–30 hours
no data
27–33 hours
30-36 hours
6-hour
light pulse
33-39 hours
36-42 hours
0 10 20 30 40 50 60 70 80 90 100 110
LENGTH OF DARK PERIOD (HOURS)

Fig. 20. Flowering response of *Chenopodium rubrum*, 60°47'N, to a single dark period of varied duration interrupted by a 6-h period of red light (115 $\mu w.cm^{-2}$) that was given at different times in darkness. Continuous 600 ft cd CW fluorescent light before and after single dark period. Control: no red light interruption. (After King, 1971.)

response (such as occurred with 12-h and 18-h light pulses), it would appear that the light-on signal of a 6-h light pulse had an effect on rhythm phasing. It is also clear that the length of the light period was being measured by the organism, since between the 6th and 12th hour of a light pulse there is a change in the influence of light on the rhythm so that its effect changes from phase dependence to one of independence – and there is then a fixed phase relationship to the light-off signal. King (1971) has suggested that the rhythm is suspended by light pulses that are 12 h or longer, and that this then allows the light-off signal to be decisive in setting the rhythm phase. I suggest that in some respects Brown's (1964) concept of autophasing may provide a model for the phase independence that appears to operate after a prolonged light period. Questions regarding uncoupling of a basic oscillator from a direct control over induction may also be pertinent here.

Leaving aside the inconclusiveness that exists over some of the foregoing basic questions, it is clear that the differences in phase response shown in the flowering of *C. rubrum* by King (1971) do provide a means of predicting the relationships between phase advances and delays that will result under different daily photoperiodic condi-

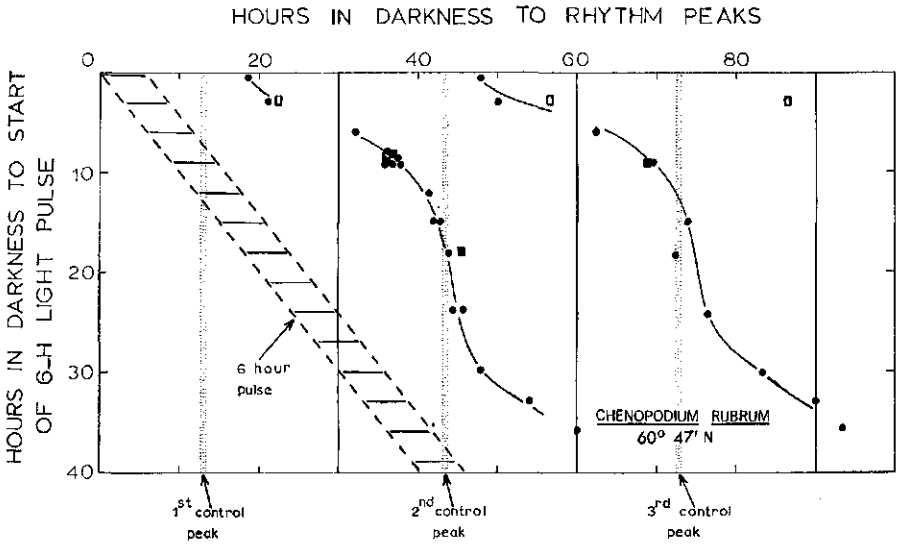

Fig. 21. *Chenopodium rubrum*, 60°47'N. The relationship between the time in the dark period when a 6-h light exposure was given and the time of maximum flowering at the first or second peaks of the rhythm of flowering. The dark period was varied to reveal the rhythmic response after 6-h light break. Experimental details given in Fig. 20. (Modified from King, 1971.)

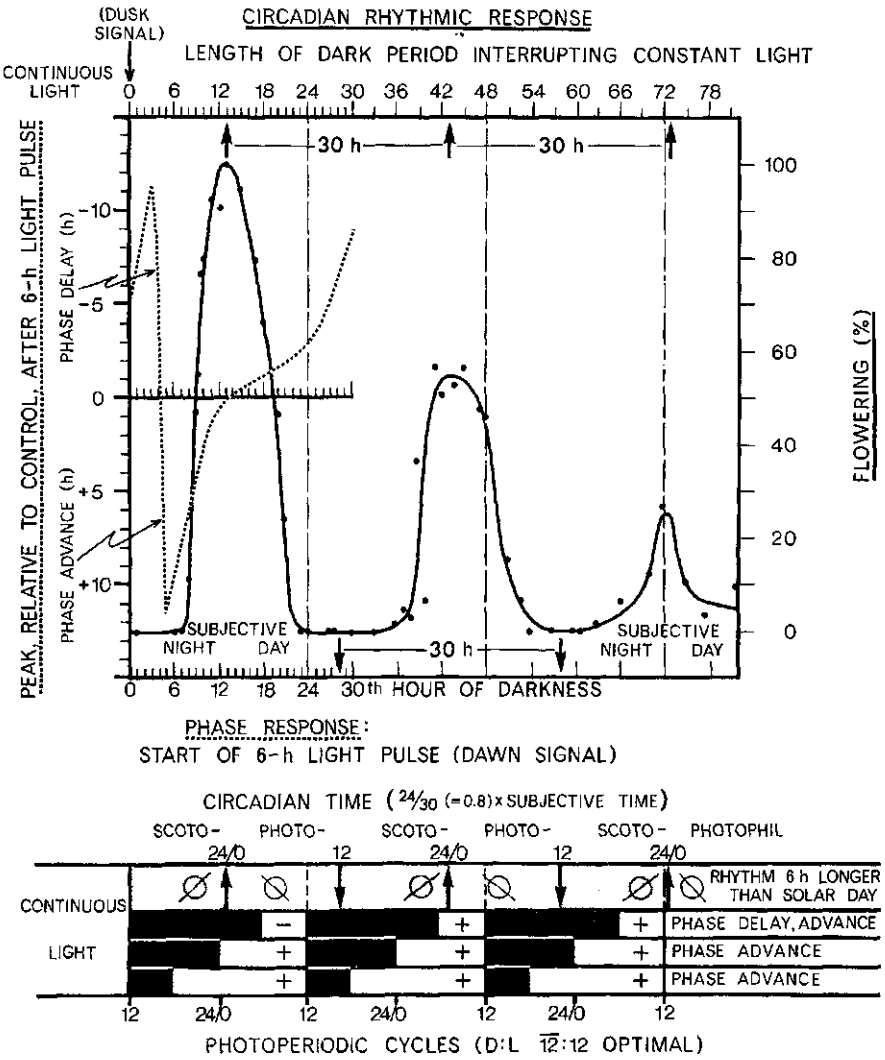

Fig. 22. Summary of role of circadian rhythmicity in photoperiodic induction in plants, using flowering of *Chenopodium rubrum*, 69°47'N, as model. Phase response and rhythmic flowering response curves based on data of King (1971). Non-graphical representation of cardinal points of sinusoidal curve based on Bünsow (1960): maximum (↑), inflection point with negative slope ⊘, minimum (↓) and inflection point with positive slope ⌀. Note that conception of scotophil and photophil phases does not follow that of Bünsow (1960).

tions. Furthermore, these results indicate that the free-running circadian rhythm can provide a timing mechanism in the photoperiodic control of flowering. It is relevant that the changes in phasing in the leaf movements of *Coleus blumei* × *C. frederici* in response to a 4-h light pulse (Halaban, 1968a) provide a phase response curve that is very similar to the *C. rubrum* phase response to a 6-h light pulse (Fig. 22). I also refer you to Sweeney's comments in this symposium regarding the similarity between the phase response of *Gonyaulax polyedra* and *Coleus blumei* × *C. frederici* (Halaban, 1968a). The early work of Zimmer (1962) on the phase response of petal movements in *Kalanchoë blossfeldiana* also shows a similarity of response in another higher plant process.

The phase shifting response can also be based on a 'subjective circadian time scale' (lower section Fig. 22; cf. Halaban, 1968a). Thus, in *C. rubrum*, 60°47'N, the subjective circadian period would be 24/30 = 0.8 of a 30-h period. This is of some interpretive value when it is realized that the free-running circadian period may be entrained to 24 hours in daily photoperiodic cycles. By the same token, the circadian rhythmic response and the phase response curve (upper section Fig. 22) could be based on a subjective circadian time scale (cf. Halaban, 1968a). It should also be noted that further simplicity can be obtained by disregarding the amplitude and reducing the sinusoidal circadian curve to its four cardinal points – as perceptively indicated by Bünsow (1960) for the rhythmic petal movements and flowering response of *Kalanchoë blossfeldiana*. Then, the maximum (↑), the inflection point with negative slope (⊘), the minimum (↓), and the inflection point with positive slope (⊘), can be indicated simply (Fig. 22).

Using Fig. 22 as our basis for predicting the role of circadian rhythmicity in photoperiodic induction, the following illustrative examples may suffice.

With transition from light to darkness, the phase of the circadian rhythm is set so that there are alternating scoto- and photophil phases (subjective night and day, respectively). While there is no flower induction (first minimum) if the dark period is less than 7-8 ('critical night length') optimal flowering (first maximum) is obtained when a single dark period of about 13 h interrupts continuous light.

An important feature to note is that a 6-h light pulse has the most effect in shifting the phase of the rhythm if it is started at some time within the first 8 h (critical night length) of darkness; during this time there is a change from a phase delay to a phase advance. The 6-h light pulse has least or no effect in phase shifting when started at the peak of the rhythm, i.e. at the time when the light-on signal is most stimulatory to flowering. A 6-h light pulse introduced at a later time than the maximum of the oscillation results in a progressively greater phase delay, and it is from this time onwards that a light-on signal results in progessively less flowering. This change in responsiveness is apparently repeated during the next approximately 30-h (circadian) oscillation (King, 1971).

From the foregoing results and conceptions, we can predict the probable effects of different photoperiodic cycles and thereby indicate the possible role of circadian rhythmicity in the photoperiodic induction of flowering. This has been discussed in detail by King (1971). I will confine my discussion briefly to the 6-, 12-, and 18-h daily photoperiodic cycles referred to in Fig. 22, with the daily dark period indicated according to conventional usage as starting at hour 12 on a solar time scale.

In the 6-h daily photoperiodic cycle, with the light-on signal starting at hour 18 of the first 24-h period of the free-running circadian rhythm (and at hour 42, 66 and 90, on succeeding days), the dark period is longer than optimal. The light-on signal starts at a time corresponding to the mid-point of the negative slope of the photophil phase of the endogenous rhythm and is less stimulatory to flowering than if given earlier in darkness. Calculating from the phase response curve (Fig. 22) the endogenous rhythm is delayed in phase by 1 h; therefore, the next peak would be at the 44th hour as compared to the 43rd for the control rhythm (i.e. at hour 20 on the next solar day). In the next 24-h photoperiodic cycle (hour 24 to 48), the 6-h photoperiod starts at hour 42, which is 2 h earlier than the peak of the delayed circadian rhythm peak. Calculating from the phase response curve (Fig. 22), there would be a phase advance of between 1 and 2 h; therefore the next rhythm peak would be at about 71-72 hours. In the next 24-h photoperiodic cycle (hour 48-72), the 6-h photoperiod starts at hour 66, which is 5 to 6 hours earlier than the peak of the rhythm; the phase response curve indicates that a phase advance of about 5 h should result.

It can thus be realized that the phase shifting capacities of the rhythmic system provide a means whereby the circadian free-running rhythm can be rephased and entrained by a daily photoperiodic cycle, and, because of this property, the oscillatory time-measuring ability of the system involving alternating changes in sensitivity to light can provide a mechanism for the quantitative control of flower induction as a function of the prevailing daylength. Observations of King (1971), using different numbers of photoperiodic cycles (before allowing the rhythm controlling flower to run free in a single dark period of varied length, as in experiments of Fig. 18–21), provide experimental evidence to support the main theoretical conceptions and calculations of the role of the circadian rhythm in the induction of flowering by varied photoperiodic cycles. There results also provide substantial evidence to support Bünning's hypothesis of the physiological clock and its dual action in photoperiodic time measurement, as has been elaborated by Pittendrigh and Minis (1964), Pittendrigh (1966), and others.

Referring now to the action of the 12-h and 18-h photoperiodic cycles on flowering on *C. rubrum*, these require less elaboration (Fig. 22). The light-off signal is dominant in setting the phase of the rhythm, so that the maximum would occur 12 to 15 h after the light-off signal, that is, the peak after both photoperiods would be advanced to hour 37–39 (24 + 13–15 hours) as compared to hour 43 for the control peak. The differences in the length of the dark period imposed by the two photoperiodic cycles would lead to quantitative differences in flowering, because, while the 12-h dark period would be more or less optimal for flowering, the 6-h dark period of the 18-h photoperiodic cycle would be less than the critical length. Thus, although plants of *C. rubrum* -60°47′N flower eventually in 18-h photoperiodic cycles, they do so much later than in 12-h daily photoperiodic cycles (Fig. 13).

Phase response in prolonged darkness and in prolonged light

The effects of a short light treatment on the phasing of a rhythm in the output of carbon dioxide persisting in darkness, as well as the effects of a short dark treatment

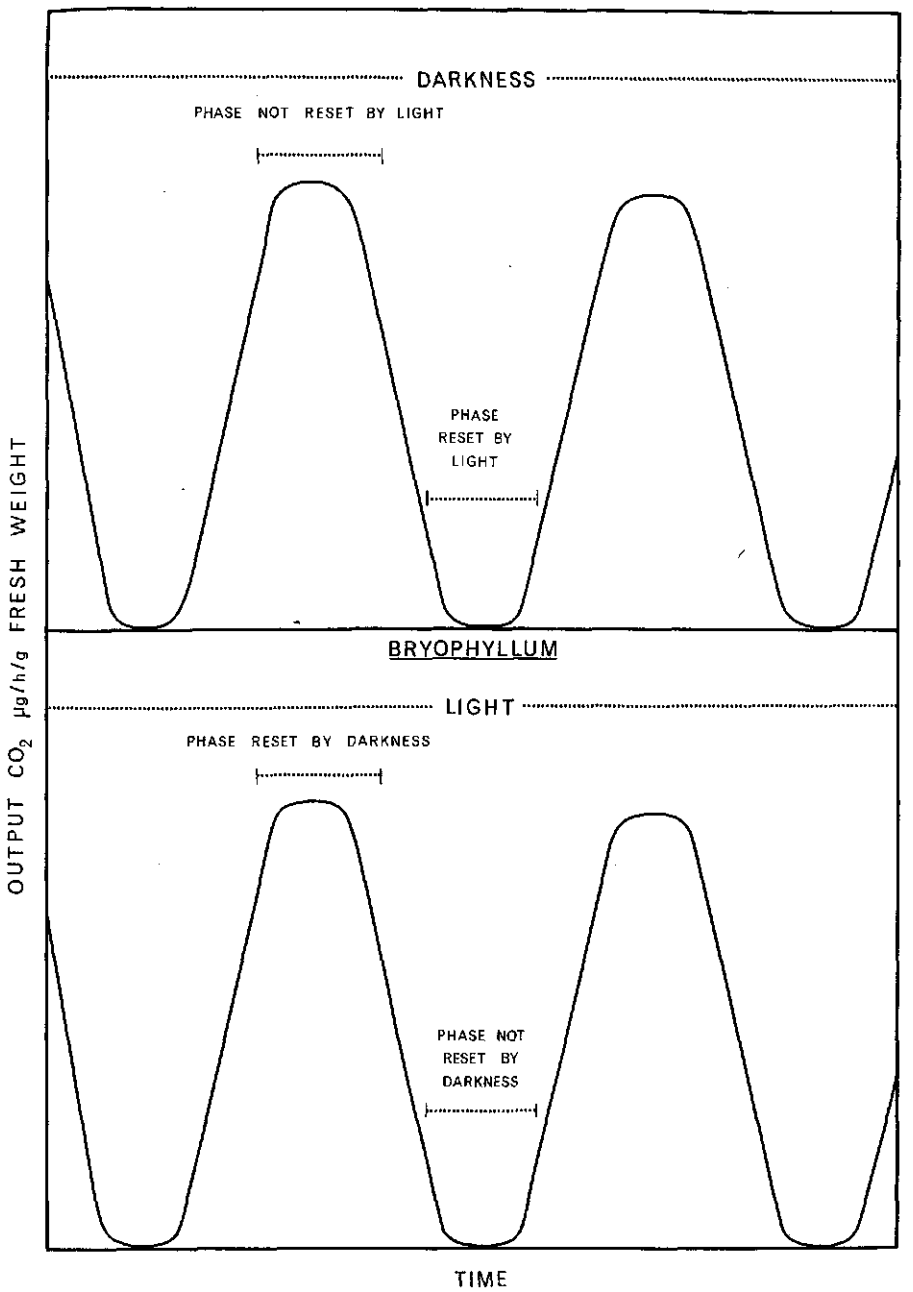

Fig. 23. *Bryophyllum fedtschenkoi.* Diagrammatic representation of the effects of short light treatments (100 lux) on the phase of a rhythm in output of CO_2 (µg/h/g fresh wt.) proceeding in darkness, with those of short dark treatments (3 or 6 h) on the phase of a rhythm persisting in illuminated leaves. (After Wilkins, 1960.)

on the phasing of a rhythm of carbon dioxide ouptut persisting in the illuminated leaves of *Bryophyllum fedtschenkoi*, have been studied extensively by Wilkins and were summarized by him in 1960 (Fig. 23). When darkness was interrupted by a short light treatment af different times in relation to a control endogenous rhythm in CO_2 output, it was found that the phase was not reset by light given at the peak of the rhythm, but the phase was reset by light given at the minimum of the rhythm in CO_2 output. This response therefore shows similarities to the phase sensitivity of other responses to the interruption of light by darkness. Conversely, when the CO_2 output of leaves was assayed in prolonged darkness, the sensitivity of the rhythm to a short light treatment varied in the opposite way to that of the rhythm in light, since the phase was reset when darkness was interrupted at the peak of the rhythm. The basis for these differences still remains obscure and it should be noted that gaseous exchange in this succulent plant is complicated by the occurrence of dark fixation of CO_2 (crassulacean acid metabolism). Nevertheless, these results do provide an example of a change in sensitivity that operates on a circadian rhythmic basis both with respect to light in darkness and to darkness in light.

Is the circadian oscillator necessary for photoperiodic induction?

Recently, Bollig and Engelmann (1971), in considering that the dual action of light in photoperiodic systems (the inductive action on the whole, and the phase regulating action on the oscillator) is a basic tenet of Bünning's hypothesis, have suggested that it may be possible to critically test this hypothesis by abolishing the circadian oscillation (or the clock postulated to measure time in photoperiodism) and then observing whether photoperiodic induction occurs. The method proposed by these workers for stopping the underlying oscillation is modified from that used by Winfree (1970) to obliterate the circadian rhythm of eclosion in *Drosophila pseudoobscura*. Winfree (1970) accomplished this by exposing the fruit flies to light of a certain strength, S* (intensity and duration), administered at a specific critical phase (T*); it was postulated that the system entered a state of singularity which was more or less phaseless – as shown by the random emergence of the flies.

The experimental methods so far used by Bollig and Engelmann with *Pharbitis nil* have been essentially those of Takimoto and Hamner (1964, 1965). The latter authors found that flowering of *P. nil* is rhythmic if scanned by 5-minute light interruption(s) over an extended dark period (e.g. 48 h); the circadian rhythm persisted even if plants were preconditioned with an exposure to an intervening light interruption of 5 min to 12 h, administered 8 h after the onset of darkness – with the exception that a light interruption of 2 hours gave no rhythmic response during the subsequent darkness. Bollig and Engelmann have explained these results on the basis of the Winfree model and suggested that the *P. nil* rhythm reaches a critical phase (T*) 8 h after the onset of darkness, from which it can be put into a singular state with a light pulse of appropriate strength (S*) – which is 2 hours (400 ft cd) under the conditions of Takimoto and Hamner's (1964) experiments.

Bollig and Engelmann have repeated the foregoing experiments with the modification

that the whole dark cycle including the intervening light period was either 58 h or 72 h (Fig. 24). Five-minute red light interruptions of 2300 erg.cm^{-2}.sec^{-1} scanned the whole dark period (after the first varied light pulse at the 8th h of darkness), at 3-h intervals. The results (Fig. 24) showed that the rhythm in flower induction was abolished by an intervening light period of 2 h, but not 4 h or 30 min. Other experiments of Bollig and Engelmann have suggested that S* is 45 to 60 min and T* is 7 to 9 hours after the first light-off signal. An indication that *P. nil* was in a singular state was shown by the fact that a new light perturbation given to the presumed arythmic system reinstated a rhythm that was independent of the time when the new perturbation was given. Bollig and Engelmann suggest that the new perturbation given to the phaseless system may bring it back to the limit cycle, as predicted in Winfree's model. Although these data suggest that the basic rhythm in sensitivity to light is essential for photoperiodic induction, Bollig and Engelmann have stressed (personal communication)

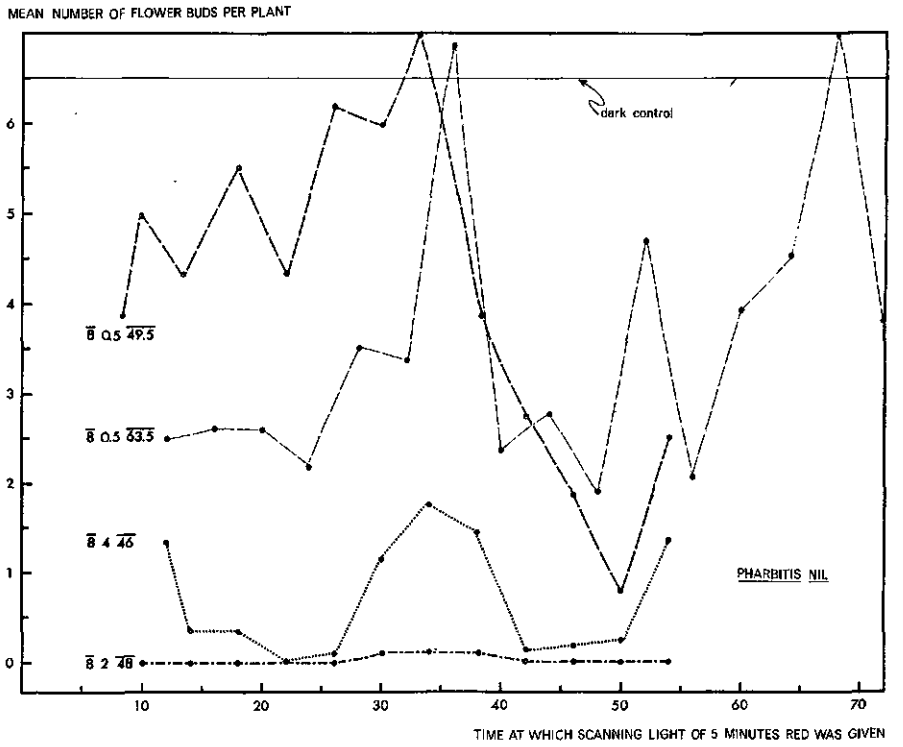

Fig. 24. Flower induction of *Pharbitis nil* after a 58-h dark period was interrupted 8 hours from start of darkness with either 0.5, 2 or 4 h of white light, and, after a 72-h dark period was interrupted with 0.5 h of white light at same time. During the rest of the 85h or 72 h dark period, the sensitivity towards a single 5-min red light pulse (2300 erg/cm^2/sec) was scanned at various times in darkness. 'Dark control' refers to flower induction in response to 72-h treatment (8 0.5 63.5) lacking a 5-min red light break. (Modified from Bollig and Engelmann, 1971.)

that they consider that their results should only be taken as preliminary until there is further corroboration or clarification of their basis. Thus, for example, it could be suggested that the system is still rhythmic in response to the cycle $\overline{8}$ 2 $\overline{48}$ (Fig. 24) and that the effect of abolishing flower induction is secondary; furthermore, the level of flowering in the cycle $\overline{8}$ 4 $\overline{48}$ is quite low. Nevertheless, these results suggest a further critical approach that is subject to experimental verification – particularly if some other rhythmic phenomenon can be found in the same organism that might provide an independent indication of the state of the postulated basic oscillator. A further question arises whether the postulated states of S* and T* could be related experimentally to changes in the phase response that are shown in the early hours of darkness in the rhythmic control of flowering of *Chenopodium rubrum* (Fig. 22).

Conclusion

This section could be called the 'C' section, since we must be circumspect in drawing conclusive conclusions concerning the diverse types of evidence available to us that do signify a role of circadian rhythmicity in photoperiodic induction in plants.

Our apparent context is that there are different rhythmic phenomena in multicellular plants that we call 'free-running' – with various frequencies and periods. Those rhythmic phenomena that we can, strictly speaking, call circadian, are relatively temperature independent, they can be rephased or entrained by light and (or) synchronized to the solar day.

One basic question is whether such phenomena are merely, in one sense, the overt expression, or 'hands' of a master 'clock' that acts as a basic time-measuring system in the daily photoperiodic induction or control of such different phenomena. Nevertheless, such hands of the clock may be of primary significance to the organism. This is shown, for example, in the photoperiodic control of flowering, in both the qualitative and quantitative characteristics of this phenomenon and its adaptive significance.

If we ask what types of evidence are available to us that signify or indicate a role of circadian rhythmicity in photoperiodic induction or control, we can roughly classify the evidence under the following headings, although I would not suggest that they are mutually exclusive: 'coincidence', 'correlation', 'coupling', 'changing sensitivity', and the 'clock mechanism and its hands'. A few examples are given under each heading.

– *Coincidence* of phasing and (or) sensitivity to light of different rhythmic phenomena, for example, in leaf movements (the hands of the clock?) and in flowering response (Fig. 5). But generalizations are not possible here, and Denney and Salisbury (1970) have suggested that in *Xanthium strumarium* there are 'separate clocks for leaf movements and photoperiodic flowering'. The evidence from *Chenopodium rubrum* (e.g. Fig. 10, 11, 12, and Table 1) also indicates that there can be a multiplicity of rhythmic phenomena with different periodicities and phase relationships, and these results also raise difficult questions regarding their possible origin from, or expression of, a basic oscillatory system.

– *Correlation*, for example, between the circadian rhythmic response and the photoperiodic response curve for the induction of flowering in specific genotypes (e.g. Fig.

13, 14, 15); or, between the phase response curves of various phenomena in different species (e.g. Fig. 22 and related discussion).

– *Coupling*, of different properties or 'parts' of the 'clock mechanism'. We can refer here to the working hypothesis of Bollig and Engelmann which suggests that there may be lack of photoperiodic induction (of flowering) if the basic oscillator is abolished or put into a phaseless state (Fig. 24); or we can refer to the specific phase relations that have been shown between leaf movements and the inductive phase for flowering (Halaban, 1968b).

– *Changing sensitivity*, as shown by the differences in response to changes in skeleton photoperiods (Hillman, 1964 and 1970), and the changing sensitivity to interruption of darkness by light (Fig. 13 and 22) and of light by darkness (Fig. 23). Whether transitions between light and darkness (in either direction) will, or can, act as dawn or dusk signals in rephasing, inhibiting or promoting a rhythmic process, is also relevant here.

– *The clock mechanism and its hands.* The evidence is sufficient to indicate that the various properties of the clock mechanism and its hands, that have been shown in specific organisms, lead us to conclude that there is indeed a positive role of circadian rhythmicity in photoperiodic induction in plants. Nevertheless, we may also conclude that various other options or alternatives are available to organisms whereby the induction or control of specific phenomena by circadian rhythmicity and (or) by photoperiod may be by-passed or eliminated. Even in these instances, however, there may be related controls. For example in the tomato plant, *Lycopersicon esculentum*, which has been classified as photoperiodically day-neutral (for flowering), there are profound effects of thermoperiod on flowering and growth, and, furthermore, photoperiod can markedly influence the general metabolism and growth of the plant (Wittwer and Aung, 1969).

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Discussion

Participants: B. G. Cumming (Author), E. Bünning, W. Engelmann, K. Hoffmann, P. J. A. L. de Lint, E. C. Wassink and M. B. Sweeney

B. M. SWEENEY (USA) stressed that it is important to distinguish between light breaks that can reset rhythms and light breaks that are too weak or short to do so, although these may have effects on photoperiodic responses. She pointed out that much confusion has resulted from the failure to make this distinction. A question related to this was asked by W. ENGELMANN (Germany) who wished to be informed of the effect on flowering of light pulses either shorter or less intense than the 6-hour pulses used by King (unpublished results).

B. G. CUMMING (Canada) referred to the responses of *Chenopodium rubrum* ecotype 60°47'N and *Lemna perpusilla* 6746 which are informative on these points. When a prolonged dark period is interrupted once by a brief red irradiation of just a few minutes, flower induction of *C. rubrum* 60°47'N is stimulated (above the dark control level) when red light is interpolated in the photophil phase, but inhibited when it is interpolated in the skotophil phase. Thus, there is a pronounced photoperiodic effect on flower induction but the phase of the rhythm is not shifted by such short light breaks (Cumming et al., 1965). King (1971) has shown in the same ecotype that rhythm phase shifting is only obtained with a light pulse of about 4 to 6 hours. Similarly, Hillman (1964, 1969) has found that, in *Lemna perpusilla* 6746, from 4 to 6 hours of light are required to reset the rhythmic flowering response. King has shown that low intensity red irradiation of 115 $\mu W/cm^2$ and higher intensity white fluorescent light of 1037 $\mu W/cm^2$ (600 ft cd) have essentially the same phase shifting effect on the rhythmic flowering response of *C. rubrum* 60°47'N. King (1971) has found that even intermittent red light pulses of 5 minutes evenly interspersed as a 6-hour skeleton light period, can be sufficient to reset the rhythmic flowering response of *Chenopodium rubrum* 60°47'N. These results suggest that the photoreversible pigment phytochrome is involved in the resetting response, since the possibility of substituting short exposures for one long one is characteristic of that system. King (1971) has also found that the phase shifting effect of such skeleton red light treatments can be partly but not completely reversed

by far-red irradiation – again implicating phytochrome action in the phasing response.

Related to the foregoing discussion, E. C. WASSINK (the Netherlands) emphasized that, in discussing light pulses, etc., it is important to consider the spectral composition of the light used, as well as the spectral quality, sequence, and duration of the light periods previously given.

P. J. A. L. DE LINT (the Netherlands) asked whether it is possible to reduce the light intensity sufficiently slowly that the rhythm (normally started or reset by the dusk signal) would not start again. If this could be accomplished in a short enough time then such a procedure could be applied in photoperiodic treatments of 'non-cyclic' plants. BÜNNING (Germany) was asked whether he could cite any work that has considered this question. Although agreeing that the question raised interesting possibilities, Bünning was not aware of any relevant experiments that had been conducted until now.

This question could be investigated in the flowering of a plant such as *Chenopodium rubrum*, that responds inductively to a single dark period interrupting continuous light, and which shows a circadian rhythm in flowering response when the duration of darkness is varied or a prolonged inductive dark period is interrupted by a brief light pulse. If there is no rhythmic induction of flowering when the light intensity is gradually reduced, then the response in daily photoperiodic cycles could be tested using the same gradual change-over from light to darkness. Then, if there is no induction, one could infer that the circadian rhythm is involved and/or required for photoperiodic induction, whereas, if there is induction, the opposite inference could be drawn.

K. HOFFMANN (Germany) noted the difficulty of reconciling Brown's postulation that all circadian rhythms with periods differing from 24 hours are based on a 24-hour (extrinsic) input. Although, theoretically, such a system could be constructed or envisaged, it would be highly complicated and, from an evolutionary standpoint, it would be most implausible. CUMMING pointed out that we should perhaps remember that as recently as the 1950's many scientists were disputing the possibility of the existance of endogenous rhythms because there was no obvious mechanistic basis for such rhythms, and because the basic conception seemed to be too 'mystical' and therefore unscientific. While some features of Brown's theory, such as the concept of autophasing, may be difficult to reconcile with our present knowledge of circadian rhythms, we should not close our minds to the possibility that some such mechanism(s) may have evolved in certain organisms that are adapted to particular environments in which the photoperiodic factor has been less decisive. An interesting example of such a mechanism, involving the interaction of a circadian oscillation with an extrinsic (lunar-day) cycle has been found in the vertical migration rhythms of the photosynthetic diatom *Hantzschia virgata* by Palmer and Round (1967). These workers have postulated an interacting dual-clock system for this organism. A clock that keeps in time with the lunar day and measures periods of 24.8 hours (summation of 12.4-hour lunar cycles); superimposed is a solar day (circadian) clock that is responsible for the suppression of the night-time suprasurface phase of the lunar-cycle migration rhythm. This suppression is evident from the fact that cells never appear on the surface (of the sand-water environment) during the time equivalent to the night phase. Even in light:

dark cycles in the laboratory, a lunar-day rhythm still persisted in approximate synchrony with the time of daytime low tide, since vertical migration was about 50 minutes later each day. Also, the same 50 minutes per day change in phase, and late afternoon rephasing to early morning, occurred even in constant light, so that there was never more than one emergence to the surface per 24.8 hours. Cumming and Wagner (1968) suggested that this organism may have acquired (through adaptation to its peculiar environment) special sensitivities to gravitational forces of the moon and, to a lesser extent, of the sun, and to correlated periodic variations in atmospheric pressure related primarily to the solar day but also to the lunar day. Such possibilities are subject to experimental investigation and are an implicit part of Brown's (1965) hypothesis.

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The role of circadian rhythmicity in photoperiodic induction in animals

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Abstract

Circadian rhythms are often regarded as playing a fundamental role in animal photoperiodism. Among the numerous models suggested for the clock, the most favoured assumes that time measurement depends on the coincidence of light with a covert circadian oscillation which is either phase-fixed to one part of the cycle or is entrained by the whole of that cycle. The supporting experimental evidence is perhaps less impressive than the model. Oscillator concepts have been applied with varying success to the regulation of testicular growth in birds, and to the photoperiodic control of diapause and polymorphism in insects. But the evidence is conflicting and sometimes entirely negative. The alternative hypothesis is that the clock is an hour-glass which measures time by means of a sequence of biochemical reactions, involving both the light and dark components of the cycle. The hour-glass is 'turned over' by light; and an additional mechanism exists for accumulating the products of effective cycles.

It seems highly improbable that photoperiodic clocks will be found to be basically alike in all animals or even in all insects. It is believed, however, that current thinking may have placed too much emphasis on oscillator mechanisms and not enough on mechanisms working on the hour-glass principle.

All responses to seasonal changes in day length involve a number of steps – photochemical, hormonal etc. – which serve to detect the environmental signals and to transmit their message to the target organs. The biological clock which each day monitors the cycle of light and dark and 'compares' the result with an innate 'standard' doubtless comes at the very beginning of this chain of command.

Most of the available information on the physiology of the photoperiodic clock in animals has come from studies on the control of diapause and polymorphism in insects and on the regulation of testis growth in birds. Insects, with their short generation time and ability to thrive in highly abnormal light cycles, including permanent darkness, have proved to be amenable subjects for the type of indirect experimental procedures which provide the usual means of approaching this problem. Unfortunately, little progress has been made in studying the clock directly. There are as yet no instances in

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which the site of the clock in the central nervous system is known with any precision, and the light-absorbing pigment receptors remain unidentified. In addition, the nature of the clock itself is in dispute. Although theories and models abound, they are all based on the assumption that time is either measured by an hour-glass system or by a system of rhythmical change – in other words, an oscillator.

For many years the hour-glass analogue was regarded as entirely consistent with the data then available. According to this view, the passage of time is measured by biochemical reactions which proceed to completion during a 24-hour cycle of light and dark. This non-cyclical act of measurement is repeated as long as other exogenous light/dark cycles follow. The effects are cumulative. But measurement ceases immediately the organism is placed in constant darkness. In insects, there was much evidence to suggest that the process of time measurement could be associated with one particular component of the cycle, namely the dark period. It was shown, for example, that in many species diapause induction requires an uninterrupted dark period of more than critical length. Moreover, the photoperiod seemed less important, since its length could be greatly increased without affecting the 'inductive' capacity of the long night component. Thus Tanaka (1950, 1951) showed that nearly all the pupae of the oak silkworm *Antheraea pernyi* entered diapause when the larvae were reared in cycles containing an 11-hour dark period and a complementary 59-hour photoperiod. Since continuous illumination was known to inhibit diapause, a dark period of more than critical length was seen as promoting diapause strongly.

The significance of the dark period in time measurement was also proved in the moth *Acronycta rumicis* (Danilevsky and Glinyanaya, 1949), in the red spider mite *Panonychus ulmi* (Lees, 1953), in the corn borer *Ostrinia nubilalis* (Beck, 1962), to mention only a few instances. Nevertheless, it also became clear that the dark response is not an entirely autonomous function. In *Antheraea*, for example, the inductive effect of a long night is considerably weakened if the duration of the daily photoperiod falls below 6 hours, either in a 24-hour or 48-hour cycle (Tanaka, 1950). And there is a similar minimal light requirement in the oriental fruit moth *Laspeyresia molesta* (Dickson, 1949) and other species. To accomomdate these facts it is necessary to assume either that both dark and light phases are being measured by separate hour-glasses or that there is only one hour-glass, and that the complementary (light) phase is concerned in 'turning over' the hour-glass and preparing it for a single act of time measurement. Results with the aphid *Megoura viciae*, which I shall consider later, fit the latter hypothesis rather exactly.

After 1960, with circadian rhythmicity attracting increased attention, it is hardly surprising that models were soon proposed in which oscillator clocks occupied a central position. Bünning had of course, introduced his hypothesis many years previously in the context of plant photoperiodism. But in 1960 the model was applied to the photoperiodic control of diapause in the cabbage white butterfly *Pieris brassicae* by Bünning and Joerrens. And soon afterwards Pittendrigh and Minis (1964) developed a version of this model based on entrainment phenomena. The idea that circadian rhythms are intimately involved in animal photoperiodism has since gained many adherents in spite of the fragmentary nature of the evidence. Indeed, the two phenomena have sometimes

been identified to an extent where overt locomotor rhythms are cited as examples of 'photoperiodism' (Beck, 1968).

Before reviewing the evidence in greater detail it may be worth considering briefly the experimental procedures that are available for testing the models. Regrettably, they are few in number. Since photoperiodic responses do not show any overt rhythmicity, it is necessary to devise some indirect means of revealing the underlying oscillation, if such is present. Inevitably this involves the manipulation of the light/dark cycle:

(i) The duration of the light and dark periods are varied within a 24-hour cycle. As with all photoperiodic treatments the cycles are repeated many times over the time span when the animal is photosensitive. The resulting 'response curve' indicates that critical points occur, but does not reveal their significance. However, important species differences are found, requiring further elucidation (Fig. 1).

(ii) The cycle length is varied systematically together with either the light or the dark component. In a well-known series of experiments using the short-day plant Biloxi soybean, K. Hamner (1960) showed that after a constant 8-hour photoperiod, flowering was promoted with cycle lengths of 24, 48 and 72 hours but was inhibited with cycle lengths of 36 and 60 hours, even though the latter all contained extended dark periods. Similar response patterns, providing evidence for circadian rhythmicity, have not been found in animal species with the exception of the house finch *Carpodacus mexicanus* (W. M. Hamner, 1963) and the parasitic wasp *Nasonia vitripennis* (Saunders, 1969) (see below). In insects it is usual to find that moderately extended dark periods function as a 'long night' irrespective of their precise length, and still longer extensions are accepted as permanent darkness. Adjusting the photoperiod length while maintaining a constant night of more than critical length, can also provide valuable information. But in many insects the photoperiodic response is attenuated or fails completely if either the photoperiod or the dark period depart by more than a few hours from their natural range. This is very noticeable in *Laspeyresia molesta* (Dickson, 1949) (Fig. 1) and in *Ostrinia nubilalis* (Beck, 1962). In general, the response curves of these species are markedly bell-shaped, indicating that diapause-free development occurs with ultra-short photoperiods or permanent darkness. This experimental limitation is somewhat less evident with species such as the pink bollworm *Pectinophora gossypiella* (Adkisson, 1964) and *Nasonia vitripennis* (Saunders, 1969) and is almost absent in the aphid *Megoura viciae* (Lees, 1965) in which the 'inductive' effect of permanent darkness is only moderately less than that of a *LD 12*:12 short-day cycle. This trend is carried still further in the Colorado beetle *Leptinotarsa decemlineata* (de Wilde, 1965).

(iii) The use of 'night interruptions' or short light pulses, which are arranged so that they scan through the dark period, has provided a tool which has long been familiar to plant physiologists. Such a cycle contains two light components, the longer 'principal' photoperiod and the shorter 'interruption'. Tests of this kind usually show that 'induction' is reduced or abolished, but that this depends on the time the light signal is introduced during the dark period. If the interruptions are applied to a night of variable length in a 24-hour cycle, the peaks of light sensitivity may or may not shift their position relative to dusk. Although this is sometimes held to indicate whether the dark period is acting as an hour-glass, no firm conclusions can be reached as long as the

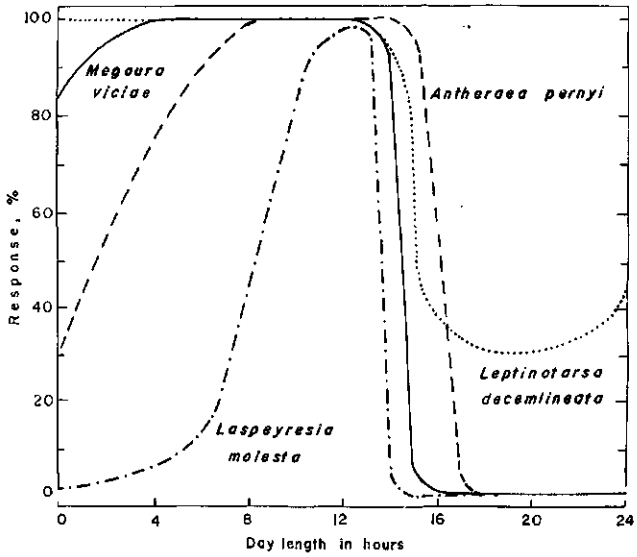

Fig. 1. Photoperiodic response curves in some insects. In *Laspeyresia, Antheraea,* and *Leptinotarsa* the curves show the incidence of diapause, in *Megoura* the percentage of parthenogenetic mothers producing oviparae. In the latter instance, the reciprocal measure – the incidence of virginopara producers – is a more appropriate index of the response.

cycle length is maintained constant.

Procedures in which the main photoperiod and the interrupted night are varied independently are more useful. With very long scanned scotophases one may hope to demonstrate the effects of the perturbation on a free-running rhythm of light sensitivity. A number of animal experiments have been based on the regimes devised by K.Hamner (1960). In Biloxi soybean, for example, pulses either stimulated or inhibited flowering according to the duration of the cycle and the point at which the pulses were positioned within the inhibitory and promotive phase of the (uninterrupted) dark period. In lengthy 2- or 3-day cycles these points tended to be separated by about 24 hours. On the whole, experiments with animals have been less successful in demonstrating a rhythmic pattern of light sensitivity although some positive instances have been recorded. The promotive effects of light breaks are much less well documented than inhibitory effects.

(iv) The temperature compensation observed in some insect photoperiodic reactions has sometimes been regarded as evidence in favour of an underlying circadian rhythm (e.g. in *Pieris brassicae* – Bünning and Joerrens, 1960). However, there are some species in which temperature compensation is much less impressive (Goryshin, 1955; Ankersmit, 1968). Alternatively, one could argue that temperature compensation may be just as desirable a feature in non-oscillator clocks.

Brief temperature treatments have been used in insects for investigating the role of light and dark period.

(v) If it is suspected that the photoperiod is measured by a circadian rhythm entrainable by the light cycle, a rather different approach may be adopted. Since the rhythm itself cannot be observed, it may well be advantageous to investigate the properties of some overt, assayable rhythm in the same species. If it is then assumed that both rhythms belong to the same circadian system and are both controlled by the same 'master clock', they may be expected to show similar determinate phase relationships to external signals. The object is then to phase-set the rhythm so that a short, 'inductive' light pulse may be made to coincide with different phase points in the oscillation. The photoperiodic effect would then be expected to vary accordingly.

Clearly, the procedures devised for demonstrating oscillator and hour-glass functions are often dissimilar. And it is one of the difficulties inherent in the subject that the type of experimentation chosen depends to some extent on the convictions of the investigator! A more comprehensive approach would often enable comparisons to be made more easily.

Oscillator models

According to Bünning's (1960) hypothesis, photoperiodic time measurement is accomplished by means of an endogenous 24-hour rhythm of light sensitivity (Bünning, 1960) (Fig. 2). The oscillation passes through alternating photophile and scotophile phases, each occupying a fixed period of 12 hours and is phase-set by dawn (or by the onset of the principal photoperiod if there is a subsidiary night interruption in the cycle). With regard to the control of diapause in *Pieris brassicae*, Bünning and Joerrens (1960) concluded that long days or interruptions in the early night inhibited diapause because light encroached on the scotophile section of the rhythm. On the other hand, light which is confined to the 12-hour photophile tended to promote diapause.

Although this model has received varying degrees of support among animal physiologists, it is unlikely that this formulation has a general validity. It is now known that in a number of insect species light sensitivity is confined to particular parts of the scotophile. Thus in *Pectinophora* (Adkisson, 1966), in *Megoura* (Lees, 1966), in *Nasonia* (Saunders, 1968) and even in *Pieris brassicae* (Goryshin and Tyshchenko, 1969), there

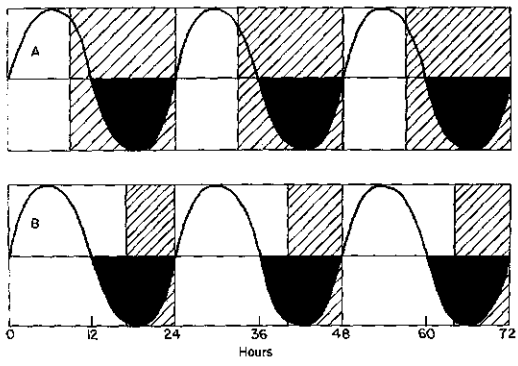

Fig. 2. Bünning's (1960) model showing the phase relationships of the hypothetical rhythm of light sensitivity under short (A) and long-day (B) conditions.

are two maxima of diapause inhibition, early and late in the night, separated by a period of complete or partial light insensitivity.

In *Megoura* it is possible to test Bünning's hypothesis more critically by greatly extending the photoperiod. In the experiment illustrated in Fig. 3, the 13.5-hour photoperiod has been lengthened to 25.5 hours, with the intention of taking the light cycle out of register with the phasing of any possible circadian rhythm. It will be seen that the response pattern to night interruptions remains virtually unchanged. Evidently time measurement in *Megoura* is more closely related to the onset of darkness.

Adkisson's (1964, 1966) observations had shown that *Pectinophora* differed from *Megoura* since the position of the two maxima of diapause inhibition, as revealed by night interruption experiments, was greatly influenced by the length of the daily photoperiod. As the night length increased, the first A peak tended to move later in the night while the second B peak remained constant relative to dusk. As a result of this fixed temporal relationship (with peak A located at ca. 14 hours after 'dawn' and the B peak 10 hours after 'dusk') the light sensitive points in the scotophile tended to move into the light as the dark period fell below critical length. Long day effects were then produced. It was not apparent, however, why the response pattern should be bimodal or why such a temporal relationship with the main photoperiod should exist. No definite evidence of circadian rhythmicity was found.

Pittendrigh's coincidence model, which he regards as one particular formulation of Bünning's general proposition, was based initially on Adkisson's results with *Pectinophora* and on his own data on the eclosion rhythm in *Drosophila pseudoobscura* (Pittendrigh and Minis, 1964; Minis, 1965; Pittendrigh, 1966). According to the theory, light has two functions: first, as an entraining agent controlling the phase of the

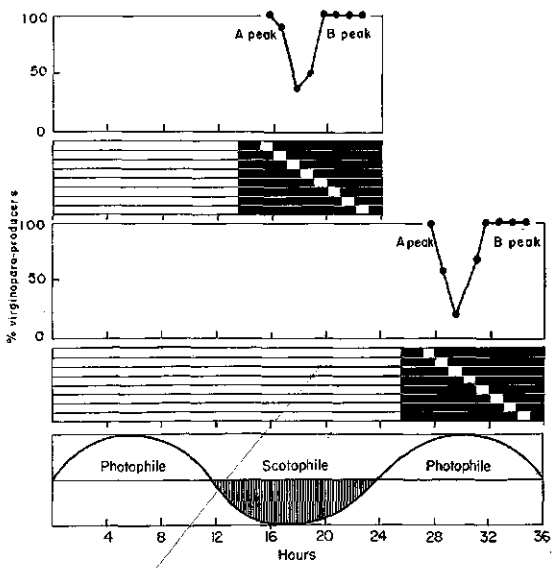

Fig. 3. Night interruption experiments in *Megoura viciae* as a test of the Bünning model.

circadian rhythm and secondly, as a photoperiodic inducer whose action may be confined to a small part of the 'scotophile'. The photoperiodic effect (e.g. diapause inhibition) will be produced when incident light coincides with the 'inducible' phase of the rhythm. This coincidence is also an integral part of the Bünning model. However, Pittendrigh has pointed out that there is no need to assume that this phase point will always occur at the same time in the scotophile since the *Drosophila* eclosion experiments have proved that the phase relations of a circadian oscillation are determined by the entire light cycle, which in the case of the *Pectinophora* experiment consisted of a longer principal photoperiod and a shorter night interruption.

When this type of regime was tested in *Drosophila* the eclosion rhythm entrained as if the two light components were joined. When the light pulse was placed soon after the principal photoperiod the latter was accepted as 'dawn' and the former as 'dusk'. But when the pulse was moved later in the night a phase jump occurred and the rhythm now entrained to a simulated photoperiod in which the light pulse functioned as 'dawn'. If this analogy is again pursued in *Pectinophora*, it is plausible to suppose that relatively slight differences in the position of the night interrruption could greatly influence the phasing of the rhythm. With interruptions in the early and late night this could mean that in the first instance the 'photoperiodically inducible[5] phase point (φ_i) of the rhythm is illuminated by the interruption itself, whereas with the later interruption this phase point could be coincident with the latter part of the principal photoperiod. (Fig. 4). According to this concept, therefore, the two light sensitivity maxima are associated with two different light functions – induction and entrainment.

Studies on the entraining capabilities of simulated ('skeleton') photoperiods were later extended to include the *Pectinophora* oviposition rhythm which in this respect was found to resemble the *Drosophila* eclosion rhythm (Pittendrigh and Minis, 1964; Minis, 1965). This rhythm was then used for testing the coincidence model. In doing so it is necessary to assume that this overt rhythm belongs to the same circadian system and therefore shows the same entrainment characteristics as the overt oscillation which is thought to be responsible for photoperiodic induction in the larval offspring. In devising a suitable test, an attempt was made to use a 15-minute light pulse both as inducer and as entraining agent. By applying similar pulses to the free-running oviposition rhythm, it was possible to derive the phase response curve. And this information was then used in deciding where the light pulse must fall in order to generate the phase advance or delay that would be required for entrainment to values of T greater or less than τ. According to this line of argument, diapause should be inhibited if the pulse (now in its capacity as 'inducer') falls in the early subjective night (corresponding to phase delays and values of T longer than τ). The results nevertheless proved inconclusive.

In recent work by Pittendrigh et al. (1970) a different approach has been adopted. The spectral sensitivity of diapause inhibition has been compared with the action spectrum for entraining the egg-hatching rhythm. The latter exhibits a broad maximum in the blue but has no red sensitivity (Bruce and Minis, 1969). However, in the diapause tests it was found that monochromatic light of all wavelengths up to 620 nm could substitute effectively for white light in typical long and short-day cycles. Since the authors

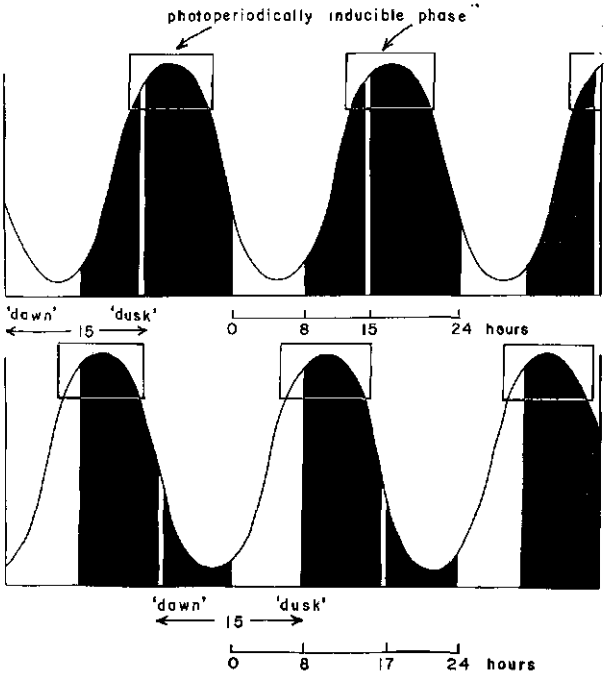

Fig. 4. Pittendrigh and Minis' (1964) entrainment model of photoperiodic action. The effect depends on the coincidence of light with a specific 'photoinducible' phase point in the entrained rhythm.

were unwilling to accept that there could be two independent circadian systems, related to different pigment receptors, they were led to the conclusion that time measurement in *Pectinophora* is not in fact governed by a circadian oscillation.

Other authors have nevertheless regarded the coincidence model as readily applicable to their experimental results. The extensive studies of Saunders (1968, 1969, 1970) on the photoperiodic control of diapause in *Nasonia vitripennis* require special comment. Diapause in this Chalcid occurs in the third instar larva but is determined maternally before the adult wasp has parasitised the fleshfly host, *Sarcophaga*. The photoperiodic response is recognised by the fact that the wasps 'switch over' to the production of diapause larvae after 5–11 short-day cycles but only do so after at least 22–30 cycles under long day conditions.

Of the two peaks of diapause inhibition which are revealed in *Nasonia* when a *LD 14*:10 cycle is scanned by night interruptions, the A peak occurs at hour 15–16 (that is, 1–2 hours after dark) and the B peak at hour 21-22. In a 24-hour cycle this means that the B peak also precedes lights-off in the subsequent daily cycle by 16–17 hours. Accordingly, Saunders relates the maxima to a simulated photoperiod of this length in which the A peak represents 'dusk' and the B peak 'dawn'. Although no studies on any overt rhythm are yet available for *Nasonia*, entrainment to 'skeleton' photoperiods of

this kind is considered probable and the fixed time relationship of 15–17 hours is taken as a reliable indicator of the phase of the rhythm controlling the photoperiodic response.

The interpretation of the position of peaks of diapause inhibition nevertheless presents some difficulties. When night interruptions were applied to two additional cycles with longer nights (*LD 12*:12 and *10*:14) the two maxima were still recognisable although their amplitude was reduced. However, the A peak had 'drifted' in the *LD 10*:14 cycle so that the 15–17 hour temporal relationship with the beginning of the main photoperiod was no longer maintained. If the A peak is related to dawn it should be possible to demonstrate this (experimental and other 'imperfections' permitting) by reducing the duration of the main photoperiod. With a cycle of *LD 11.3*:10 the A peak was delayed by about 2 hours, in agreement with the entrainment model. But the B peak remained relatively stable in relation to the onset of darkness, and could be more easily accounted for by an hour-glass hypothesis.

Experiments on *Nasonia* in which 4-hourly periods of chilling (at 2 °C) were applied to different parts of a short day *LD 14*:10 cycle have also provided evidence on the photoperiodic mechanism (Saunders, 1967). If the biochemical processes associated with the clock are slowed down or arrested at this temperature, a timing event depending mainly on the dark period should be correspondingly curtailed by night cooling. Low night temperatures did in fact convert the short day (or long night) response into a long day one. However, when chilling was applied at the beginning or middle of the photoperiod in a long day *LD 16*:8 cycle, the response was again reversed. Clearly, therefore, the photoperiod is also involved in the *Nasonia* clock, as the oscillator model would predict. Nevertheless this cannot be regarded as decisive evidence for rejecting the proposition that the *Nasonia* clock may function on the hour-glass principle. As will be shown later in *Megoura*, the dark period timing process runs more slowly if this phase is preceded by a photoperiod of reduced length. The result of such a deficiency is that the very potent 'long day' effect normally associated with an 8-hour night, is much reduced, and the response is partially reversed. This effect is not apparent in *Megoura* until the principal photoperiod is reduced to about 6 hours, whereas the chilling treatment in *Nasonia* would presumably leave an effective photoperiod of 12 hours. However, it is worth emphasising that *Megoura* seems to be relatively insensitive to deficiencies in the photoperiod. Insects such as *Laspeyresia molesta* (Dickson, 1949) and *Ostrinia nubilalis* (Beck, 1962) – whatever the nature of their clocks – are evidently more susceptible.

Night interruption experiments employing ultra-long cycle lengths of 48 and 72 hours have perhaps provided the strongest evidence for the participation of some kind of rhythmical process in the *Nasonia* photoperiodic clock. With the 48-hour cycle (*LD 10*:38) two peaks of light sensitivity appeared at hours 19 and 43. With the 72-hour (*LD 10*:62) cycle there was a third peak at hour 67. Saunders (1970) has concluded that the A peak, which is found at hour 15–16 in the 24-hour cycle (see above), is displaced to hour 19 and is then repeated with circadian frequency (Fig. 5A, D). The disappearance of the B peak is difficult to account for in terms of the coincidence model.

In the codling moth *Carpocapsa pomonella* Peterson and Hamner (1968) and Hamner

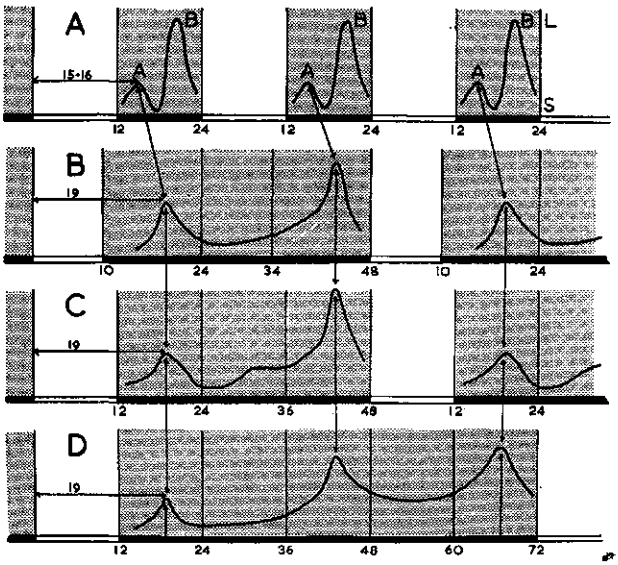

Fig. 5. Photoperiodism in *Nasonia vitripennis* (from Saunders, 1970). The diagram shows the pattern of response (peaks of diapause inhibition) elicited by night interruptions in cycles of differing length. 1-hour light pulses (which are not shown) were used in the 24-hour cycle, 2-hour pulses in the 48- and 72-hour cycles.

(1969) consider that the control of diapause involves both rhythmic and hour-glass timers. Night interruption experiments revealed only one peak of diapause inhibition in 24-hour cycles whereas two maxima were found in 48- and 72-hour cycles. The photoperiod was held constant at 8 hours. In the 2- and 3-day cycles diapause inhibition was strongest 8 hours after dusk (the hour-glass function) and 8 hours before dawn. This association with dawn is regarded as indicative of circadian rhythmicity. However, a possible alternative explanation might be that the interruption is again defining the beginning of an 8-hour scotophase with the main photoperiod acting as the 'light break'. The effect would then be a product of the same hour-glass timer. An analogous situation in *Megoura* is illustrated in Fig. 9.

Models with two oscillators have sometimes been invoked, Goryshin and Tyshchenko (1969) (see also Danilevsky et al., 1970) have been principally concerned with the differing form of the photoperiodic response curves in *Lepidoptera*. In the species studied – *Pieris brassicae*, *Barathra brassicae* and *Acronycta rumicis* – the incidence of diapause was reduced at very short daily photoperiods, although to a varying extent. The existence of a second 'critical photoperiod' is held to indicate that an independent act of time measurement is taking place during the photoperiod. Night interruption experiments, within a 24-hour cycle, again revealed one or two maxima of diapause inhibition but their position within the dark period depended on the temperature, on the relative length of the light and dark and, of course, on the species. *Acronycta* was notably less sensitive to light breaks than the other two.

Goryshin and Tyshchenko considered that their observations could best be explained if there were two 'oscillators'. Oscillator A begins to function immediately at lights-on but ceases spontaneously before lights-off, the duration of activity varying with the species or geographical strain. Oscillator B is temperature-sensitive and begins to function at lights-off although there is a latent period before an effective level of 'activity' is reached. Diapause occurs when the 'active' components of A and B fail to overlap in time.

It is noteworthy that in this model the oscillators do not appear to have a periodic function and the duration of their activity seems to bear no relation to the circadian cycle. Indeed, the 'oscillators' appear to have the properties of hour-glasses. Since, however, the principal light and dark periods were not varied independently, no definite conclusions can be reached.

The photoperiodic control of diapause in the corn borer *Ostrinia nubilalis* is attributed by Beck (1964) to the interplay of two oscillating systems whose sites have been defined anatomically. One centre, located in the epithelium cells of the ilium (hind-gut) contains osmophilic, fluorescent granules which appear and disappear cyclically with a period of about 8 hours. The granules are believed to be the precursor of a hormone which acts on the second system. The latter consists of certain unspecified neurosecretory cells in the brain which are also said to show an 8-hour cycle of secretory activity. Development only occurs if the two rhythms are held in phase by the appropriate long-day light cycle. This work has not yet been confirmed in other insects. The fact that the fluorescent granules are probably lysosomes, containing mitochondrial fragments (McLeod et al., 1969), does not of course exclude the possibility that they are in some way connected with the cyclical production of a hormone.

Photoperiodism in birds

Not surprisingly, the photoperiodic mechanisms in birds seems to differ materially from those in insects. It is noticeable that photoperiod plays a more important role than the dark period. For example, Wolfson (1959) found that testicular maturation in *Junco hyemalis* occurs in long days even if the cycle contains a long 16-hour night. And there is evidence that light components of short duration show the kind of cumulative effects that are uncommon in insects.

Some of the most striking evidence concerning the role of rhythms in animal photoperiodism was secured by W. M. Hamner (1963, 1964) who worked with the house finch *Carpodacus mexicanus*. In spite of some variability, testis growth was stimulated when the birds were placed on 12, 36 or 60 hour cycles (all with 6-hour photoperiods); in contrast, testis maturation was inhibited with cycle lengths of 24, 48 or 72 hours (Table 1). These results, with their clear periodicities, suggested that the 1, 2 or 3-day non-inductive cycles were accepted as 'short', even when they were whole multiples of 'normal' short-day cycles (e.g. *LD 6*:18). Night interruptions placed at hours 12 or 36 tended to be promotive, indicating that light was influencing the same phase points as in a completed cycle of this length.

Hamner and Enright (1967) then proceeded to test Bünning's proposition that there

Table 1. The effect of cycles with differing night lengths on testis maturation in the house finch *Carpodacus mexicanus* (From Hamner and Enright, 1967).

Bird and channel No	Cycle duration (h)			
	36	48	60	72
1	49.1*	1.7	79.8	36.5
2	33.0	64.0	25.0	42.8
3	39.0	2.2	0.5	11.1
4	32.6	3.8	27.5	9.7
5	32.6	3.6	38.2	2.2
6	42.7	3.8	53.1	4.2
7	–	7.3	33.2	2.1
8	41.4	2.7	75.9	1.0
9	–	2.4	29.8	3.6
10	–	4.1	35.6	5.3

* Weight of left testis in mg. Photoperiod 6 hours.

is only one 'master clock' in the circadian system and that the 'hands' can be 'read' by examining any other daily overt rhythm. This is clearly of considerable importance as there appear to be no other instances in animal photoperiodism where this assumption has not been made tacitly, if indeed, overt rhythms have been used at all in the experimental analysis. Hamner and Enright first examined the closeness of the linkage between the circadian rhythm controlling gonad development and that of locomotor activity. Results with the non-stimulatory cycles *LD 6*:42 and *6*:66 indicated that 6 hours of light presented every other circadian cycle or even every third cycle are sufficient to synchronize and entrain the waking-sleeping rhythm. Locomotor patterns with ahemeral cycles that stimulated gametogenesis (*LD 6*:30 and *LD 6*:54) were more complex but could be readily explained by assuming that the photoperiod was causing alternate phase advances and delays. So far, these observations were consistent with Bünning's hypothesis. In the first place, the patterns of activity were proved to be the products of entrainment to these two types of ahemeral cycle (inductive and non-inductive); and secondly, the patterns for inductive and non-inductive cycles were characteristically different.

A further test, which is similar to one conducted by Pittendrigh and Minis (1964) (see above), was based on a knowledge of the phase response relationships of the locomotor rhythm. With this information it was possible to state that when the birds were entrained by a 22-hour cycle (including a 6-hour photoperiod) their activity would be synchronized with the light phase in the cycle. But with a 26-hour cycle the entrained rhythm would be such that the birds would receive light late in their subjective day. If the activity records of the birds do, in fact, reveal the position of the 'hands', and if the photoperiod rhythm is driven by the same clock, the 26-hour cycle should prove stimulatory and the 23-hour cycle inhibitory. Although this prediction was partly fulfilled, the examples of anomalous testis growth or lack of growth, were sufficiently numerous to suggest to the authors that Bünning's hypothesis in its simplest form was

inadequate to account for the results. Additional experiments in which a 6-hour light pulse was administered either during the early or late subjective day of a free-run, also produced inconsistencies. Hamner and Enright therefore concluded that there were either two circadian systems or that if the rhythms were manifestations of a single master clock, they were not phase-locked, at least under laboratory conditions.

The coincidence model has been tested by Menaker and Eskin (1967) in the house sparrow *Passer domesticus*, again using the rhythm of locomotor activity as an indicator of the circadian system and the maintenance of testis size as the photoperiodic response. The object was to control the phase setting of the rhythm underlying the response so that different phase points could be illuminated by a short daily light pulse. For this it was necessary to devise a lighting schedule that would entrain without provoking a photoperiodic response. A 14-hour period of dim green light was found to perform this function satisfactorily, entraining the locomotor rhythm yet failing to prevent the regression of the testis. A 75-minute pulse of intense white light (by itself, non-inductive) was then used to 'probe' different parts of the cycle. The testis response was only evoked when the pulse was positioned near the end of the green photoperiod. Presumably it was then illuminating the appropriate 'inductive' phase point.

Photoperiodism in aphids: an hour-glass timer

The idea that circadian rhythms are involved in animal photoperiodism – either as an integral part of the clock or more peripherally – has proved so attractive in the last decade that other possibilities have largely escaped attention. Yet there are species in which no evidence for any rhythmical function has been discovered, despite intensive search. The vetch aphid *Megoura viciae* falls into this category (Lees, 1960, 1965, 1966 and 1971). The accumulated evidence suggests that the *Megoura* clock is indeed an hour-glass, although it is an hour-glass of a rather sophisticated character, involving both the light and dark moieties of the illumination cycle. It may well be that similar timing mechanisms are much more prevalent than is generally supposed, particularly among insects.

In *Megoura* photoperiod controls the determination of the developing embryos as oviparae or virginoparae through a centre located in the brain of the mother. The site of this centre – which presumably includes the photoreceptor and the clock – has not yet been identified. In the clone used, the cycle *LD 14.5*:9.5 produces a response almost exactly intermediate between long- and short-day. At this critical length, about half the parent virginoparae produce some virginoparous daughters (and are therefore classified as 'virginopara-producers') while the others give birth exclusively to the short-day oviparous form. No virginopara-producers at all are formed in the short day cycle *LD 13.5*:10.5.

I have already referred to night interruption experiments in which a 10.5-hour dark period was scanned by 1-hour light pulses (Fig. 3). Using the appearance of virginopara-producers as an index, there are two points of light sensitivity, the first extending from about 1 to 3 hours after the onset of darkness and the second from about 5.5 hours to the critical night length of 9.5 hours. From hours 4 to 5 there is an almost

complete insensitivity to light. I have also mentioned that these time relations are very precisely maintained if the principal photoperiod is extended by 12 hours to 25.5 hours, indicating that in these circumstances the position of the two maxima is not related to the beginning of the photoperiod, as Bünning's model predicts. On the contrary, the pattern of changing light sensitivity is clearly determined by the onset of darkness – an hour-glass effect. This conclusion has nevertheless been questioned by Pittendrigh (1966) who has pointed out that the *Drosophila* eclosion rhythm becomes fully damped if the daily photoperiod exceeds 12 hours; and that if an analogous oscillation in *Megoura* were concerned in measuring the 'scotophile', the longer and shorter photoperiods might be expected to exert the same effect. However, this argument does not take into account the fact that the night interruption peaks also remain fixed to dusk when the principal photoperiod is *reduced* to 8 hours and is only slightly delayed if it is still further reduced to 4 hours (Fig. 6). Presumably, (by analogy with *Drosophila*) the oscillation would not damp out under these circumstances, so that a changed phase relationship between the oscillation and the light cycle would be expected. The light sensitivity peaks should then take up different positions within the dark period. These results also present difficulties of a more general nature if entrainment is regarded as playing an essential part of photoperiodic time measurement. For in *Megoura* the dark period hour-glass continues to function normally in light/dark cycles ranging from 36 hours (*LD 25.5*:10.5) to 16.5 hours (*LD 6*:10.5). Yet these are well outside the limits of entrainment observed in most higher organisms.

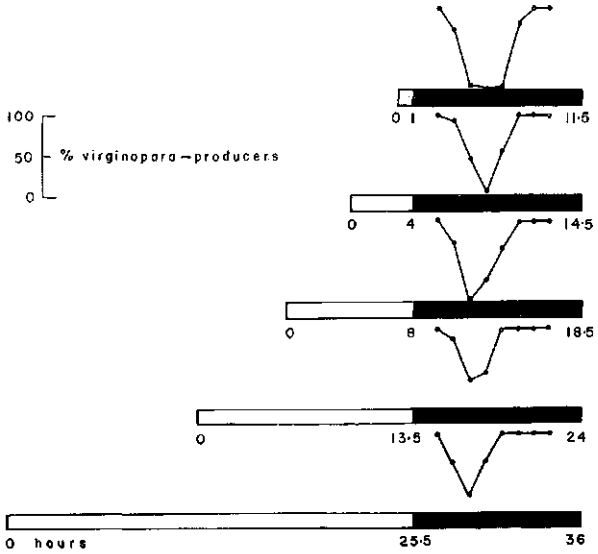

Fig. 6. The dark period hour-glass timer in *Megoura viciae*. Note that the pattern of response to interpolated 1-hour light breaks (which are not shown) is hardly influenced by the principal photoperiod, even when its duration is less than 12 hours.

We have already seen that regimes which include long or very long scanned dark periods have been widely used for testing the oscillator hypothesis. A long series of experiments, based initially on Hamner's (1960) design, has been carried out with *Megoura*. In all the cycles used, an 8-hour main photoperiod was combined with a variable dark period which ranged from 20 to 72 hours in length; the 1-hour night interruptions were positioned at 4-hourly intervals, the first 4 hours after lights-off, the second 8 hours, and so on. Since all the cycles contained an uninterrupted night of more than critical length, a dark reaction, working on the hour-glass principle, should always have ample time to proceed to completion. According to this simple view, night interruptions should exert no effect. This proved to be correct for most cycles (Fig. 7). But a striking and consistent reversal in the response was noted when the light break was placed 8 hours after the beginning of darkness (in addition, a low incidence of virginopara-producers was sometimes found when the break preceded the termination of the night by 11 hours). Yet although the dark period was particularly photosensitive at hour 8, there was no evidence that this sensitivity was repeated with circadian frequency at hours 32 and 56. This does not therefore provide any support for the oscillator hypothesis. At the same time, the unexpected reversal of the response following a light pulse at hour 8 clearly required further evaluation. It seemed that a long night was not necessarily 'inductive' under these circumstances.

The similar effect of a night interruption placed 8 hours after the beginning of a

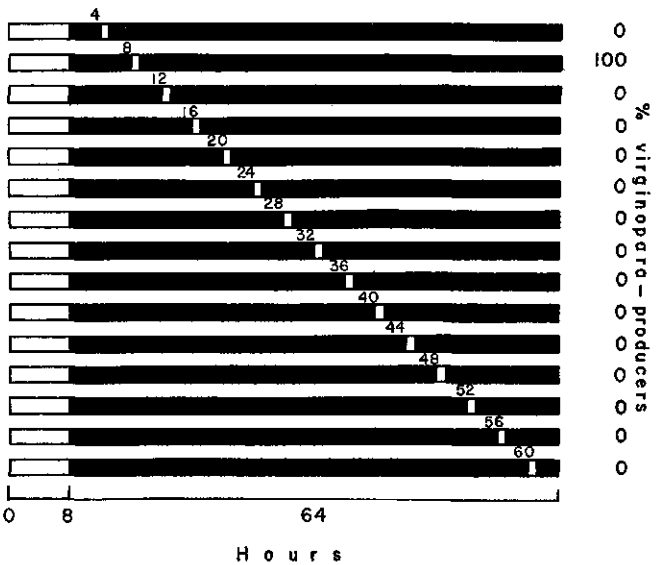

Fig. 7. Night interruption experiment in *Megoura viciae* employing a very long cycle. In the control series the uninterrupted 64-hour dark period acted as a normal long night, no aphids producing virginoparae.

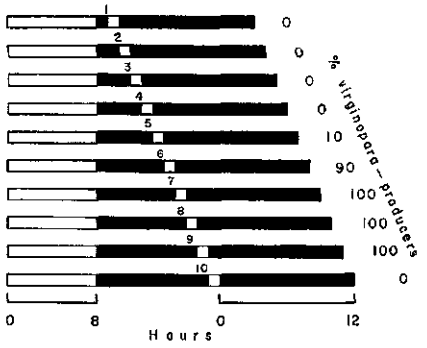

Fig. 8. Regimes used in *Megoura viciae* for demonstrating the different effects of 'early' and 'late' night interruptions on the response to a subsequent dark period (12 hours) of more than critical length.

10.5 hour dark period has already been noted (Fig. 3). The mode of action of such a 'late' interruption was examined in greater detail by positioning 1-hour light breaks closer together during the important earlier hours of the dark period and by interpolating a constant 12-hour dark period after the interruption (Fig. 8). The results again showed that the dark period can exceed the critical length (12 hours in this case) and, can still be 'non-inductive' when the preceeding night is interrupted between hours 6 and 9.5 (corresponding therefore to the B maximum in Fig. 3). For these and other reasons the term 'inductive' is no longer applied to long night effects in *Megoura*. It is thought more likely that the photochemical products associated with a 'late' interruption (or a short night) provide the positive stimulus governing embryonic development. If this is so, it is appropriate to classify the maternal response in terms of the production of virginoparae.

To return to Fig. 8: it is also clear that whereas a 'late' interruption overrides the 12-hour dark period, an 'early' light interruption corresponding to peak A, does not. This must mean that after interpolating a light pulse 1–3 hours after dusk, the photoperiodic clock continues to 'measure' the subsequent 12 hour dark period and responds appropriately when the critical length is exceeded. In contrast, no effective measurement of the night length takes place after the 'late' interruption.

The role of light when introduced early in the dark period can be gauged more precisely by obtaining the critical night length. Table 2 shows that it is just 9.5 hours, which is exactly the same as the critical night length in a 24-hour cycle containing only one light and one dark component (i.i. *LD 14.5*:9.5). The inference must be that the 'dark reaction' is reversible in its initial stages: the 'early' light break evidently causes the timing mechanism to revert to hour zero, where it remains until measurement again starts in darkness. In contrast, as we have seen, no critical night length can be demonstrated after a 'late' light break. Indeed, there is no obvious way of telling whether a timing process is proceeding or not. What is certain is that a light break in the late night does not cause a reversal of the previous dark reaction. An irreversible photochemical process is initiated which, as other evidence shows, leads to the formation of stable products which can be accumulated during successive cycles (see below).

We have so far established that time measurement takes place during darkness but

Table 2. Regimes used in *Megoura viciae* to define the duration of the critical night length after a 1-hour light break in the early night.

Exp. No	Cycle(h)				Virginopara-producers (%)
	L	D	L	D	
1	13.5	1.5	1.0	9.0	100
2	13.5	1.5	1.0	9.25	90
3	13.5	1.5	1.0	9.5	60
4	13.5	1.5	1.0	9.75	20
5	13.5	1.5	1.0	10.0	5

that the 'dark reaction' (or hour-glass) consists of at least four constituent processes – presumably chemical reactions – which take place in a definite sequence. We have seen that the first step, lasting about 2–3 hours is reversed by light; moreover, action spectra show that the photoreaction is blue – but not red-sensitive and is comparitively slow (Lees, 1971). The second, light insensitive, stage lasts from hour 4 to hour 5. Little is known about it except for one very interesting fact. If a light break, positioned at hour 4, is extended to hours 6 or 7 so that it now overlaps the third stage in time, a strong 'late' interruption effect is obtained. This conveys the impression that during the light-insensitive stage 2, the underlying 'dark reaction' is proceeding on its normal time course, irrespective of the environment. This culminates in the spontaneous recovery of light sensitivity from hour 5 onwards.

The effects of incident light during stage 3 which extends from hour 5 to the critical night length of 9.5 hours has already been described. This irreversible photoreaction is comparatively fast and requires lower energy levels than the stage 1 photoresponse (Lees, 1971). Both blue and red wavelengths are effective. Finally, the stage 4 of the 'dark reaction' supervenes as the hours of uninterrupted darkness pass beyond the critical night length and light ceases to have the promotive effect characteristic of stage 3.

One further point might be made in connection with the photosensitive stages 1 and 3. The response to night interruptions obviously recalls the 'bimodal' pattern observed in other insect species. It has been noted that the different amplitudes of the A and B peaks of diapause inhibition have been attributed to the dual effects of light, which are regarded as qualitatively different by supporters of the oscillator hypothesis. The first, namely photoperiodic 'induction', is said to require higher energies than the second or entraining function. An entirely different interpretation is offered for *Megoura* in which peak A represents a physiological process (reversal of the dark reaction) which could never take place in nature; while peak B represents the normal response to a natural long day (or more appropriately, short night). Their differing amplitudes merely reflect the differing kinetics of the 'dark reaction' at these two points in time.

I have for some years regarded the dark timing process as the central component in the *Megoura* photoperiodic clock. The reason is that time measurement is 'within wide

limits' independent of the duration of the main photoperiod (Lees, 1965, 1966). In this context, 'wide limits' referred to photoperiods of 8 and 25.5 hours. Unfortunately, some authors have gained the impression that I attributed no function at all to the main photoperiod, apart from its obviously important role in marking the beginning and end of the dark period in natural cycles of illumination (Pittendrigh, 1966; Danilevsky et al., 1970). This was not my intention. Indeed the contrary can very easily be demonstrated by restricting the length of the principal photoperiod to under six hours.

If the night interruption experiments are repeated, using main photoperiods of 8, 4 and 2 hours, the most striking feature in the response pattern is that the critical night length becomes longer as the principal photoperiod is reduced. It is 9.5 hours with a main photoperiod of 8 hours; 10.5 hours with one of 4 hours and 11.5 hours with one of 2 hours (Table 3). The position of the B peak is also displaced later in the dark period. Indeed, it is as if the main photoperiod serves to turn over the hour-glass. If the main photoperiod is too short the hour-glass is only tilted and therefore appears to measure time slowly.

This main photoperiod requirement can also be detected in other situations. If the series of night interruption experiments shown in Fig. 7 is inspected, it will be seen that in the penultimate cycle the 1-hour light pulse precedes the next 8-hour photoperiod by 7 hours. But this does not stimulate virginopara-production as might have been expected. The reason for this is simply that the light pulse is too short to prepare the clock mechanism for a normal timing act during the ensuing 7-hour night. This

Table 3. Experiments illustrating the role of the principal photoperiod in the operation of the dark period hour-glass in *Megoura viciae*. Note that the critical night length increases as the photoperiod diminishes.

Exp. No	Cycle (h)				% Virginopara-producers when X =		
	L	D	L	D	8	4	2
1	X	1	1	12	0	0	0
2	X	2	1	12	0	0	0
3	X	3	1	12	0	0	5
4	X	4	1	12	0	0	10
5	X	5	1	12	10	0	11
6	X	6	1	12	90	4	57
7	X	7	1	12	100	4	67
8	X	8	1	12	100	85	80
9	X	9	1	12	100	95	100
10	X	10	1	12	0	100	100
11	X	11	1	12	0	0	100
12	X	12	1	12	0	0	30
13	X	13	1	12	0	0	5
14	X	14	1	12	0	0	4
15	X	15	1	12	0	0	0

can readily be demonstrated by increasing the duration of the 1-hour pulse: a strong 'late interruption' effect then appears (Fig. 9). Under these circumstances the widened light break acquires the status of a principal photoperiod and the 8-hour photoperiod becomes the 'interruption'.

A principal photoperiod of rather more than 4 hours is obviously essential for preparing the dark period hour-glass for a single timing act. This preparatory stage is itself time-dependent. Yet this is only incidental to the procedure of time measurement. In the response curve it is the 9.5 hour dark period that is critical and not the 13.5 hour light phase, since this photoreaction has already proceeded to completion some time between 4 and 8 hours.

We have noted that in *Megoura* light can have three separate effects depending on whether it is experienced as a relatively long principal photoperiod or as a short interruption in the 'early or 'late' night. Although they are believed to represent distinct photochemical processes it is worth emphasising that 'early' light breaks can also acquire the status of a principal photoperiod if their length is extended. And both have maxima at 450–470 nm. It therefore seems possible that the early events which take place at photoreversal represent the initial stages of a response which can be completed during a more extended principal photoperiod. The type of responses envisaged in the *Megoura* clock are shown diagramatically in Fig. 10 which also includes some typical cycles and their effects.

It is, of course, difficult enough to construct a set of 'rules' consistent with a large body of data. But such a model obviously acquires a highly speculative flavour if an interpretation is attempted in more fundamental terms. Nevertheless, it is worth recalling that the visual responses of animals involve the combination and dissociation of a protein and a light-absorbing pigment. This could also be true of the *Megoura* clock which is regarded as a comprising series of slow biochemical reactions linked together in sequence. The early reversibility of the 'dark reaction' perhaps suggests that in its initial stages, the protein-pigment complex can be dissociated by light.

No animal species are known in which one single cycle is photoperiodically effective. An hour-glass capable of no more than a single act of time measurement therefore

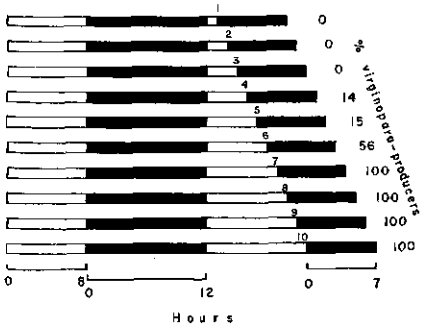

Fig. 9. Series of cycles illustrating the role of the principal photoperiod in *Megoura viciae*. Normal time measurment during the 7-hour dark period is resumed as the light pulse is made longer, with the result that the original 'main' photoperiod functions as a 'late' night interruption.

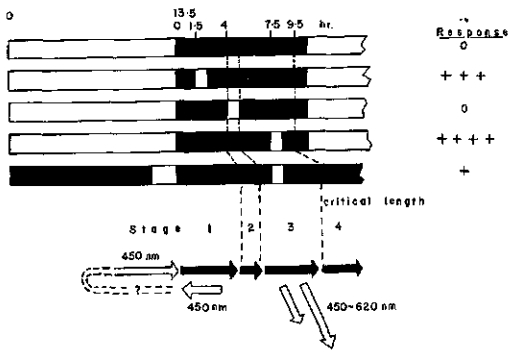

Fig. 10. Model for the *Megoura* hour-glass clock. The dark timing sequence and the possible photoreactions are indicated respectively by black and white arrows; and the differing spectral sensitivites of stages 1 and 3, corresponding to 'early' and 'late' night interruptions, are also noted. The effects of some typical cycles are shown above.

requires two additional mechanisms: some means of 'turning over' the hour-glass; and a device for accumulating the products of successive cycles. The first operation is accomplished during the principal photoperiod; the second capability is much in evidence when *Megoura* is exposed to increasing numbers of long-day (styled more appropriately short-night) cycles (Table 4). It is particularly striking that the promotive effects are virtually proportional to the numbers of short nights given, regardless of whether they are placed consecutively or are separated by varying numbers of long-night cycles (Table 5). The impression is strongly conveyed that a long night – the result of a completed timing process – is inactive and is certainly not inhibitory. On the other hand, it may well be that each short night produces a quantal amount of 'active substance' which persists even through intervening long-night cycles.

It remains to be decided what features the *Megoura* photoperiodic clock has in common with those of other insect species. In the aphid *Dysaphis plantaginea* in which photoperiod controls the production of the winged gynoparae, there are some similarities as well as some differences. Night length is particularly important in time measurement, but not exlusively so. On the other hand night interruptions show a marked B peak of light sensitivity, but no well defined A peak. Bonnemaison (1968, 1970) finds no evidence that any rhythmical process is involved but considers it necessary to invoke the presence of two hour-glass clocks, one to measure the dark and the other the light.

Hour-glass mechanisms may well play a highly significant part in the photoperiodic responses of other insects. It is clearly wrong to dismiss this possibility merely because the peaks of light sensitivity move their position within the night as the duration of the principal photoperiod is changed. Both light and dark components of the cycle are implicated in the operation of the timer. Although dark period time measurement is particularly striking in *Megoura* because it is *relatively* independent of the duration of the photoperiod, there is no reason why this should always be so. Indeed, an inspection of the 24-hour response curves of many species suggests a much greater dependency on photoperiod.

If, as one supposes, responses to day length have been evolved repeatedly, it would not be surprising to find that the types of clock are highly diverse, embracing both

Table 4. Cumulative effects of long day (i.e. short night) cycles in *Megoura viciae*. L = long day (*LD 16*:8); S = short day (*LD 12*:12). The regime was applied during the time of the parent aphid's greatest sensitivity to photoperiod. Day of birth of parent virginoparae indicated by vertical bars.

		Virginopara-producers (%)
L S	S S S S S S S S S S	0
S L	S S S S S S S S S S	0
S S	L S S S S S S S S S	0
S S	S L S S S S S S S S	0
S S	S S L S S S S S S S	0
S S	S S S L S S S S S S	0
S S	S S S S L S S S S S	0
S S	S S S S S L S S S S	0
S S	S S S S S S L S S S	0
L L	S S S S S S S S S S	0
S L	L S S S S S S S S S	5
S S	L L S S S S S S S S	13
S S	S L L S S S S S S S	28
S S	S S L L S S S S S S	23
S S	S S S L L S S S S S	17
S S	S S S S L L S S S S	9
S S	S S S S S L L S S S	4
S S	S S S S S S L L S S	0
L L	L S S S S S S S S S	43
S L	L L S S S S S S S S	51
S S	L L L S S S S S S S	80
S S	S L L L S S S S S S	91
S S	S S L L L S S S S S	83
S S	S S S L L L S S S S	79
S S	S S S S L L L S S S	12
S S	S S S S S L L L S S	4
S S	S S S S S S L L L S	0

Table 5. Treatments showing that the accumulation of long-day (L) effects in *Megoura viciae* is not inhibited by intervening short-day (S) cycles. Compare with Table 4.

		Virginopara-producers (%)
S L	S L S L S L S L S L	100
S L	S S L S S L S S L S	96
S L	S S S L S S S L S S	54
S L	S S S S L S S S S L	35
S L	S S S S S L S S S S	36
S L	S S S S S S L S S S	33
S L	S S S S S S S L S S	8
S L	S S S S S S S S L S	8

rhythmical and hour-glass mechanisms. Nevertheless, it is difficult to avoid the impression that, on present experimental evidence, the role of circadian rhythms in animal photoperiodism has been exaggerated. It may well be that in terms of the evolutionary process, there are selective advantages to be gained in separating the circadian and photoperiodic systems.

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Discussion

Participants: A. D. Lees (Author), G. Becht, J. Brady, K. Hoffmann, P. J. A. L. de Lint, D. S. Saunders, H. U. Thiele and E. C. Wassink

With regard to the 100% response to a light break placed 8 hours after the beginning of darkness, does this effect depend specifically on the length of the subsequent dark period? (WASSINK.) The response is not influenced by the subsequent dark period, provided this exceeds the critical length. The explanation I offer is that a quantal amount of 'virginopara-producing substance' is formed by a light break at 8 hours (or

for that matter, by a natural short-night), whereas this hypothetical substance is not produced in a night of more than critical length (AUTHOR).

Does the night interruption at hour 8 have a double effect when it is followed by a shorter dark period? Can it then function both as a 'break' and as a 'main' photoperiod? (DE LINT.) Probably yes. Normally, the response is 'saturated' (100% virginopara-producers) even when there is only one 8-hour dark period every two or three days. Since a 1-hour break, as used in the experiments, weakens the 'late interruption' effect and causes the critical night length to increase, it is necessary to follow the interruption with a rather longer dark period, one, say of 10 or 11 hours. Then cumulative effects can be detected (AUTHOR).

Have you any evidence that the cumulative effect of long-day illumination is temperature-compensated as in *Nasonia vitripennis* and *Sarcophaga argyrostoma?* (SAUNDERS.) No (AUTHOR).

In *Pieris brassicae* which develops without diapause under long-day conditions Bünning and Joerrens (1960) have shown that dormancy is similarly avoided in skeleton photoperiod conditions which simulate a long photoperiod. Is it possible that a dark period hour-glass mechanism could be operating when the 24-hour cycle contains only two 1-hour light pulses, separated by an uninterrupted dark phase of more than critical length? (THIELE.) With such very short photoperiods it is necessary to be certain that the insect is actually responding to the light pulses and is not experiencing the equivalent of continuous darkness. In Bünning and Joerrens' response curves for *Pieris brassicae* the incidence of diapause was about 20–40% in permanent darkness and 18–55% with a daily 2-hour photoperiod. This variability is typical of the reaction of insects to darkness or ultra-short photoperiods. The range of diapause incidence was much the same in Bünning and Joerrens' experiment with skeleton photoperiods (AUTHOR).

Pittendrigh has criticised the hour-glass interpretation of your experiments with long photophases on the grounds that the length of the photophase is irrelevant since he finds that in *Drosophila* the clock is stopped by more than 12 hours of light. I presume, however, that this criticism is not applicable to your experiments using very long nights when in *Drosophila*, the clock would not be stopped (BRADY). That is so (AUTHOR).

Photoperiod can act as a developmental blocking agent. Are there other means, either surgical or chemical, that will accomplish the same thing? (BECHT.) Insects can, of course, be kept in permanent diapause by extirpating the brain, thereby removing the whole light receptor-effector system. Chemical blocking agents have not been much studied (AUTHOR). In mammals there is evidence that melatonin has the same effect as short-day treatment. For example, Rust and Meyer showed that melatonin administered to the weasel in summer causes the coat to turn white and the testes to regress (HOFFMANN).

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Circadian rhythms and physiology with special reference to neuroendocrine processes in insects

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Introduction

The manner by which the output of a circadian clock is translated into the overall behavior of an animal is one of the intriguing aspects of the study of biological rhythms. But the very complexity of these pathways present a challenge to those who attempt to unravel the basis of the clock. For, as stated by Bünning (1967): 'The chain of physiological events from the molecular basis of the clock to the overt rhythm is so complicated that the features of the overt rhythm do not necessarily reveal the features of the central clock.'

The determination of the site of a biological clock has served as a primary goal in the study of the physiological basis of rhythms. With the notable exceptions of the house sparrow (Menaker, 1968) and *Aplysia* (Strumwasser, 1965; Jacklet, 1969) the major studies in this area have used insect systems, which serve as the focal point of this paper.

Of special interest here is the control of the eclosion rhythm. This rhythm in *Drosophila* has been extensively studied by Pittendrigh and co-workers for almost 20 years. It stands, without question, as the best known of all animal rhythms. Although it has yielded many insights into the theory of circadian clocks, the size of *Drosophila* has precluded extensive work on the location of the clock and the physiological basis of the rhythm. Consequently, the eclosion rhythm of the giant silkmoths has provided the experimental data for the following consideration of some physiological aspects of animal rhythms.

Localization of biological clocks in insects

The cockroach activity rhythm

The first report of the localization of a biological clock was the claim by Harker (1956, 1960) that the subesophageal ganglion of the cockroach contained the clock which controlled the activity rhythm. Her experiments showed that decapitation abolished the locomotor activity rhythm of the cockroach, but after implantation of a subesophageal ganglion, the rhythmicity returned. The phase of the newly established rhythm

was in accord with the previous phase of the donor. But these results have not been confirmed, and subsequent work has directed attention to the brain as the site of the activity clock.

Since there are recent reviews concerning the development of the research on the cockroach activity rhythm during the past 10 years (Brady, 1969, 1971), only a summary of the pertinent experiments will be given here. The site of the cockroach clock is apparently the optic lobe region of the brain (Nishiitsusuji-Uwo and Pittendrigh, 1968b). Extirpation of this area, while not drastically interfering with normal locomotor activity, renders the animal arrhythmic. This result does not appear to be simply a matter of trauma since other operations that would be expected to give equal or greater trauma (e.g. bisection of the brain or extirpation of one cerebral lobe) do not have any consistent effect on the activity rhythm.

The activity clock receives all of its light information via the compound eyes. Consequently, if the eyes are completely blacked over or the optic nerves severed, the cockroach displays a free-running activity rhythm in a light-dark cycle (Nishiitsusuji-Uwo and Pittendrigh, 1968a). This uncoupling of the clock from the photoperiod occurs even if the nerve pathways leading to the ocelli are left intact (Roberts, 1965; Nishiitsusuji-Uwo and Pittendrigh, 1968a).

The way in which the optic lobe clock controls activity is not yet clear. It was first thought that the clock caused the release of material from the neurosecretory cells of the brain (Roberts, 1966; Nishiitsusuji-Uwo and Pittendrigh, 1968b). But later work indicates that the rhythm apparently is driven nervously because any operation which

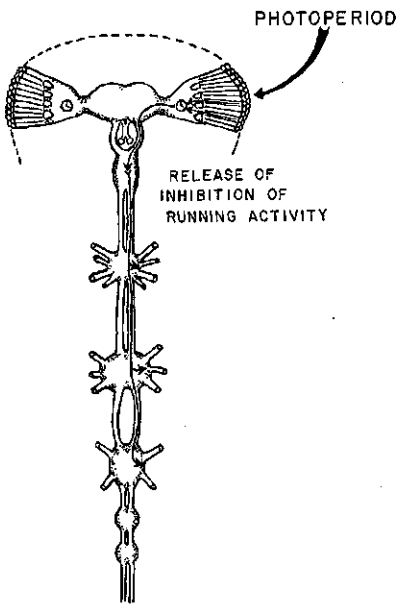

Fig. 1. A summary of the physiological steps in the control of the cockroach activity rhythm.

bilaterally disrupts nervous continuity between the optic lobes and the thoracic motor centers yields an arrhythmic condition (Brady, 1969; Roberts et al., 1971). Fig. 1 summarizes the steps involved in the cockroach activity rhythm. Photoperiod information is received by the eyes and synaptically transmitted to the clock which seems to be in the optic lobes. The nervous outflow from the clock then acts on the locomotor centers to produce the overt activity rhythm.

The eclosion rhythm

Location of the clock. In the giant silkmoths, the time of eclosion is dictated by the photoperiod and varies from species to species. As seen in Fig. 2B, when the brains were removed from developing moths, their subsequent emergence was no longer gated and eclosion occurred at random. This effect of brain removal could be reversed simply by implanting a brain into the abdomen of the brainless animal (Truman and Riddiford, 1970). The eclosion of such 'loose-brain' moths then occurred during the gate which was typical for the species (Fig. 2C). In addition, these moths showed a typical free-running rhythm of eclosion when transferred into continuous darkness (Truman, 1971c). The brain, therefore, is indispensible for the gating of eclosion. If this ganglion is transplanted into the tip of the abdomen of a brainless animal, it restores both the proper phase-setting under photoperiod conditions and the ability to free-run in continuous darkness.

Although the above results clearly show that the brain served as an important link

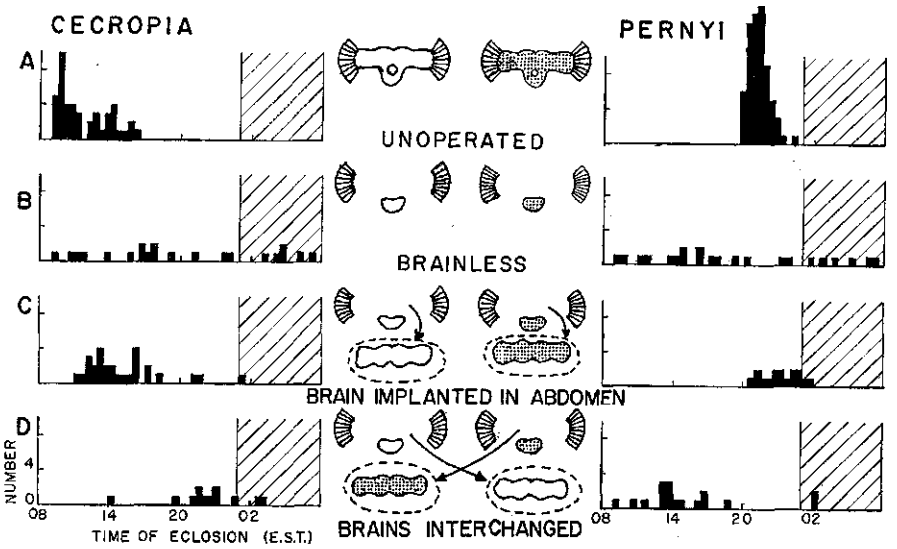

Fig. 2. The eclosion of Cecropia and Pernyi moths in a 17L:7D regimen showing the effects of brain removal, the transplantation of the brain to the abdomen, and the interchange of brains between the two species.

between the reception of light information and the gating of eclosion, its role in this chain of events was undefined. To clarify this role, brains were interchanged between *Hyalophora cecropia* and *Antheraea pernyi* pupae (Truman and Riddiford, 1970). As seen in Fig. 2D, the eclosion of both experimental groups was gated. But, more importantly, the respective gates were interchanged. Manifestly, the phase-setting of the eclosion gate could be transferred from one species to another simply by transferring the brain. Subsequent experiments have shown that the implantation of cerebral lobes is sufficient to gate the eclosion of a brainless animal (Truman, 1971c).

The fact that the cerebral lobe area of the brain controls the phase-setting of the eclosion rhythm strongly suggests that it also contains the circadian clock. Conclusive proof of this hypothesis awaits further experimentation.

The pathway of light information. With an eyeless strain of *Drosophila*, Engelmann and Honegger (1966) first showed that the eclosion clock did not receive light information via the eyes. Zimmerman and Ives (1971) arrived at a similar conclusion by a comparison of the action spectrum of the eclosion clock with the spectral sensitivity of the compound eyes. Also, in silkmoths Truman and Riddiford (1970) showed that the surgical extirpation of the compound eyes did not interfere with the proper gating of eclosion.

The light information necessary for the phase-setting of the eclosion clock is perceived directly by the brain (Truman and Riddiford, 1970). This was shown by using a modification of a technique developed by Williams and Adkisson (1964). As shown in Fig. 3, Cecropia pupae were used to plug holes in an opaque partition which separated two photoperiod chambers. The anterior end of each animal was exposed to a 12L:12D regimen and the posterior end to the inverted 12D:12L photoperiod. In ten animals the brain had been removed from the head and implanted into the tip of the abdomen. Another group of 10 consisted of animals which had their brain removed and then immediately reimplanted into the head. Thus both experimental groups were exposed to exactly the same photoperiod conditions except for the photoperiod to which the brain was exposed. Nine animals in each group survived the operation and

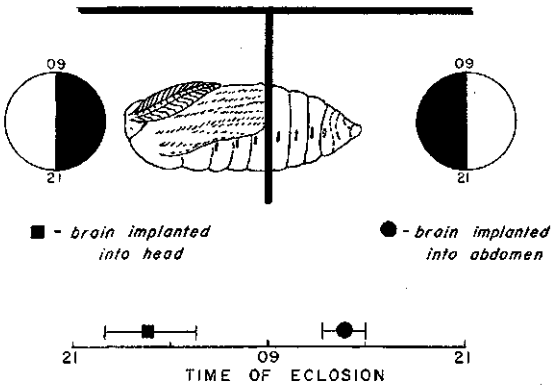

Fig. 3. The eclosion of two groups of 'loose-brain': Cecropia moths which differed only in the site of brain implantation. The anterior end of each was exposed to light from 09:00 to 21:00; the posterior end from 21:00 to 09:00. The time of eclosion was determined solely by the photoperiod to which the brain was exposed. The mean and standard deviation of each group is given.

subsequently emerged. As seen in Fig. 3, the time of eclosion was dictated by the photoperiod to which the brain was exposed. These results clearly show that the brain itself is photosensitive.

Analysis of the eclosion clock

A major problem in the analysis of a biological clock is the determination of the relationship of the underlying driving mechanism to both the photoperiod cycle and the overt rhythm. In this respect the eclosion rhythm proves to be especially convenient. After a long exposure to light, the eclosion clock stops; a subsequent onset of darkness then serves to restart the clock from a constant phase point of the cycle (Pittendrigh, 1966). This point can be thus considered as the beginning of a circadian cycle. From this reference point one can determine the effects of light and darkness upon the clock. This analysis is further simplified by the finding in *Drosophila* that under many photoperiods, the eclosion clock stops during the photophase and is, therefore, restarted anew at each lights-off (Pittendrigh, 1966).

Components of the clock: the scotonon and the photonon

This hour-glass behaviour of the eclosion clock was exploited in a preliminary analysis of the eclosion rhythm of the Pernyi silkmoth (Truman, 1971a). In this species the clock controls the secretion of the 'eclosion hormone' by the brain (see p. 127); eclosion follows about 1½ hours later. It is important to note that if Pernyi are transferred from light to continuous darkness, the hormone is released about 22 hours after the light-dark transition. Since the eclosion rhythm of the moth has a free-running period which is also 22 hours, hormone release has a phase relationship of 360° to the initiation of the circadian cycle (i.e. it occurs at the end of the cycle). Thus lights-off indentifies the start of the cycle and the release of hormone pin-points the end. With these two reference points the effect of light on the kinetics of the cycle was determined for a range of photoperiods. After the onset of darkness, the eclosion clock commences a cycle according to its free-running kinetics (the *scotonon* portion of the cycle). With the onset of the photophase, light causes a defined change in the kinetics of the remainder of the cycle (the *photonon* portion: Truman, 1971a). The interaction of these two sets of kinetics describes the phase-setting of hormone release for a wide range of light-dark cycles (Fig. 4).

Of special interest to this paper is the relation of the photoperiod to the width of the eclosion gate (Truman, 1971a). The gate width in a given photoperiod was taken as at measure of the accuracy of the clock in that regimen. In photoperiods with very short nights, the accuracy was poor. As the dark period was extended, accuracy rapidly increased until, after a certain amount of darkness, maximal accuracy was attained. Further lengthening of the dark period had little or no additional effect. Since in these photoperiods, the night coincides with the beginning of the scotonon, it was clear that the completion of some event in the early part of the scotonon was necessary for maximal accuracy. On the basis of this observation the scotonon was divided into two

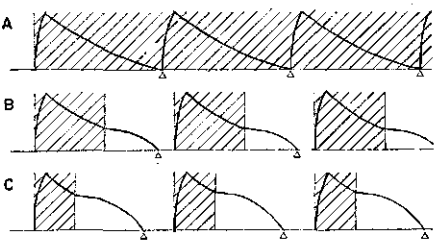

Fig. 4. A schematic representation of the behavior of the Pernyi eclosion clock. A: transfer from continuous light to continuous darkness initiates the free-running, or *scotonon* kinetics of the clock. B and C: in light-dark regimens, lights-on causes a switch to the *photonon* kinetics and thus alters the time-course for the remainder of the cycle. The open traingles identify the median time of eclosion hormone release.

periods: the *synchronization period* which occurs during the beginning hours of the cycle and the *dark decay period* which encompasses the remainder of the scotonon. The completion of the first period is necessary for the attainment of maximal accuracy. If light interrupts the second period, a change in kinetics is provoked but accuracy is not markedly affected (Truman, 1971a). From the *Drosophila* data obtained with skeleton photoperiods (Pittendrigh, 1965), the switch in kinetics from the scotonon to the photonon appears to be extremely rapid. But an exact determination of the relationship of the shape of the photonon to the duration and intensity of light exposure has yet to be done.

The significance of the synchronization period of the circadian cycle

It is of importance at this point determine the significance of the increase in accuracy with the completion of the synchronization period. The graphical representation of this process as the steep rising limb of the scotonon, while being a convenient conceptual device, nevertheless does not show the mechanism for the increase in accuracy. Of pertinence to this determination are the data provided by Lees (1971) on the photoperiodic response of *Megoura viciae*. Lees has shown that the photoperiodic mechanism of this aphid is completely reset by a light pulse given in the early night. When the pulse is applied at a later time in the night, resetting does not occur. The eclosion clock behaves similarly (Truman, 1971d).

Fig. 5 shows the effect of light pulses given during the synchronization period of the circadian cycle. After transfer from a 17L:7D regimen to continuous light, the scotonon began at lights-off and therefore the subsequent rhythm of eclosion hormone release showed a 360° phase relationship to lights-off. When a ½-h light pulse was given after the first hour of darkness, the resultant rhythm had a phase of 360° to the lights-off of the pulse. Thus, the pulse served to reset the circadian cycle. This resetting can apparently be carried on indefinitely. Even after a series of 20 pulses during succeeding synchronization periods, the eventual steady-state rhythm had a phase of 360° to the last pulse (Fig. 5).

The phase delay which occurs after a light interruption of the synchronization period is explained by the finding that the occurence of light during this period serves to immediately stop the clock (Truman, 1971d). This was demonstrated by experiments similar

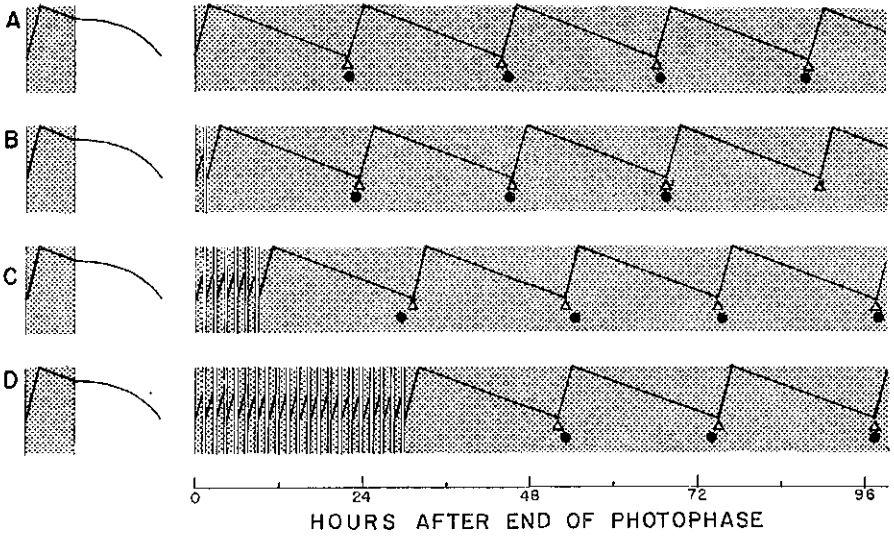

Fig. 5. The response of the Pernyi eclosion hormone rhythm to ½ hour light pulses given during the *synchronization period* of the circadian cycle. A: moths transferred from a 17L:7D regimen into continuous darkness; B: one light pulse applied after one hour of darkness; C: six successive pulses given – one hour of darkness between pulses; D: twenty successive pulses given – one hour of darkness between pulses. Open triangles indicate median times of hormone release expected if the light pulse reset the clock. Filled circles are observed medians. Curves show the behavior of the eclosion clock under the experimental conditions. (From Truman, 1971d).

to those used to show that the *Drosophila* clock stops in continuous light (Pittendrigh, 1966: Fig. 7). *Antheraea pernyi* were exposed to a 17L:7D regimen during adult development. As summarized in Fig. 6, each population was then exposed to a light pulse beginning 1 hour into the night. At the end of the pulse, the animals were transferred to continuous darkness. If the clock was stopped during the pulse, then lights-off of the pulse should always act as an 'absolute phase-giver' (Pittendrigh, 1960). Thus, the steady-state eclosion rhythm should always have a phase relationship of 360° to the end of the pulse. Alternatively, if the clock was running, then a lengthening of the pulse should eventually cause a phase advance in the eclosion rhythm. As is evident in Fig. 6, the resulting rhythms always showed a phase of 360° relative to the end of the pulse. If instead of one hour, 23 hours (the time necessary for one complete circadian cycle plus 1 hour) elapse before the light interruption, the resultant steady-states all have a phase of 360° to the end of the pulse (Truman, 1971d). These data strongly support the interpretation that the occurrence of light during the synchronization period serves to stop the driving mechanism of the clock.

A dark obligatory process at the beginning of the circadian cycle would demand a slope of 1 in the phase-response curve at the beginning of the subjective night. Such a slope occurs in the function determined for *Antheraea pernyi* eclosion (Truman, 1971d) and for *Drosophila* eclosion (Pittendrigh, 1965: Fig. 5). It is of interest that this

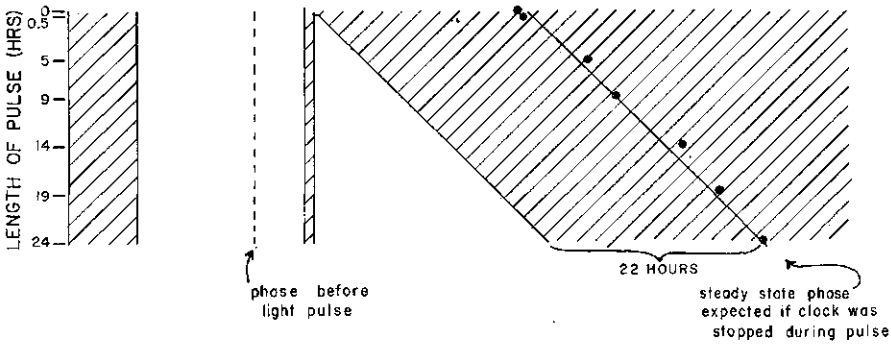

Fig. 6. Experiment showing that a light interruption during the synchronization period of the circadian cycle stops the driving mechanism of the clock. After the interruption the steady-state rhythms always showed a phase of 360° to lights-off, regardless of the length of the pulse. The observed phase of the steady-state rhythm is given as closed circles. For further explanation see text.

characteristic slope is also evident in the *Drosophila* phase-response curve obtained by a flash of high intensity light of 1/2000 second duration (Pittendrigh, 1960). Thus, in some cases this process can be instantaneously reversed.

The effects of light on the scotonon are summarized in Fig. 7. In photoperiods having very short scotophases, the synchronization period is not completed during the night and the driving mechanism of the clock stops at lights-on. The broad spread of eclosion under these conditions must then occur by the uncoupling and fade-out of any driven components in the system. As darkness is increased, increasing numbers of animals complete the synchronization period and thus the peak narrows. After attainment of the dark decay portion of the scotonon, the onset of light only serves to alter the kinetics of decay as defined by the relation of the photonon to the scotonon (Truman, 1971a).

Involvement of a photosensitive molecule in the mechanism of the eclosion clock?

It is common phenomenon in plants that rhythms fade out in continuous bright light (Wilkins, 1960). To restart the rhythm, a definite number of hours of interrupted darkness is necessary (Bünning, 1967). The above results show that the need for darkness is also a characteristic of the eclosion clock, but more importantly this period of

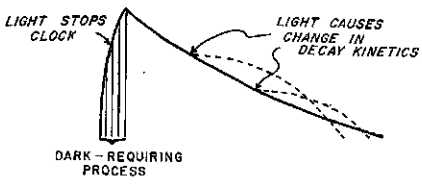

Fig. 7. Summary of the response of the driving mechanism of the eclosion clock to light. The onset of light during the *synchronization period* stops the clock. A light interruption during the *dark deday period* alters the kinetics of the remainder of the cycle. (From Truman, 1971d).

darkness is an indispensible requirement for each cycle of the driving mechanism. Without it, the driving mechanism is stopped. The existence of a definite 'dark process' in the clock cycle suggests that a photosensitive molecule may participate in the clock mechanism. Moreover, the fact that at the end of the pulse the clock restarts from the beginning of the cycle and not from the point that it stopped shows that light can rapidly reverse all of the happenings during the first few hours.

The kinds of circadian clocks in animals

The presence of a dark-dependent process in the driving mechanism of the eclosion clock is in marked contrast to the cockroach activity clock. In the latter case the activity rhythm persists for weeks in continuous light (Roberts, 1960). This ability to continue to oscillate in continuous light is achieved because the clock mechanism is completely insensitive to light. As discussed above, phase-setting of this clock is accomplished only through the participation of the compound eyes and, thus, only through synaptic interaction. In the absence of this input, such as occurs when the optic nerves are severed, the clock cannot be entrained by a light-dark regimen (Nishiitsusuji-Uwo and Pittendrigh, 1968a). Indeed, even if the light is allowed direct access to the brain by replacing part of the head cuticle with a transparent window, the rhythm still free-runs (Nishiitsusuji-Uwo and Pittendrigh, 1968a). The persistence of the rhythm in continuous light and the failure of the clock to be entrained without the participation of a peripheral photoreceptor precludes the involvement of a photosensitive molecule in the cockroach clock mechanism.

Differences in the two clocks are further underlined by a comparison of their phase-response curves. This function, obtained by subjecting the free-running clock to single pulses of light, has been used as a parameter of the circadian mechanism (Pittendrigh, 1966). In *Drosophila* a light pulse applied at certain points of a cycle can generate phase shifts of up to 10 hours in the eclosion rhythm (Pittendrigh, 1965). By contrast, interruption of the cockroach activity rhythm gives phase shifts which are about two hours at a maximum (Roberts, 1962).

In general, the clocks which have been studied in animals fall into one of two groups, similar either to the eclosion clock or to the cockroach activity clock. Unfortunately, for most of the systems reported, there are gaps in the information as to the location of the clock, the manner of reception of light information, the effect of continuous light, and the phase-response curve. Therefore, some assignments to one type or the other must be considered tentative.

Type I clocks

Clock mechanisms which stop in moderate intensities of continuous light are included under Type I clocks. To date the only animal clocks which have been adequately examined and shown to stop in continuous light are the eclosion clocks of *Drosophila* (Pittendrigh, 1966) and *Antheraea pernyi*. But as indicated in Table 1 several other rhythms have characteristics similar to the eclosion rhythm. It is of interest that these

Table 1. Rhythms controlled by Type I clocks.

Organism	Rhythm	Location of clock	Site of photosensitivity	Length of max. $\Delta\varphi$	Effect of LL	Authors
Unicellular						
Gonyaulax	luminescence	–	–	8 h	arrhythmia above 100 lux	Hastings and Sweeney, 1958
Euglena	phototaxis	–	–	> 8 h		Bruce and Pittendrigh, 1958
Paramecium	mating reaction	–	–	> 6 h	arrhythmia arrhythmia	Bruce and Pittendrigh, 1957 Ehret, 1959
Multicellular						
Drosophila	eclosion	?	brain (?)	10 h		Zimmerman and Ives, 1971 Pittendrigh, 1965
					arrhythmia	Pittendrigh, 1966
Silkmoth	eclosion	cerebral lobes of brain (?)	brain			Truman and Riddiford, 1970; Truman, 1971c
				4 h	arrhythmia	Truman, 1971d
Pectinophora	oviposition	?	?	$2\frac{1}{2}$ h[1]	?	Minis, 1965
Carausius	activity	cerebral lobes of brain (?)	cerebral lobes (?)[2]	?	arrhythmia	Eidman, 1956

[1] The phase response curve of *Pectinophora* has a slope of 1 at the beginning of the subjective night.
[2] The data are not sufficiently detailed to determine whether the clock was entrained or free-running after the eyes were removed.

rhythms include those of unicellular organisms. Of the three listed here, a complete phase-response curve has been determined only for *Gonyaulax polyhedra* (Hastings and Sweeny, 1968). As with the eclosion rhythm, the slope of the *Gonyaulax* phase-response curve at the beginning of the subjective night is 1. The fragmentary data available for *Euglena* and *Paramecium* are also of great interest. In *Euglena* an eight-hour light pulse started at the beginning of the subjective night gives a phase delay of approximately 8 hours (Bruce and Pittendrigh, 1958: Fig. 9). Similarly, if the *Paramecium* mating rhythm is interrupted by a light pulse early in the subjective night, the resultant phase delay (Ehret, 1959) is consistent with that expected if the clock was restarted by the pulse. Thus, the clocks of all three unicellular organisms are apparently reset by light interruptions occurring in the early part of the night.

In one-celled organisms, of course, the clock and photoreceptive mechanism must reside in the same cell. The strong similarities between these unicellular rhythms and some rhythms in higher animals suggest that perhaps in the latter cases, also, the light-sensitive gating mechanism may be contained entirely within the same cell.

Type II clocks

This type of clock has yet to be described from any protist and is apparently the product of a complex nervous system. Characteristically, rhythms controlled by this clock free-run in both DD and LL (but see section 'The interaction of photoreceptors...)'. In a number of cases the clock has been surgically isolated from its site of photosensitivity; therefore, the complete gating mechanism is clearly not unicellular. Besides the above-mentioned case of the cockroach activity rhythm, the dissociation of the photoreceptor from the clock is clearly evident in the sea hare, *Aplysia californica*. Strumwasser (1965) has demonstrated that a nerve cell in the parieto-visceral ganglion shows a circadian rhythm of electrical activity even after the ganglion is placed in organ culture. This rhythm is evident after the intact animal has been exposed to a week of continuous light. It is important to note that the clock cannot be phase-shifted or entrained by light in vitro but only in vivo. The ganglion contains the clock but not the photoreceptor for the clock. Thus, in *Aplysia*, as in the cockroach, the clock is sensitive to, and phase-shifted by, synaptic input but not light.

Table 2 summarizes the characteristics of many type II clocks. As is evident from the Table, there are large gaps of information in most systems. The phase-response curves show that in general these clocks are more stable to interrupting light perturbations since the maximum phase-shifts are usually small. But the phase-response curve of *Passer domesticus* (Eskin, 1969) indicates that this need not necessarily be so.

Roles of the two types of clocks in insects

Fig. 8 serves as an oversimplified summary of the roles of these two types of clocks in insects. Type I clocks appear to be associated primarily with developmental rhythms. Thus, events such as hatching, eclosion, and the release of the brain hormone are probably controlled by Type I clocks. Type II clocks are generally involved with activity

Table 2. Rhythms controlled by Type II clocks.

Organism	Rhythm	Location of clock	Site of photosensitivity	Length of max. $\Delta \varphi$	Effect of LL	Authors
Aplysia	electrical activity	Cell 3 of parieto-visceral ganglion	external to parieto-visceral ganglion	?	rhythm persists	Strumwasser, 1965
Cockroach	locomotor activity	Optic lobes of brain (?)				Nishiitsusuji-Uwo and Pittendrigh, 1968b
			Compound eyes			Roberts, 1965, Nishiitsusuji-Uwo and Pittendrigh, 1968a
				2 h		Roberts, 1962
					rhythm persists	Roberts, 1960
Gryllus	activity	?	Compound eyes and ocelli	?	rhythm persists	Nowosielski and Patton, 1963
Passer	activity	Pineal (?)				Gaston and Menaker, 1968
			extra-retinal receptor (not pineal)		arrhythmia above 500 lux[1]	Menaker, 1968
				8 h		Eskin, 1969
Glaucomys	activity	?	?	2 h	rhythm persists	De Coursey, 1961
Mesocricetus	activity	?	?	1½ h	?	De Coursey, 1964
Rat	activity	?	eyes	?	rhythm persists	Browman, 1937

[1] Arrhythmia is due not to the stoppage of the clock but the masking effect of light (see p. 125).

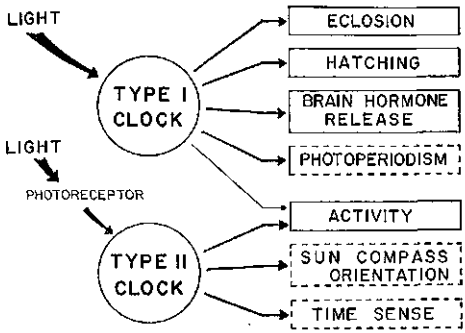

Fig. 8. A generalized summary of the roles of the two types of circadian clocks in insects.

rhythms. But it should be noted that the activity rhythm of the walking stick may be controlled by a Type I clock; so, also, the oviposition rhythm in the pink bollworm, *Pectinophora* (Table 1).

In addition to the obviously rhythmic functions, phenomena such as photoperiodism, the 'time-sense,' and sun compass orientation may have developed from one or the other type of clock. In the Pernyi moth the photoperiodic termination of diapause has many similarities with the eclosion rhythm (Truman, 1971c). Similarly, many parallels can be drawn between photoperiodism and oviposition in *Pectinophora* (Pittendrigh and Minis, 1964, 1971). Additionally, as indicated above, the hour-glass behavior of the eclosion clock has many points of similarity with the system described by Lees (1971) for *Megoura*. At present there are no data from insects which indicate from which type of clock the time sense and sun compass orientation were derived. They have been included with Type II clocks solely because of their association with locomotor activity.

The interaction of photoreceptors in the expression of a rhythm

According to Aschoff (1960), light can influence a rhythm in two ways: it can act as a 'Zeitgeber' to determine the phase of the rhythm relative to the photoperiod, and it can exert a masking effect. As an example of the latter, Aschoff cites his work on the activity rhythm of the green finch. The activity of this bird is inhibited by darkness. Consequently, when light was alternated with darkness, the bird always began activity at lights-on and stopped at lights-off, irrespective of the photoperiod. By contrast, when dim light was used rather than darkness, the effect of the photoperiod in determining the phase of the rhythm became evident. The onset of the bird's activity could then be seen to vary with the photoperiod as is characteristic of clock-controlled events (Aschoff, 1965). Thus, the phase setting of the clock was masked by the overriding inhibitory action of darkness.

Role of the eyes in the partial masking of the eclosion rhythm

Light also exerts a masking effect in the eclosion rhythm of some species of silkmoths. As shown in Fig. 9A the distribution of eclosion of Cecropia moths shows a strong skewing towards lights-on. This effect is more striking when one remembers that, in this species, eclosion is the result of the release of the eclosion hormone $1\frac{1}{2}$ hours earlier (Truman and Riddiford, 1970). Thus, in much of the population the response to lights-on is practically immediate. As described above, the photosensitive mechanism responsible for the phase-setting of the Cecropia eclosion rhythm is located in the median portion of the moth brain. Therefore, it is of interest to determine if this stimulatory effect of lights-on is mediated through the same photosensitive system (Truman, 1971e).

The sensory basis of the lights-on effect was determined by isolating the Cecropia brain from different sources of nervous input. As seen in Fig. 9C after transection of the optic nerves, the response to lights-on was markedly reduced. The severing of the circumesophageal connectives (Fig. 9B) did not have this effect.

Although these results suggested that the lights-on effect was mediated through the eyes, their interpretation was complicated by the possibility of regeneration of the cut nerves. In order to rule out regeneration, the brain was transplanted to the tip of the abdomen. As seen in Fig. 9D, these loose-brain animals showed a well-defined eclosion peak which was not skewed towards lights-on. But, again, this experiment was not completely satisfactory because a loose-brain animal differed from an intact moth in more respects than just the absence of connections with the compound eyes. Therefore, I attempted to reestablish the stimulatory effect of lights-on in brainless moths by implanting the brain with its attached eye imaginal discs. After metamorphosis, the brain implants were supplied with well-formed, albeit inverted, compound eyes.[1] As

[1] Eichenbaum and Goldsmith (1968) have demonstrated that in *Musca* the retinular cells of 'inside-out' eyes give normal electrical responses to illumination.

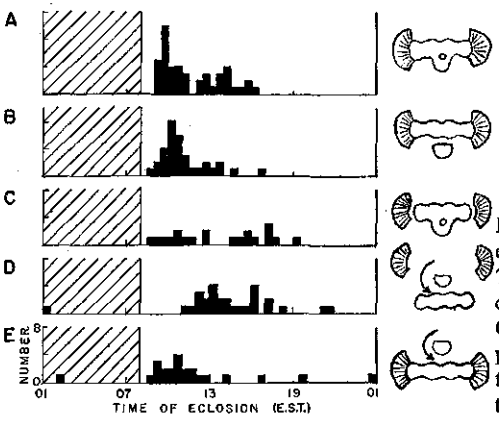

Fig. 9. The influence of the eyes in the emergence of Cecropia moths in a 17L: 7D regimen: A: Normal moths; B: circumesophageal connectives transected; C: optic nerves severed; D: brain transplanted to abdomen; E: brain with attached eye imaginal discs transplanted to the abdomen.

seen in Fig. 9E, the immediate response to lights-on was restored in these preparations. Thus, we see that this immediate response occurs only through the mediation of a second photoreceptor – the compound eyes.

Masking by continuous light

In the discussion of the two types of circadian clocks, it was noted that Type II clocks could free-run in both DD and LL. But there are examples of rythms which are apparently controlled by Type II clocks but which become arrhythmic under continuous bright light. However, in those cases which have been studied in sufficient detail, it is clear that the arrhythmia is not due to a stoppage of the clock. Rather, in a manner similar to what we have seen above, this condition is secondarily produced by the overriding influence from another photoreceptor. This can be most clearly seen in the work of Menaker (1968) on the perching rhythm of *Passer domesticus*.

In this bird constant light of 500 lux or above causes continuous activity and no perching rhythm is evident. Menaker has shown that this rhythm is entrained through an extraretinal photoreceptor and, thus, blinded birds perceive and entrain to photoperiod cycles in an essentially normal fashion. It is then of interest that these blinded birds continue to show a free-running rhythm of activity at intensities of constant light as high as 2,000 lux. Thus, the activity clock is capable of free-running in continuous bright light, but its output is normally masked by a stimulatory effect of light mediated through the eyes.

In mammals one finds similar examples. Normally, in the rat a rhythm of pineal serotonin persists in continuous darkness but not in continuous light. After removal of the eyes, the rhythm persists under both conditions (Snyder et al., 1965). In this case both the phase-setting and masking effects of light are apparently mediated through the same receptor, the eyes.

The physiological basis for the two effects of light are schematically summarized in Fig. 10. The photoperiod can phase-set the clock either by acting directly on it (Type

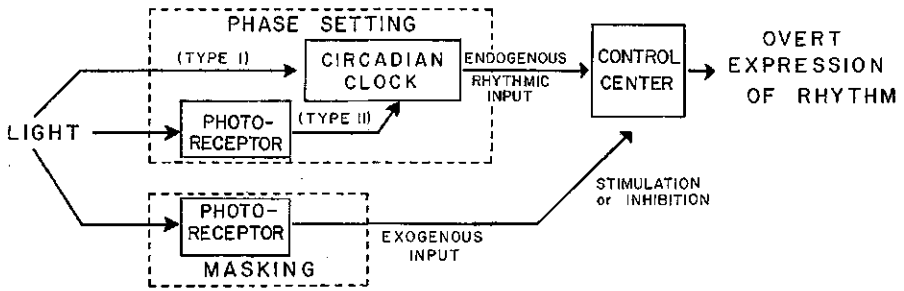

Fig. 10. A hypothetical scheme showing the physiological basis of the phase-setting and the masking effects of light.

I clocks) or through a peripheral photoreceptor (Type II clocks). A rhythmic daily input is then fed into a hypothetical control center for the particular rhythm. Information on contemporary conditions of illumination is also supplied to this control center. These two inputs then interact to produce the overt expression of the rhythm.

Role of clocks in the physiology of insects

The insect brain

As the major integrative center of sensory information, the brain a priori becomes the main candidate for the location of biological clocks in insects. In the cockroach, we have seen that the activity rhythm is controlled by clocks centered in the optic lobes of the brain (Nishiitsusuji-Uwo and Pittendrigh, 1968b). The rhythm is then apparently enforced through a rhythmic outflow of electrical activity from the clock (Brady, 1969). Rhythmical daily increases in electrical activity have been recorded from insect brains (Azaryan and Tyshchenko, 1969), but the significance of such recordings has yet to be proved. But, contrary to the assertion by Brady (1969), all major insect rhythms are not mediated electrically; and the emphasis of this paper has been on clocks which are expressed hormonally.

The brain hormone – ecdysone axis. Brain hormone and ecdysone are two of the three classical insect hormones and constitute the prime movers in the periodic molting of the insect cuticle (Schneiderman and Gilbert, 1964). Most of the studies of rhythmic secretion of the brain hormone have been based on histological correlations of changes in nuclear volume of the brain neurosecretory cells (Rensing et al., 1965). But in the insect brain there are at least 4 histologically distinct types of neurosecretory cells in the median area (Herman and Gilbert, 1965), and the specific cell type involved in brain hormone synthesis is yet to be determined. Perhaps more pertinent studies on rhythms of brain hormone secretion have involved the daily fluctuations in the size of an ecdysone-sensitive puff in the salivary glands of *Drosophila*. From these rhythmic changes one can infer a daily rhythm of ecdysone release and accordingly, a daily rhythm of brain hormone release (Rensing, 1971).

A direct determination of the time of brain hormone and ecdysone release was performed on the last instar larva of the sphinx moth, *Manduca sexta* which were exposed to a 12L:12D regimen. At two-hour intervals through the day, larvae were ligatured behind the head and between the second and third abdominal segment. According to Williams (1952), the subsequent pupation of the thoracic compartment indicates sufficient quantities of blood-borne brain hormone were present to activate the prothoracic glands; pupation of the abdominal compartment occurs only after sufficient ecdysone has been secreted from the prothoracic glands. Using this technique, the data in Fig. 11 were obtained (Truman and Riddiford, 1971). It is clear that the brain hormone is released only during a specific period of the day. Moreover, the rhythmicity of release of brain hormone has enforced upon the prothoracic glands a rhythmic secretion of ecdysone.

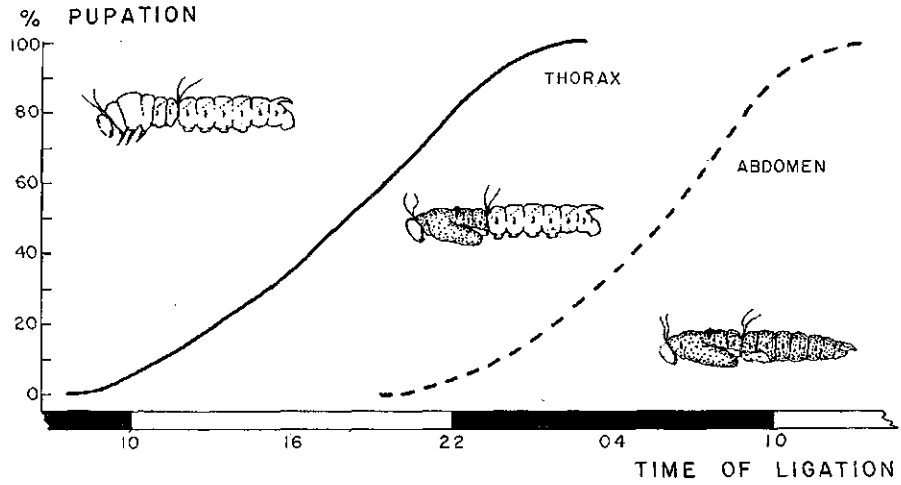

Fig. 11. The timing of brain hormone and ecdysone release by prepupal *Manduca sexta* in a 12L:12D regimen. Pupation of the thorax (solid line) indicates sufficient brain hormone was released to activate the prothoracic glands. Pupation of the abdomen (dashed line) occurs after secretion of a sufficient titer of ecdysone from the prothoracic glands.

The eclosion hormone – bursicon axis. The finding that the brain is capable of gating eclosion even when implanted into the abdomen indicated that the output of the eclosion clock was hormonal. This inference was confirmed by the demonstration that homogenates prepared from the brains of pharate moths serve to stimulate eclosion precociously (Truman and Riddiford, 1970; Truman, 1971b). In the saturniids eclosion follows $1\frac{1}{2}$ to 2 hours after hormone injection; *Manduca* has a lag period of 4 to 6 hours.

The eclosion hormone can be extracted from the brain of Pernyi moths during the latter two-thirds of adult development. During this period hormone is apparently shipped down the axons of the neurosecretory cells and stored in the corpora cardiaca, the neurohaemal organs of the brain. On the day of eclosion, the hormone titer in the corpora cardiaca reaches a maximum. Then at the moment prescribed by the clock, the corpora cardiaca empty and hormone appears in the blood (Truman, 1970). The effects of the liberated hormone will be considered in the next section. Of special interest here is the relation between the eclosion hormone and another insect hormone, bursicon. The latter hormone is responsible for the tanning of the cuticle of freshly emerged insects (Fraenkel and Hsiao, 1962; Cottrell, 1962) and, accordingly, provokes the tanning and hardening of the wings of a freshly emerged moth (Truman, 1971f).

In the sphinx moth, bursicon is produced in the abdominal portion of the nerve cord and is secreted into the blood within 15 minutes after eclosion (Truman, 1971f). As would be expected by the relationship of bursicon release to emergence, the former is dependent upon the prior secretion of the eclosion hormone. Thus, if the pupal cuticle is removed from a pharate sphinx moth during the afternoon preceding its normal eclo-

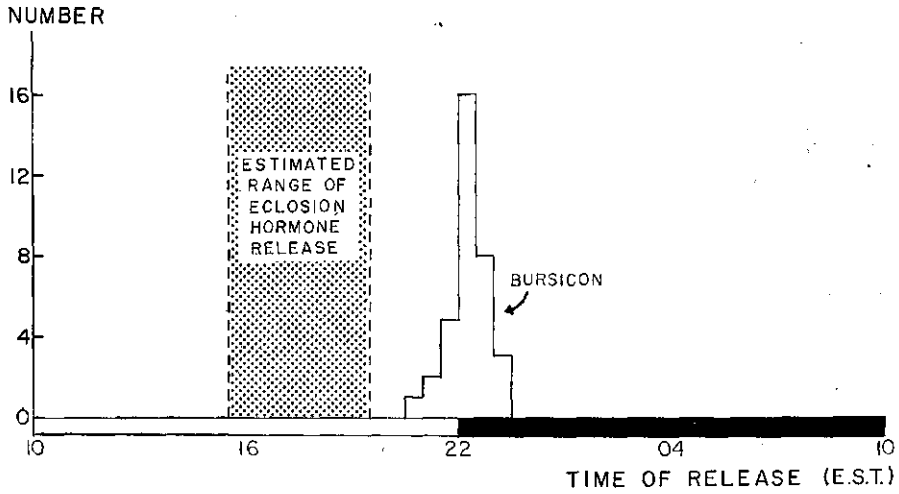

Fig. 12. Timing of eclosion hormone and Bursicon release by *Manduca sexta* in a 12L:12D regimen. The estimation of eclosion hormone release was based on the response of moths to injected hormone. The time of bursicon release was determined by behavioral markers.

sion gate, busicon will not be released at that time. But after its gate that evening, and therefore after exposure to the eclosion hormone, bursicon is liberated. Accordingly, the early injection of the eclosion hormone causes the precocious release of the tanning hormone (Truman, 1971f).

As shown in Fig. 12, the secretion of eclosion hormone and bursicon occurs only during specific periods of the day; as with the above case of brain hormone and ecdysone, only the brain-centered system is controlled by a clock. The timed release of the eclosion hormone then enforces a rhythmic release of bursicon from the abdominal nerve cord.

The physiology of eclosion

Adult eclosion: a gated event. Pittendrigh and Skopic (1970) have termed adult eclosion a gated event. They have shown that in *Drosophila* the act of eclosion is not simply the terminal step in the developmental sequence which transforms the maggot into the fly. Apparently, development is completed without respect to time of day, but eclosion occurs only in periods dictated by the clock. Thus, flies which finish development too late to exploit one gate must then wait until the gate on the following day.

The separation of the timing of eclosion from the developmental schedule can also be demonstrated by injecting the eclosion hormone into pharate Pernyi moths 8 to 10 hours before their normal gate. The fact that these injections invariably provoke eclosion within two hours clearly demonstrates that these moths were developmentally

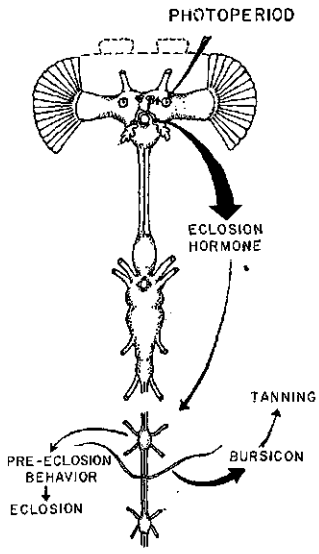

Fig. 13. A summary of the steps involved in the control of moth eclosion.

competent to emerge prior to their normal time. Manifestly, these moths were simply 'waiting' for the hormonal signal from the clock.

The normal sequence of events during adult eclosion. The changes accompanying eclosion are primarily behavioral. These have been recently reported elsewhere (Truman, 1971b) and thus will only be summarized here. After release of the eclosion hormone, the moth begins a stereotyped series of abdominal movements the pre-eclosion behavior. In the silkmoths, these patterns are species-specific and last approximately 1¼ hours. At the end of this behavior, eclosion occurs. The pupal cuticle is ruptured along the dorsal mid-line by a vigorous 'shrugging' of the wing bases. The moths then sheds the pupal skin through vigorous peristaltic-like movements of the abdomen. It is of interest that if an isolated pharate abdomen is injected with the eclosion hormone, this fragment will perform the pre-eclosion behavior and then shed the surrounding pupal cuticle (Truman, 1971b). After eclosion, the moth struggles through the surrounding cocoon, comes to rest and expands its wings.

The necessity of passing through the gate. The hormonal output of the eclosion clock is essential for the proper functioning of the adult moth. One way of demonstrating this is by manually removing the pupal cuticle from a pharate Pernyi prior to its eclosion gate. These peeled moths do not spread their wings, make little attempt to move, and in essence display little of the behaviour typical of the moth. But at the arrival of their gate, the entire sequence associated with eclosion and the escape from the cocoon is read off. At its termination, the animals then display the normal moth behaviour. Thus, even though development is complete and the pupal cuticle removed, these peeled

pharate moths cannot assume the moth behavior until exposed to the eclosion hormone.

Another way of demonstrating the necessity of the gate is by removing the gating mechanism – i.e., by extirpation of the brain. In this case some parts of the behavioral sequence associated with eclosion still occur, but other parts are absent or occur out of sequence. In brainless Cecropia the pre-eclosion behavior is rarely displayed. Moreover, eclosion often occurs prior to the complete resorption of the molting fluid so that many moths emerge soaking wet. A disastrous occurrence is the occasional degeneration of the intersegmental muscles of the abdomen (an event which normally takes place after eclosion; Lockshin and Williams, 1965) prior to eclosion. Since these muscles are needed for emergence, the moth is trapped inside its own cuticle.

The chaotic series of happenings which are observed during the emergence of brainless moths are not evident in 'loose-brain' animals. In the latter moths, events associated with eclosion proceed in an orderly fashion. Thus, the hormonal output from the clock serves to co-ordinate various processes related to eclosion and ensures a smooth transition from the pharate to the adult condition.

Summary

The activity rhythm of the cockroach is apparently controlled by a clock located in the optic lobe area of the brain. This clock is entrained not by light but by synaptic input from the compound eyes. The output of the clock is probably electrical since intact nerve pathways from the optic lobes to the thorax are prerequisite for the expression of the activity rhythm. The eclosion rhythm of silkmoths is also controlled by a brain-centered clock, although in this case the clock is probably in the cerebral lobes. Unlike in the cockroach, the phase-setting of the eclosion clock is accomplished by light perceived directly by the brain itself. The clock, in turn, controls the release of the 'eclosion hormone' from the brain.

A preliminary analysis of the silkmoth eclosion clock demonstrated that the free-running cycle of the clock could be divided into two parts: an initial *synchronization period* and a *dark-decay period.* Under photoperiod conditions, the completion of the first part is essential for the maximal accuracy of the eclosion clock. Further experiments have shown that the synchronization period has an obligatory dependence upon darkness. A light interruption during this period serves to stop the driving mechanism of the clock. Thus, the beginning of each cycle of the eclosion clock involves a dark-dependent step which must be completed in order for that cycle to be completed.

The presence of a dark-dependent step in the eclosion clock is in marked contrast to the cockroach activity clock which can free-run for months in continuous light. This ability of the latter is due to the fact that light sensitivity is external to the driving mechanism itself, i.e., light information is transmitted synaptically to the clock by the eyes. These two differences in the mode of action of light on the clock has led to the division of animal clocks into two categories. The first type includes clock mechanisms which are stopped in continuous light and thus must have a 'dark process'. Interestingly, the unicellular organisms which have been studied to this point also appear to

possess this type of clock. The second type involves clocks which can free-run in continuous light. These mechanisms are entrained by synaptic input from photoreceptors which are external to the clock mechanism. The second type of clock is the major clock found in higher animals.

The rhythmic output from a biological clock can be drastically modified through the influence of other photoreceptors. In the case of the eclosion rhythm of the Cecropia moth, the onset of light stimulates emergence, and thus the eclosion peak is skewed towards this signal. Removal of the compound eyes abolishes this immediate response to lights-on, although the ability of the clock to perceive the photoperiod remains. A similar system exists in birds. In the sparrow continuous light enforces continuous activity and thus abolishes the activity rhythm. After removal of the eyes, an operation which does not interfere with entrainment of the rhythm by photoperiod, a well-defined activity rhythm persists even under continuous bright light. Thus in both examples the output of the biological clock is masked to a greater or lesser degree by the overriding effect of another photoreceptor.

The output of the brain-centered eclosion clock is a neurosecretory hormone, the eclosion hormone. In response to this hormone the moth begins a program of abdominal movements which leads to eclosion, the escape from the cocoon, and the spreading of the wings. Injection of the eclosion hormone into an animal prior to its normal eclosion time, provokes the precocious emergence of the animal. Thus, eclosion is truly a 'gated' event. Prior to the gate the moths are developmentally competent to emerge, but are 'waiting' for the signal from the clock.

The eclosion hormone is indispensible for the proper transition from the pharate adult to the adult state. If the pupal cuticle is removed from an animal before the release of the eclosion hormone, the animal continues to behave in a 'pupal' fashion until the passage of its gate. After this event adult behavior is assumed. Also, the removal of the gating mechanism by the extirpation of the brain yields a moth which, although it can eclose, cannot complete the transition to the adult condition.

Therefore, we see that the clock controlling eclosion is brain-centered and interacts with photoperiod in a defined manner to set the time of the eclosion gate. The output of the clock can be modified by other sensory input, and is eventually manifest by the release of the eclosion hormone from the brain. This chemical messenger then serves to activate a program in the nervous system which leads to the eclosion behavior and the co-ordinated transition from the pharate adult to the adult condition.

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Discussion

Participants: J. W. Truman (Author), G. Becht, W. Engelmann, K. Hoffmann, M. Raabe, D. S. Saunders, H. Schooneveld and B. M. Sweeney.

The location and nature of the photoreceptor for the eclosion clock is still an unanswered question. Studies with *Drosophila* (Zimmerman and Ives, 1971) have excluded the eyes from possible involvement in the entrainment of this clock, and the silkmoth work has localized the receptor to the cerebral lobe area of the brain. Its action spectrum has been published for the *Drosophila* rhythm, but such a study has not been pursued for the moth eclosion rhythm. In regards to a possible receptor pigment, the saturniid brain is colorless with the exception of dark pigment spots at the tip of each optic lobe. These aggregates are the remnants of the larval ocellar pigment and may be surgically removed without imparing the ability of the brain to perceive light.

It is obvious that the eclosion clock is not entrained through a conventional photoreceptor and, indeed, it is the contention of this paper that the photoreceptor may be part of the driving mechanism of the clock itself. As covered in the text, this conclusion was derived from the apparent photoreversibility of the first part of the clock cycle – the synchronization period. The exact relationship of duration and intensity of exposure to light to the stopping of the clock has yet to be worked out for the Pernyi moth.

In the case of subsaturating light of low intensity, one would expect that the driving

mechanism of the clock would still run but that the synchronization period would be lengthened. At first glance this appears to be counter to the observation that in *Gonyaulax* the free-running rhythm shortens in constant dim light. But since the dark decay period is also sensitive to light, the overall shortening of the cycle can be accounted for by invoking a compensatory change in kinetics during the decay part of the cycle.

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Circadian rhythms in unicellular organisms

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Abstract

The occurrence of circadian rhythms in unicellular organisms is documented. The properties of these rhythms are compared with those of rhythms in multicellular organisms with respect to the effects of physical and chemical factors on period and phase, and it is concluded that the same phenomenon is responsible for the generation of all circadian oscillations. What we know concerning this process from the study of unicellular rhythmic organisms is discussed.

Introduction

Circadian rhythms may be defined as oscillations with periods of about 24 hours which do not disappear in constant temperature and illumination. Such rhythms were recognized several hundred years ago from studies of the leaf movements of legumes. The presence of a similar phenomenon in unicellular organisms was discovered much more recently, probably because the observation of such small creatures requires more sophistocated methodology. In the last twenty years however, the list of unicellular autotrophs displaying rhythms has constantly grown longer, and a number of rhythms in heterotrophs have been recognized. A handful of these rhythmic organisms have been investigated in as much detail as any multicellular plant or animal.

It has been tacitly assumed that circadian rhythms in unicellular organisms are the same as those in multicellular. It seems to me that it might be useful to compare rhythms in unicells with those in multicellular organisms to see whether they are indeed wholly comparable. This is an important question, since an affirmative answer will restrict the kinds of theories which can be formulated to explain the generation of circadian oscillations. In addition, if we can accept unicellular organisms as truly representative of the phenomenon of circadian rhythmicity, it will be possible to take advantage of mass culture techniques and uniform material provided by many cultures of unicellular protists.

Examples of rhythms in unicellular organisms

Let us first briefly review what is known concerning rhythmic behavior in protists. Of all the unicellular organisms in which rhythms can be detected, perhaps *Euglena gracilis*

has been studied the longest and the most thoroughly. In 1948, Pohl reported that this motile green flagellate collected more rapidly in a light beam during the day than at night. Fluctuations in phototaxis continued in constant darkness interrupted briefly at intervals with a test light. A method for automatically recording the phototactic rhythm of *Euglena* was devised by Bruce and Pittendrigh (1956) and employed by them to investigate the effects of temperature level and temperature changes as well as other factors on periodicity. Recently, Brinkman (1966) has shown that the phototactic rhythm in *Euglena* as measured by Bruce and Pittendrigh is actually a composite of two rhythms, one in cellular motility and the other in responsiveness to light direction, more properly phototaxis (Fig. 1). These two rhythms do not have maxima at the same point in time. The amplitude of the rhythm in motility is the more pronounced. The motility of a colorless strain of *Euglena* is also rhythmic (Kirschstein, 1969).

Edmunds and his colleagues have studied cell division in *Euglena* and have demonstrated that this process is under the control of the circadian system. Cell division synchrony in *Euglena* can be attained by the proper choice of a light-dark cycle, usually close to LD 12:12. Cell division will continue to take place every 24 hours in continuous dim light, although the generation time for any one cell is longer than 24 hours, due to the low light intensity (Edmunds, 1966). In *Euglena*, a rhythm in cell division can also be maintained by exposing the cells to random light and dark periods (Edmunds and Funch, 1969). Cell division in a mutant strain of *Euglena* with defective photosynthesis is also rhythmic in continuous darkness or continuous light (Jarrett and Edmunds, 1970). In this mutant, the cell division rhythm can be observed after a single transition from continuous darkness to continuous light (Fig. 2).

A large proportion of the rhythms in unicellular photoautotrophs concern processes which involve light, like phototaxis, photosynthesis, or bioluminescence. This is not surprising if rhythmicity confers any selective advantage in regulating metabolism,

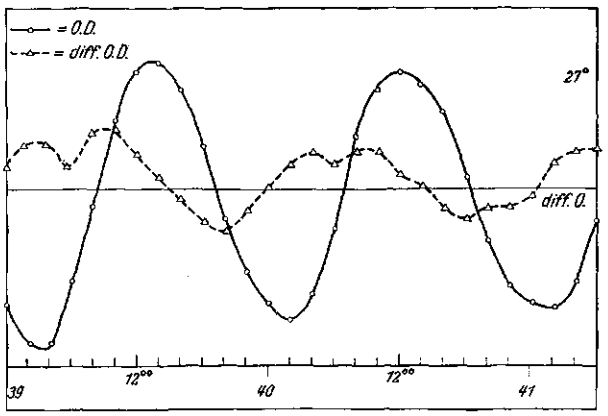

Fig. 1. The rhythm in motility (solid curve) and phototaxis (broken curve) in *Euglena gracilis* at 27°, in a light-dark cycle of 20 min test light, followed by 100 min darkness. (From Brinkmann, 1966.)

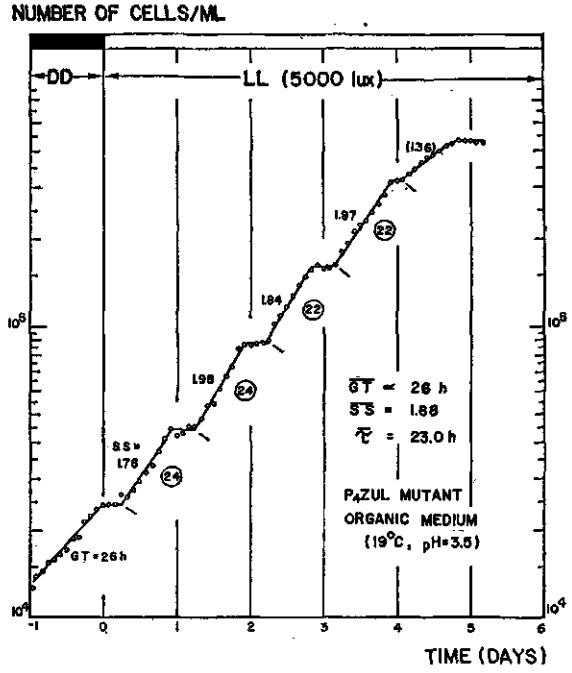

Fig. 2. The rhythm in cell division in a photosynthetic mutant of *Euglena gracilis*, initiated by a transfer from constant darkness to continuous light at 19°. The average period is 23.0 h and the average step size of the successive division bursts is 1.88. (From Jarrett and Edmunds, 1970).

since the processes dependent on light are the obvious candidates for circadian control. A number of examples of photosynthetic rhythms are found among the phytoplankton diatoms and dinoflagellates. So general in fact is this phenomenon that it has been possible to detect it in mixed samples of phythoplankton collected at sea, not necessarily of uniform species composition (Doty and Oguri, 1957). A rhythm in photosynthesis under laboratory conditions has been documented in the diatom *Phaeodactylum tricornutum* (Palmer et al., 1964) and in several dinoflagellates, most thoroughly in *Gonyaulax* (Fig. 3) (Hastings et al., 1961).

A number of the phytoplankton dinoflagellates are luminescent. Of these, at least three photosynthetic species, probably more, display bright luminescence only at night. (Biggley et al., 1969). In *Gonyaulax*, this behavior proves to be a true circadian rhythm which can continue for long times in continuous light and constant temperature, provided the light intensity is held below a critical value (Fig. 4) (Hastings and Sweeney, 1958). On the other hand, the non-photosynthetic dinoflagellate *Noctiluca* is brightly luminescent at all times of day, with no trace of rhythmic control (Nicol. 1958).

The brackish water dinoflagellate *Gyrodinium dorsum* responds phototactically to blue light. To be effective, the blue light must be preceeded by brief exposure to red

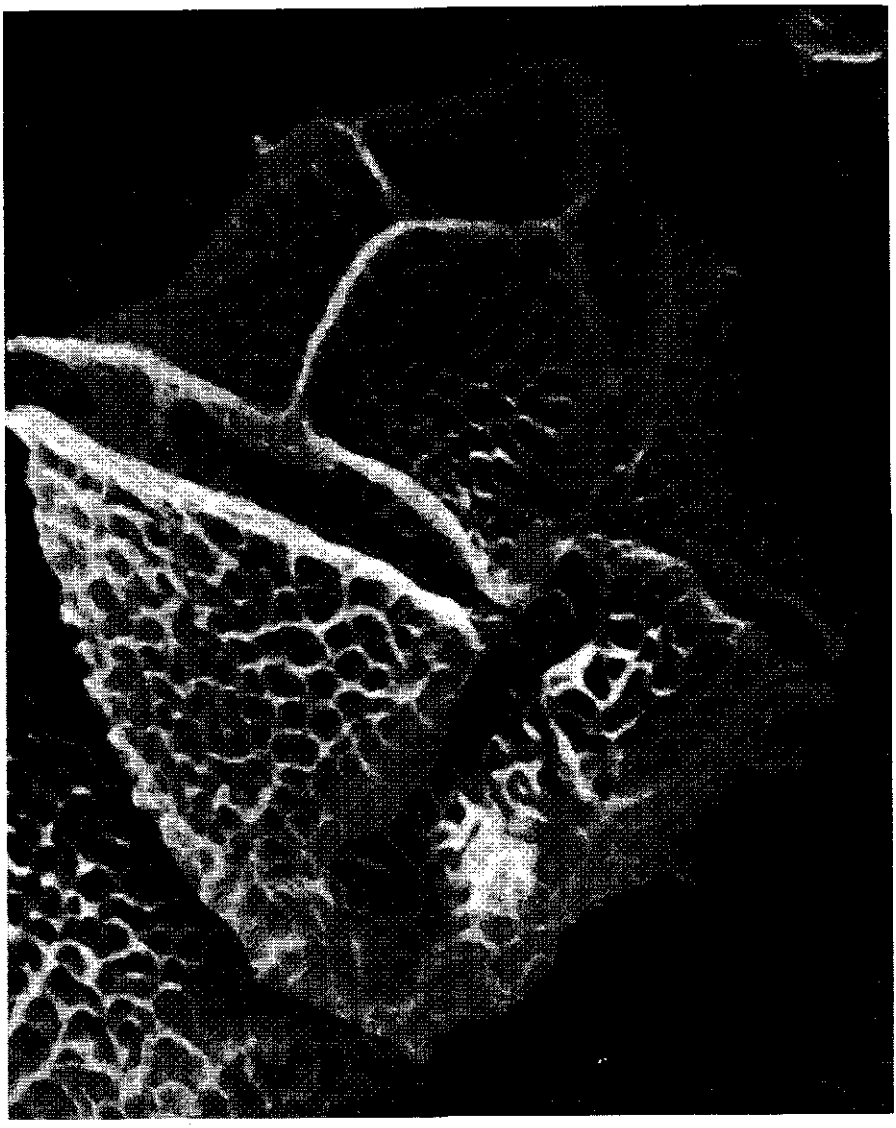

Fig. 3. *Gonyaulax polyedra* photographed with the scanning electron microscope by the author in the laboratory of Dr Preston Cloud, University of California, Santa Barbara. Magnification × 3500.

light. However, this red pretreatment is not effective at all times of day (Forward and Davenport, 1970). When *Gyrodinium* is grown in LD 12:12, it is not possible to potentiate phototaxis with red light during most of the dark period. In cultures maintained

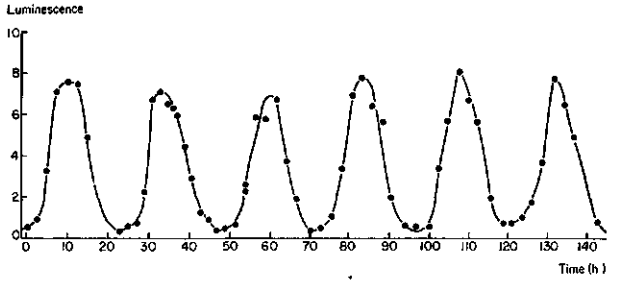

Fig. 4. The rhythm in stimulated luminescence in *Gonyaulax poleydra* in continuous light (1000 lux), 20°, data from previous publications, Sweeney and Hastings.

in continuous darkness, red light is only effective for a relatively short time close to dawn on the previous LD cycle, and two maxima in effectiveness 24 hours apart can be observed (Fig. 5). There is thus a circadian rhythm in activation of phototaxis by red light in *Gyrodinium*.

There is a very interesting class of circadian rhythms which are adapted to conform to tidal changes. These quasi-tidal rhythms are found in diatoms and a species of *Euglena* which inhabit tidal mud flats where the environment changes drastically with the state of the tide, (Round and Palmer, 1966). These organisms are found on the surface at low tide but disappear before the return of the water as high tide approaches. Transferred to the laboratory, they migrate but now with a typical circadian periodicity (Fig. 6), phased however by the last tide experienced in nature. Thus their phase is not primarily determined by light-dark changes but by factors changing with the tide, such as wetting of the substrate. The resetting effect of wetting has been demonstrated in the diatom *Surirella* by Hopkins (1966). Wetting the mud substrate when this diatom is on the surface causes it to migrate downward, not only immediately but also at about the

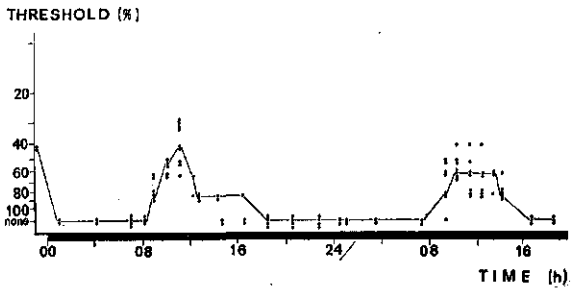

Fig. 5. The rhythm in responsiveness of phototaxis to red light activation in *Gyrodinium dorsum* in continuous darkness. Phototaxis is stimulated with blue light (470 mm). The light periods in the previous light-dark cycle were from 12.00 to 24.00 o'clock. Red preillumination was for 45 sec 620 nm light (8.1×10^{15} quanta cm^{-1} sec^{-1}). (From Forward and Davenport, 1970.)

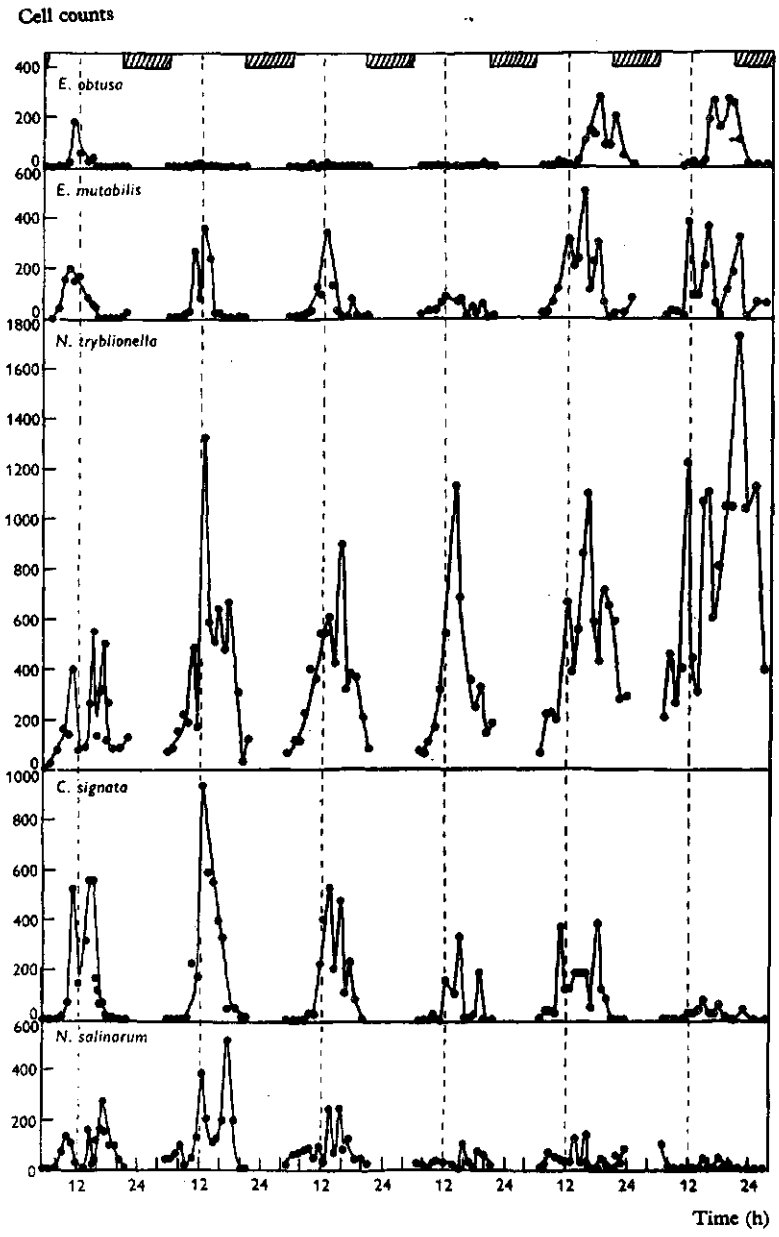

Fig. 6. The rhythm in migration of a number of organisms from the mud flats of the River Avon, measured in the laboratory in continuous light at 20° for 4 days and then at 12°, showing the circadian nature of the rhythm in the absence of tidal changes. Cross hatching on the upper abscissa indicates the natural dark periods where organisms were collected. E. = *Euglena*; N. = *Nitzschia*; C = *Cylindrotheca*. (From Round and Palmer, 1966.)

same time on the following day. Resetting only occurs however if *Surirella* has been illuminated during or immediately preceeding illumination. After a time in darkness, the downward migration can be determined by a light-dark cycle, taking place now toward the end of the light period. *Surirella* shows a circadian rhythm in both geotaxis and phototaxis, both of which contribute to its migratory behavior.

Another mud flat diatom *Hantzschia* also migrates up and down in time to the tides (Palmer and Round, 1967). In the laboratory, it continues to appear at the time of low tide, a little later each day, showing a rhythm with a period of about 24.8 hours. The phase is not dependent on the light-dark cycle and the rhythm is identical in LD and continuous light (Fig. 7). *Hantzschia* never surfaces at night, however. When it would be expected to show an upward migration at night, one period of the migration is omitted and a new migration begins in the morning (Fig. 7). Palmer and Round have interpreted this as evidence that migration is determined by a true tidal rhythm with a period of 12.4 hours which cannot be expressed during the night phase of the circadian rhythm.

The rhythms discussed so far are measured with populations of unicellular organisms. However, it is possible to demonstrate rhythms in single isolated cells. Photosynthesis in the large single celled alga *Acetabularia* is rhythmic, being several times more rapid during the day than at night even though in continuous artificial illumination (Sweeney and Haxo, 1961; Schweiger et al., 1964, Terborgh and McLeod, 1967;

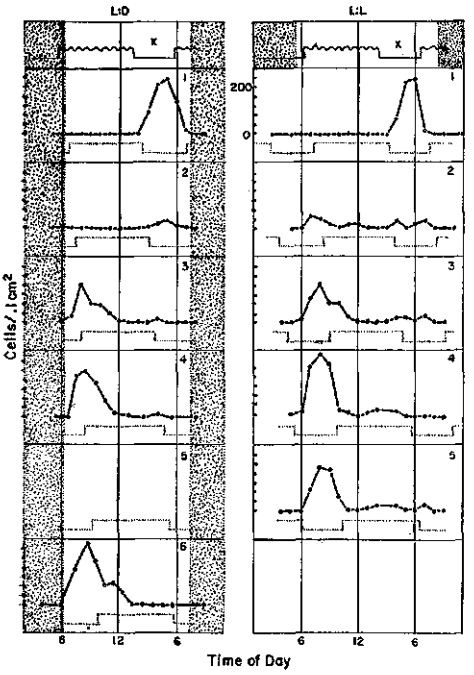

Fig. 7. The migration rhythm of the diatom *Hantzschia virgata* in the laboratory on LD 12:12 (left curves) and in continuous light (right curves), showing the 28.4-hour period under both conditions and the transition from evening to morning maximum between day 2 and 4. (From Palmer and Round, 1967).

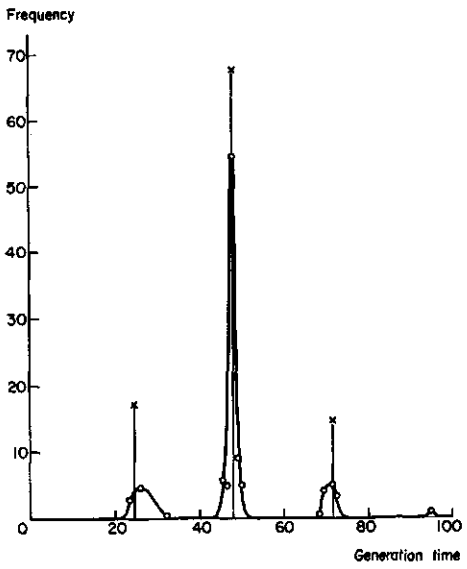

Fig. 8. The frequency (%) of different generation times (h) in single cells of *Gonyaulax polyedra* isolated in capillaries in LD 12:12. (From Hastings and Sweeney, 1964).

Hellebust et al., 1967). Since *Acetabularia* is such a large cell and sensitive methods for measuring gas exchange are available, it is quite possible to demonstrate a rhythm in oxygen evolution in a single *Acetabularia*. The special interest in this rhythm stems from the fact that *Acetabularia* can tolerate enucleation, and even grow and develop normally without a nucleus. The photosynthetic rhythm continues in enucleated cells. In *Gonyaulax* too, a photosynthetic rhythm can be demonstrated using a single isolated cell and the Cartesian reference diver technique (Sweeney, 1960). Gating of cell division by a circadian rhythm may also be seen in isolated cells in capillary tubes (Fig. 8) (Hastings and Sweeney, 1964). It is clear that a population is not necessary for the expression of rhythmicity in unicellular organisms.

Circadian rhythms have been found in several protozoans which are unable to carry out photosynthesis. For example, the mating of some *Paramecia* occurs periodically, even in constant conditions. (Ehret, 1959; Karakashian, 1968). One interesting species, *Paramecium multimicronucleatum*, changes its mating type with a circadian rhythm, being type IV during one phase and type III 12 hours later (Barnett, 1966). *Plasmodium*, the parasitic protozoan which causes malaria times its life cycle inside the tissues of the host so that the stage infecting mosquitos appears in the blood in the evening when mosquitoes are biting (Hawkins, 1970).

A comparison of rhythms in unicellular and multicellular organisms

These examples of circadian rhythmicities in unicellular organisms, while not an exhaustive list, should suffice to demonstrate that such rhythms are by no means rare or exotic. What of the mechanism by which these rhythms are generated? Is it the same as

in multicellular organisms? We know that oscillations are not generated as part of the biochemistry of photosynthesis, luminescence or any of the other process by which we measure rhythms, because changing the rates of these processes or modifying their biochemistry does not alter rhythmicity itself. Since we do not yet know by what process oscillations are produced, the detectible properties of the oscillator are restricted to its period and phase. In the circadian rhythms in multicellular organisms there is a considerable body of knowledge concerning how both period and phase may be altered by changes in physical and chemical factors. Knowledge of this kind is also available for unicellular organisms. A comparison of these experiments should answer the question whether rhythms in unicellular and multicellular organisms are fundamentally similar or different.

One of the most striking features of rhythms multicellular organisms, be they plants, insects, arthropods, or reptiles is the marked temperature independence of the period. (Sweeney and Hastings, 1960). It is of course more difficult to demonstrate this feature in mammalian rhythms because of their accurate regulation of body temperature, but experiments with hibernators such as bats suggests that this generalization holds (Rawson, 1960). Temperature independence is also characteristic of circadian rhythms in *Euglena*, *Gonyaulax* and *Paramecium*. In all types of rhythms, absolute temperature independence is the exception rather than the rule, however. In *Gonyaulax* for example, there is a small but consistent increase in the period with increasing temperature. (Hastings and Sweeney, 1957). Period length passes through a maximum at about 28°, an observation which suggests that temperature compensation accounts for the temperature independence of the period and that it is not quite perfect. Table 1 (Sweeney and Hastings, 1960 p. 90–91) shows the Q_{10} of the inverse of the period, the frequency, of a number of rhythms from both multicellular and unicellular rhythms. It can be seen that the variation of period with temperature is no more wide in unicells than in multicellular organisms.

Less completely studied than the dependence of the period on the ambient temperature is its dependence on light intensity under constant conditions. Aschoff (1960) has called attention to the generalization that the rhythms of nocturnal animals show longer periods in constant light than in continuous darkness, the length of the period being a function of the light intensity, while in diurnal animals, periods are shorter in light than in darkness. Unicellular organisms cannot be divided into diurnal and nocturnal species in this way. *Gonyaulax* for example might be termed 'night-active' with respect to luminescence and 'day-active' in its photosynthesis. The period of both rhythms shortens as the light intensity in constant light is increased, being longest in continuous darkness as any given temperature. (Hastings and Sweeney, 1958). 'Aschoff's rule' then applies to unicellular organisms only so far as dependence of the period on light intensity can be shown as in multicellular animals.

Although it would be both interesting and informative to compare the rhythms in unicellular and multicellular organisms with respect to their sensitivity toward biologically-active molecules, data is very scare. Only molecules which alter properties unequivocally linked to rhythmicity such as period and phase are of interest, since the suppression of a rhythm by an inhibitor may well be a secondary effect which makes the

expression of the rhythm impossible, rather than stopping the clock itself. There are very few substances among a large number which have been assayed which show any effect on period, and none which consistently alter phase. Of the few active molecules, none have been assayed on a wide variety of rhythms, with perhaps one exception, heavy water. The substitution of deuterium oxide for a part of the intracellular water has been shown to lengthen the period of such divers rhythms as phototaxis in *Euglena* (Bruce and Pittendrigh, 1960), leaf movement in *Phaseolus* (Bünning and Baltes, 1963; Bünning and Moser, 1968) and activity in *Peromyscus* (Suter and Rawson, 1968) and several species of birds (Palmer and Dowse, 1969). Recently, Enright (1971) has also demonstrated a similar effect on the tidal rhythm of the sand-beach isopod, *Excirolana chiltoni*.

A considerable number of studies have been devoted to the characterization of stimuli which cause phase changes in rhythms, generally known as 'Zeitgeber' since they serve to synchronize rhythms to external time. Changes in light intensity, and to a lesser extent temperature changes are the principal zeitgaber for circadian rhythms. A single short exposure to light is sufficient to change the phase of the *Drosophila* eclosion rhythm, (Pittendrigh and Bruce, 1957) the activity rhythm of the flying squirrel (DeCoursey, 1960) or leaf movement of *Phaseolus* (Bünning and Moser, 1966) or *Coleus* (Halaban, 1968). The amount and direction of the phase shift is dependent on the phase of the rhythm when the stimulus is given in a very characteristic way. This is best represented by plotting the phase shift as a function of the circadian time when the change occurred in a 'phase response' curve. Such a curve for *Coleus* leaf movement is shown in Fig. 9a. (Halaban, 1968), while beside it in Fig. 9b is the curve for *Gonyaulax* luminescent rhythm. In both, a single light exposure in otherwise continuous darkness is the stimulus. Phase shifting can also be brought about by interposing a dark period in a constant light regime. In this case, the sensitive phase of the cycle is the day phase

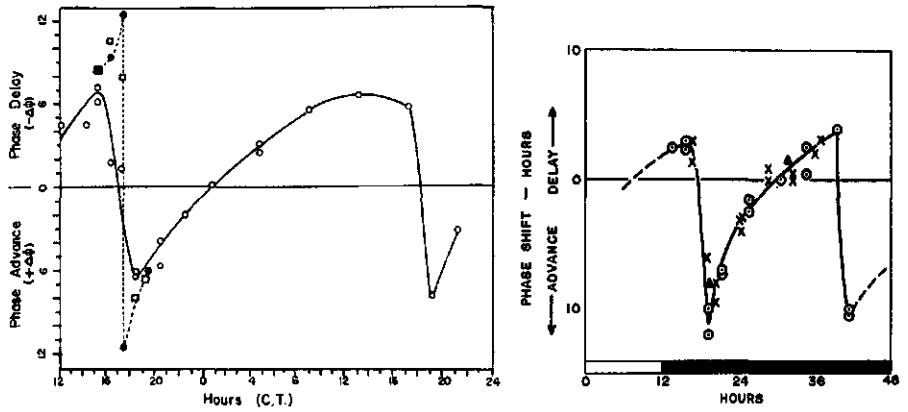

Fig. 9. Phase response curves for the leaf-movement rhythm in *Coleus blumei* × *C. frederici* (left, from Halaban, 1968) and the rhythm in stimulated luminescence in *Gonyaulax polyedra* (right, from data from previous publications, Sweeney).

and the characteristic phase response curve is displaced by 12 hours.

The action spectra for the effect of light on phase are easier to determine in unicellular organisms than in the more complex multicellular creatures where non-functional tissues may shade the cells where oscillations are generated. In *Gonyaulax*, (Hastings and Sweeney, 1960) the effective wave lengths are blue (maximum at 475 nm) and red (maximum at 650 nm). Action spectra have not been determined in detail in many organisms. Blue light is effective in moth (Bruce and Minis, 1969) and in fungal rhythms, (Sargent and Briggs, 1967), while red light shifts phase in the bean leaf movement rhythm (Bünning and Moser 1966). Halaban (1969) made the interesting observation that red light advances phase in *Coleus* while blue light delays phase. It is not clear yet what the receptor pigment is in vertebrates, but it does not appear to be in the eyes, Menaker, 1968). Rhythms in *Gonyaulax* (Sweeney, 1963) and *Paramecium* (Ehret, 1959) can be reset by ultraviolet light.

It seems safe to conclude from the close similarity between rhythms in unicellular and multicellular organisms that the underlying oscillation is generated in a similar way in all circadian systems. Thus unicells can be used in studies of the mechanism involved. There is, however, one feature of unicellular organisms which can be troublesome. If a population becomes synchonous with regard to the cell division cycle, mitosis and cytokinesis will occur periodically. As discussed below, there is good reason to consider the cell division cycle a different periodicity from circadian oscillations. However, the cell cycle in a number of populations of unicellular organisms can be synchronized by appropriate light-dark cycles, and even by single changes in light intensity. Once synchronized, cell division can continue in synchrony for several cycles in constant conditions, and hence resembles a circadian rhythm if the generation time is of the order of 24 hours. Varying the conditions will usually cause a continuous variation in the generation time in the absence of circadian control, however. In *Tetrahymena* for example, (Wille and Ehret, 1968), populations synchronized by light changes may show generation times of 21, 27.8 and 40.8 hours under various conditions (Fig. 10).

Coincident with the cell cycle there are many changes in metabolism, DNA synthesis, changing rates of photosynthesis and changing pigment concentrations, to cite only a few. Extreme care must be exercised to distinguish metabolic variables derived from the cell cycle from those with significance for the circadian oscillator.

What is known about the generation of circadian oscillations from studies of unicellular organisms

Armed with a unicellular rhythmic organism, able to grow in mass culture in a reproducible way, what can we discover about the nature of the generation of circadian oscillations? This process is obviously complex, and has built into it temperature independence and resettability. The very existence of rhythms is isolated cells of *Gonyaulax* and *Acetabularia* exclude the possibility that oscillations are generated by feedback between cells or tissues, although it is possible that in multicellular organisms rhythmicity may be propagated from one tissue to another in this manner, perhaps through hormone interactions.

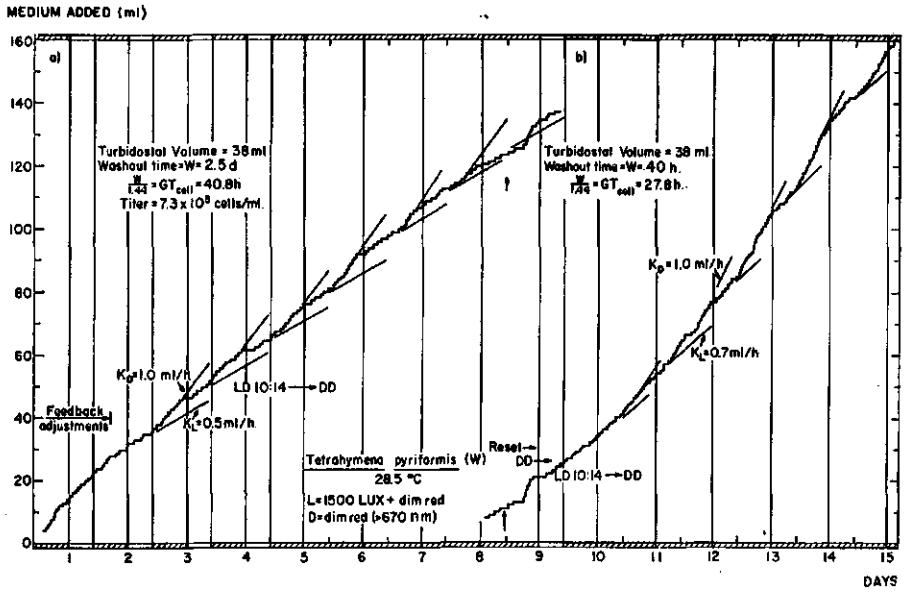

Fig. 10. Cell division synchrony in *Tetrahymena pyriformis* in a turbidostat at 28.5°, Synchrony is established in LD 10:14, and continued after transfer to red light (670 nm). Generation times, 27.8 h (right) and 40.8 h (left). Ordinate is ml. medium added to maintain a constant cell density. (From Wille and Ehret, 1968).

In several protists, *Euglena* and *Gonyaulax* for example, more than a single physiological process is rhythmic. In both these cases, the phases of the different processes are different. However, the different overt rhythms all have the same period and change phase together, in a way that is consistent with a single oscillator. This is not always the case in multicellular organisms where evidence for two simultaneous oscillations with different periods has occasionally been obtained.

Is there any evidence concerning what part of the cell houses the oscillator? Since non-photosynthetic cells can be rhythmic, the chloroplast is not the unique site. Nor is the nucleus necessarily present in the cell for rhythms to be generated, as shown by *Acetabularia*. The failure to demonstrate a circadian rhythm in any procaryote lends credence to the supposition that rhythms will not be found in isolated chloroplasts or mitochondria, but may instead depend on interactions between organelles, taking place across membrane barriers. As yet we have no knowledge as to how this might occur. Studies of the exchange of radioactive isotopes of calcium and sodium in *Gonyaulax* (Fig. 11) do not show any difference in the rates of uptake or efflux of these ions associated with phase, therefore it does not seem likely that permeability changes in the cell membrane can be responsible. This does not exclude intercompartmental changes in permeability however.

From time to time suspicion has been cast on the cell cycle as the generator of cir-

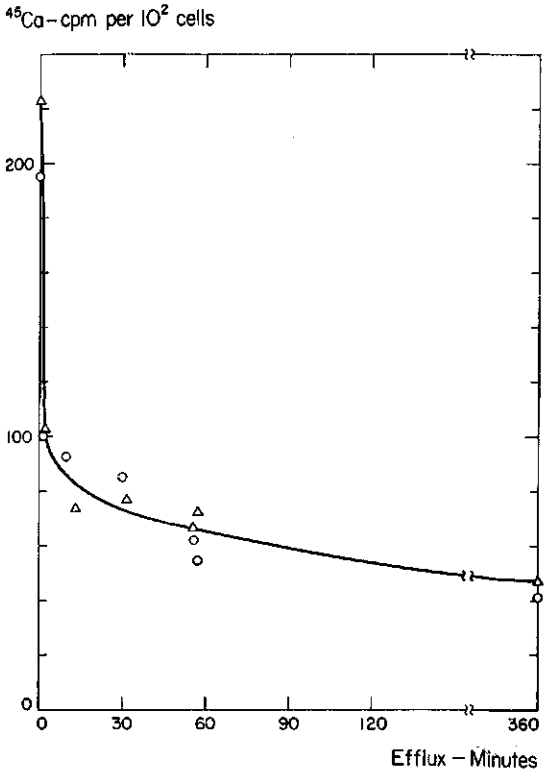

Fig. 11. The rate of efflux of ^{45}Ca from 5 ml cells of bacteria-free *Gonyaulax polyedra* in the day phase (circles) and the night phase (triangles) at 22°. Cells on LD 12:12 were labelled for 3 days in ^{45}Ca, (0.05 μc, and 0.35 μg per 5 ml cell suspension). Day phase culture, 1930 cells per ml; night phase culture, 1780 cells per ml. Cells were transferred to unlabeled medium for the times indicated.

cadian rhythms. However, the properties of the cell cycle clearly distinguish it from the circadian cycle, in some organisms at least. The cell cycle is temperature sensitive while rhythmicity is all but temparture independent. Furthermore, the circadian rhythms of luminescence and photosynthesis in *Gonyaulax* are most distinct in populations in the stationery phase, in which there are few if any dividing cells. Chloramphenicol immediately halts cell division, but has no effect on these rhythms.

The lengthening of the period of rhythms in deuterated *Euglena* and other organisms implicates cell metabolism in the generation of rhythmicity. Metabolism as a whole is clearly not involved however. Experiments in which 8-hour pulses of a large number of metabolic inhibitors were assayed for effects on the phase of the glow rhythm in *Gonyaulax* (Hastings, 1960) gave essentially negative results. No detectible changes in ATP level were found in *Gonyaulax* in continuous light at an intensity permitting rhythmicity in luminescence and photosynthetic capacity. Oxygen consumption and electron flow in photosynthesis are not rhythmic in *Gonyaulax* (Sweeney, 1969). These results point to a discrete oscillation-generating system, which does not involve all of cell metabolism.

Studies of the effects of inhibitors of protein synthesis on rhythms in *Euglena*,

Gonyaulax and *Acetabularia* have yielded perplexing results. Feldman (1967) has shown that cycloheximide brings about a concentration-dependent lengthening of the period of the phototaxis-motility rhythm in *Euglena*, (Fig. 12), although the effect is not quantitatively equivalent to the inhibition of protein synthesis. Preliminary studies indicate that cycloheximide has no effect on the photosynthetic rhythm in *Acetabularia*. Chloramphenicol is without effect in *Euglena* (Feldman, 1967) *Acetabularia* (Sweeney et al., 1967) and *Gonyaulax*. Recently, rifampicin, too has been shown not to affect the rhythm of photosynthesis in *Acetabularia* (Vanden Driessche, 1969).

The results with actinomycin D are not consistent with a direct involvement of DNA-dependent RNA synthesis in the generation of circadian oscillations. In *Gonyaulax* (Karakashian and Hastings, 1962) actinomycin D stops rhythmicity of the luminescent glow, but not of photosynthesis and stimulated luminescence. Moreover, the effect on the glow rhythm does not occur immediately, but only after one normal cycle. After the rhythm has disappeared, a low intensity continuous glow remains, indicating that the target of the inhibitor may be the rhythmic mechanism. The transduction steps between oscillator and luminescent glow could be the site of inhibition however. In *Acetabularia*, the first effect of actinomycin is a reduction of the amplitude of the rhythms in photosynthesis and chloroplast shape (Sweeney et al., 1967; Vanden Driessche, 1966). Only after a long treatment of more than a week is rhythmicity lost, and then only in *Acetabularia* containing a nucleus. Enucleated cells continue to be rhythmic in the presence of actinomycin D.

If not protein synthesis, then perhaps the activity of proteins is the basic rhythmic variable. The activity of some enzymes has been shown to change with the phase of the circadian cycle, the soluble luciferase in *Gonyaulax* for example (Hastings and Bode, 1962). However, it is clear that the changes in the luciferase activity alone cannot

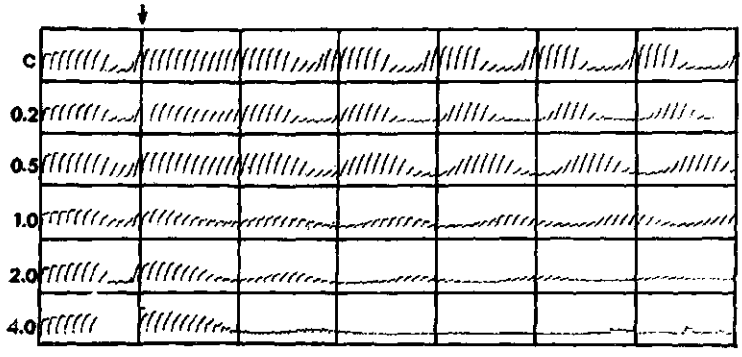

Fig. 12. Lengthening of the period of the phototactic rhythm in *Euglena gracilis* by cycloheximide. Records of phototaxis for seven days in continuous darkness interrupted by a test light at 25°. Abscissa, time, the vertical lines being 24 h apart; ordinate, cycloheximide concentration in μg per ml. 'C' is control without cycloheximide. Each ascending line in the record is the response of *Euglena* to a test light, accumulation in the light being represented as increased lenght of the line. (From Feldman, 1967).

account for the rhythm observed. The soluble luciferin also changes in activity. Even combined, however, the variations in the activities of luciferin and luciferase are insufficient to account for the amplitude of the rhythm in mechanically stimulated luminescence, which varies 50–100 fold during one cycle in alternating light and darkness. In *Gonyaulax*, it is possible to stimulate luminescence chemically as well as mechanically by adding to the cell suspension small amounts of acids, calcium and magnesium salts, procaine, alcohols, acetone and other fat solvents (Fig. 13). The amount of light produced on chemical stimulation by any one of these agents during the day phase is much greater than when stimulation is mechanical, while in the night phase, both chemical and mechanical stimuli elicit the same amount of light (Fig. 14). Thus two components appear to contribute to the rhythm in mechanically-stimulated luminescence, one seen on chemical stimulation, of the right magnitude to be derived from changes in luciferin and luciferase, and a second rhythm which is only evident on mechanical not on chemical stimulation, and which may involve the normal mechanism for stimulation of a flash. The nature of this rhythm is under investigation at the present time. Preliminary results suggest that it is not a variation in the threshold of the response to mechanical stimuli with phase. The particulate 'scintillon' luminescence is not greater in extracts from night phase cells. Its contribution to cellular luminescence is still unclear.

The study of rhythms in unicellular organisms has turned up several other examples of rhythms composed of more than one componant. The contribution of motility changes to the rhythm in phototaxis in *Euglena* has already been mentioned. Another example was discovered by Kelly and Katona (1966), during a study of the luminescence of phytoplankton samples from the Woods Hole area which contained several species of dinoflagellates. These samples showed a rhythm in luminescence augmented by a rhythm in light inhibition, maximal during the day phase.

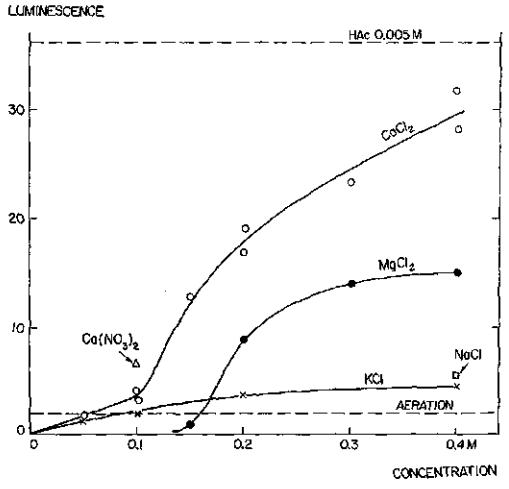

Fig. 13. The stimulation of luminescence in *Gonyaulax polyedra* by the salts indicated compared with that by acetic acid and by aeration, during the light period of a 12:12 LD cycle at 22°. Luminescence is given in relative units. The concentrations (molar) are those present in the cell suspension after mixing.

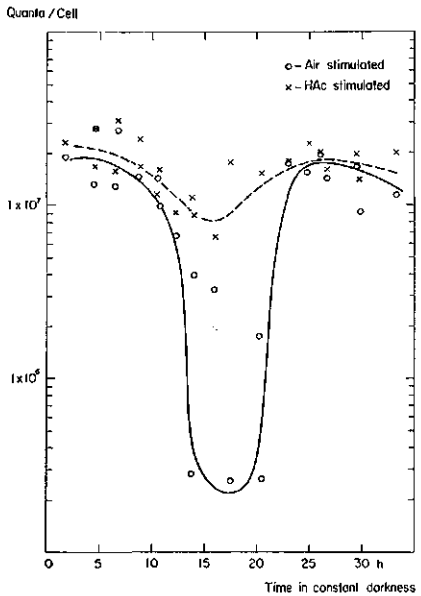

Fig. 14. The rhythm in luminescence in *Gonyaulax polyedra* stimulated mechanically (circles) and by the addition of acetic acid (final concentration 0.005M) (crosses). Ordinate, light emitted in quanta per cell. (From Sweeney, 1969).

It was reported by Sweeney (1969) that the activity of ribulose diphosphate carboxylase in *Gonyaulax* changed in step with the photosynthetic capacity. The finding has not proven repeatable when cells are extracted and assayed in 50% glycerol, in which this enzyme is much more stable than in the previous extraction medium which contained tris buffer, magnesium, EDTA and glutathione without glycerine. In the presence of glycerine, the specific activities of ribulose diphosphate carboxylase from day and night phase *Gonyaulax* grown in continuous light or on a light-dark cycle are the same. The explanation for the discrepancy between former and present experiments is as yet unexplained. However, it seems unlikely that the circadian differences in photosynthetic capacity can be explained as simple differences in the amount of this enzyme.

There is one interesting consequence of the study of rhythms in unicellular organisms. It is quite simple in such systems to detect the absence of rhythmicity, as distinct from asynchrony of individual cells of a population, since isolated cells can be studied (Sweeney, 1960). Investigation of this question has shown that cells can be in every respect arhythmic and still survive without apparent harm. In fact, *Gonyaulax* grows fastest when arhythmic in continuous bright light. Thus cellular organization and function are not absolutely dependent on the control function of the circadian oscillator. Furthermore, no theory for the mechanisms of rhythmicity which cannot be bypassed, such as transcription of essential parts of the genome can be considered seriously.

From this discussion it is clear that studies of circadian rhythms in unicellular organisms have not as yet yielded definitive knowledge concerning the generation of

oscillations with a circadian period. However, it is my intuitive feeling that these unicellular rhythms offer by far the most promising material for progressing toward such an understanding.

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Discussion

Participants: B. M. Sweeney (Author), K. Hoffmann, J. Rombach, H. G. Schweiger and E. C. Wassink

You mentioned different reactions in photosynthesis that do not show rhythms. If CO_2-uptake and O_2-evolution show a rhythm, would it be possible that the difference is in intake of the HCO_3-ion? (WASSINK.) I don't think so, since rhythms in photosynthesis can be measured in high bicarbonate buffers (AUTHOR).

It seems interesting that, in relation to pressure, there is no saturation of luminescence with increasing pressure at night, whereas during the day-phase there is. Is there an explanation for this? (WASSINK.) The results of experiments with the pressure induction of luminescence are incomplete as yet, and I should prefer to leave this point undiscussed until then, to be sure that this difference in saturation behaviour is real (AUTHOR).

Were there any high frequency oscillations in ATP content in *Gonyaulax*? Are there any changes in the pH after addition of salts? (SCHWEIGER). There appear to be no changes in external pH but of course one does not know about the pH inside the cell (AUTHOR).

Was the reset caused by an ultraviolet light pulse phase-dependent? (HOFFMANN.) Yes. In the night phase, larger resets are obtained with ultraviolet pulses than during the day. However, all phase changes with ultraviolet are advances (AUTHOR).

In multicellular organisms cultivated in constant conditions, an overt rhythm is often

started only after an external impulse. The supposition has been made that cells in these organisms were originally desynchronized and that the impulse served to synchronize the rhythmic processes in the cells. Is it possible to measure a rhythm in a single cell of the type you used and, if so, do you know whether there are conditions in which a single cell shows no rhythmicity and are these conditions similar for cultures and single cells? (ROMBACH.) In populations of *Gonyaulax*, the circadian rhythm disappears in continuous light of intensities higher than about 5000 lux, Single cells can be removed from such an apparently arhythmic population and can be placed in a Cartesean reference diver apparatus suitably arranged for measuring the production of oxygen and such single isolated cells are also arhythmic. This argues against the cause of population arhythmia being asynchrony of rhythmic members and for the stopping of the clock by bright continuous light (AUTHOR).

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Circadian rhythms: subcellular and biochemical aspects

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The phenomena of circadian rhythms have been thoroughly examined from different aspects in unicellular organisms, plants, animals and human beings, and a series of properties has been described (Bünning, 1967). The unsolved problem of the underlying molecular mechanisms remains the central issue of circadian rhythms. Molecular mechanisms means necessarily that proteins, i.e. enzymes, are involved in these processes.

With a complete understanding of the molecular mechanism of the circadian rhythm it will be possible to generate similar phenomena in completely defined biochemical systems. To obtain such systems, different approaches are possible (Fig. 1). The knowledge derived from observations on whole multicellular organisms, has been used mainly for constructing mathematical, physical or physicochemical models. So far little use has been made of isolated organs, isolated tissues and homogenates to gain insight into the mechanism of circadian rhythms.

Multiplicities of single cells or of unicellular organisms have been useful for studying the mechanism of circadian rhythms. The next logical step, namely the preparation of cell-free systems from such multiplicities of single cells, does not yet play an important part in the study of circadian clocks but it has proved to be extremely useful in the study of higher-frequency oscillations.

The third approach uses individual single cells and cell biological methods. The number of studies with single cells is still small, most probably because of experimental

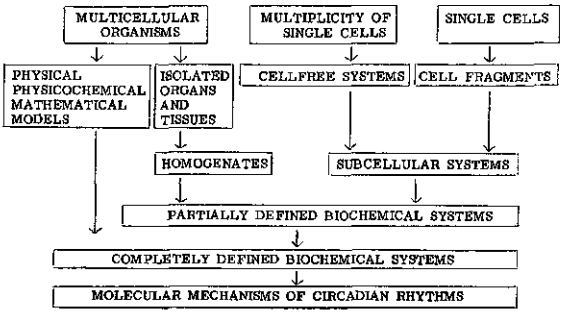

Fig. 1. Different approaches for the investigation of circadian rhythms.

difficulties. However the results obtained by these methods should contribute to the understanding of the molecular mechanims of the biological clocks.

The work with individual single cells makes the use of cell fragments practical. The major issue is the interrelation of cell nucleus and cytoplasm. In the near future it should be possible to prepare surviving subcellular systems from cell fragments and then gain further insight into the mechanisms of biological clocks by reconstitution of the cytoplasm. The next logical step will be to prepare partially defined biochemical systems and then to find access to the molecular mechanisms of circadian rhythms by a completely defined biochemical system.

There are many difficulties when working with individual single cells, mainly because of the insensitivity of the methods available. However other properties make individual single cells well suited for the work on circadian rhythms (Table 1). In contrast to multicellular animals and multicellular plants, intercellular regulatory mechanisms such as contact and neural regulation and humoral regulation can be excluded in single cells. In single cells the whole problem is reduced to the enzyme level.

Since there are particular technical difficulties in working with single cells, the organism to be used for such studies must have a certain minimum size and metabolic activity for measurements of sufficient accuracy. Furthermore, the organism must be easy to culture. Finally, it has to have a life cycle significantly longer than 24 hours, to exclude the overlapping of any metabolic events and oscillations based on cell division rhythms. With these three conditions, and several others of minor importance there is only a very limited number of species which can be considered for such studies. One of these species is *Acetabularia* (Hämmerling, 1963; Schweiger, 1969).

Acetabularia is a unicellular and uninuclear green alga that grows to a length of up to 5 cm. The full-grown cell of *Acetabularia mediterranea* consists of a stalk and an apical cap. The rhizoid on the basal part of the stalk contains the cell nucleus for most of the life cycle. One life cycle takes about 15 weeks under optimum conditions in the laboratory but two years in the wild.

Mainly because of size and resistance to techniques of cell surgery, *Acetabularia* has proved suitable for cell biology. Techniques readily applicable to *Acetabularia* include removal of the rhizoid, which contains the cell nucleus. The resulting anucleate fragments are viable. They continue to grow for weeks and undergo the same characteristic morphogenetic differentiation as is seen in intact cells. *Acetabularia* can also be used for preparing subcellular systems, called cytoplasts, which are viable for weeks.

Another cell biological method for which *Acetabularia* is well suited is the transfer

Table 1. Regulatory mechanisms.

Type of regulation	Multicellular organisms	Multiplicity of unicellular organisms	Single cells
Contact (neural)	+	(+)	–
Humoral	+	(+)	–
Enzyme	+	+	+

of the cell nucleus from one cell to another one. For this purpose two techniques have been developed. The first one, called the transplantation technique, includes the grafting of a nucleate rhizoid to another stalk. In the second, the implantation technique, the cell nucleus is isolated and purified from other cytoplasmic constituents and finally injected into the cytoplasm. Results obtained by using these techniques together with results from other organisms have given rise to some ideas on the molecular mechanism of circadian periodicity.

Using a most elegant method of measuring ocygen evolution based on the principle of the cartesian diver, Sweeney and Haxo (1961) have shown that the photosynthetic capacity in *Acetabularia* is subject to substantial changes during day and night. These oscillations are retained if the cell is kept under constant light. Furthermore, the authors have shown that the endogenous oscillations in photosynthetic activity are preserved even in the absence of the nucleus. Finally it was demonstrated that both the nucleate and anucleate cell can reset the phase under the influence of an exogenous light-dark change. The majority of these experiments were carried out on *Acetabularia major*.

Another method, used to estimate oxygen evolution, is based on the principle of the platinum electrode (Schweiger et al., 1964a). With this method, measurements on *Acetabularia mediterranea* showed that circadian oscillations are maintained under constant conditions, for more than 40 days (Fig. 2). This holds for nucleate and anucleate cells. As anucleate cells preserve their endogenous circadian periodicity for a number of weeks the cell nucleus is therefore not essential for the maintenance of the rhythm.

Besides the methods which are based on the cartesian diver and the platinum electrode, the classical technique of manometric measurement of oxygen, developed by Otto Warburg, has been used succesfully to study the rhythmic behaviour of photo-

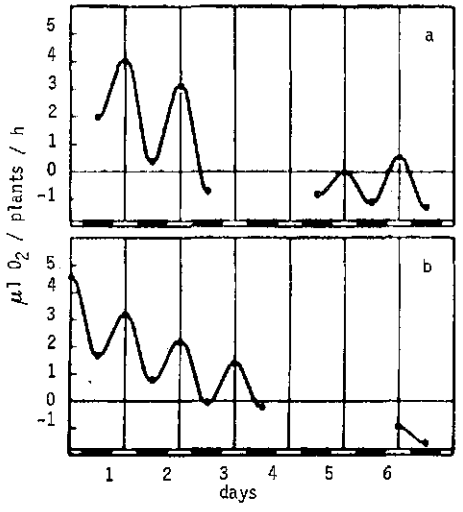

Fig. 2. A circadian rhythm in anucleate cells 36 and 42 days resp. after enucleation (Schweiger et al., 1964b).

synthetic activity in *Acetabularia* (Vanden Driessche, 1966). However, this method is not sensitive enough for measurements in an individual cell.

By developing the original technique of the platinum electrode and increasing its sensitivity, oxygen evolution in an individual cell can now be measured and recorced over weeks (von Klitzing and Schweiger, 1969; Mergenhagen and Schweiger, 1971). Evaluation is carried out by analogue and digital methods. With the flow-through system the oxygen concentration can be recorded continuously and substances such as antibiotics and other specific inhibitors can be added to the medium (Fig. 3, 4, 11 and 12). Thus the influence of these materials on the photosynthetic capacity can be measured in a more direct way. Moreover, this method enables us to study in detail fast effects in a single cell.

It is obvious that chloroplasts must be involved in circadian rhythms of photosynthetic activities. Since oscillations of photosynthesis are retained in anucleate cells, it would be of interest to investigate a possible correlation between this autonomous feature of the chloroplasts and their other genetic features.

Molecular genetic autonomy is indicated by the ability of anucleate cells of *Acetabularia* to increase the number of chloroplasts (Clauss et al., 1970). Furthermore molecular genetic autonomy of chloroplasts is confirmed by the fact that anucleate cells of *Acetabularia* can increase their RNA content (Schweiger and Bremer, 1961) and that isolated chloroplasts are able to synthesize ribosomal and transfer RNA (Schweiger and Berger, 1964; Berger 1967). However the molecular genetic autonomy of chloroplasts is obviously limited. This has been clearly shown for the RNA synthesis in anucleate cells. An increase in RNA can be demonstrated in anucleate *Acetabularia*

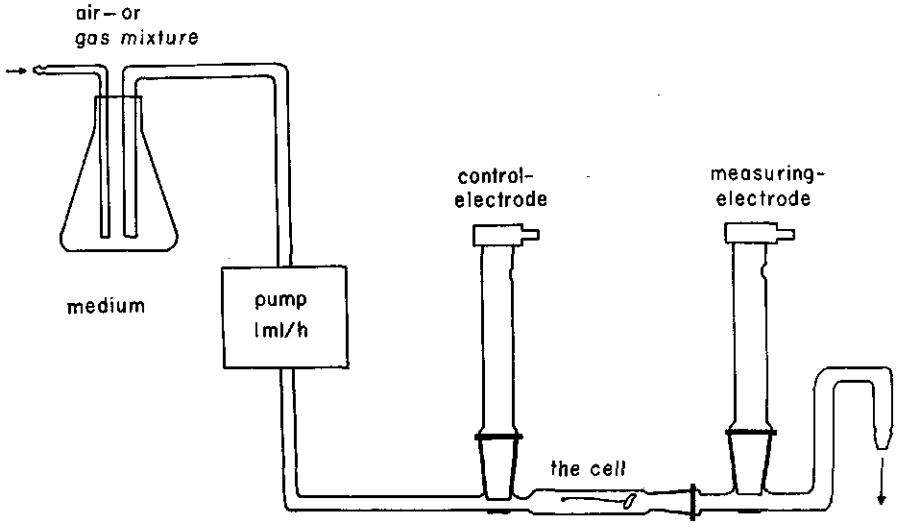

Fig. 3. Flow-through-system for recording a circadian rhythm in an individual cell (Mergenhagen and Schweiger, 1971).

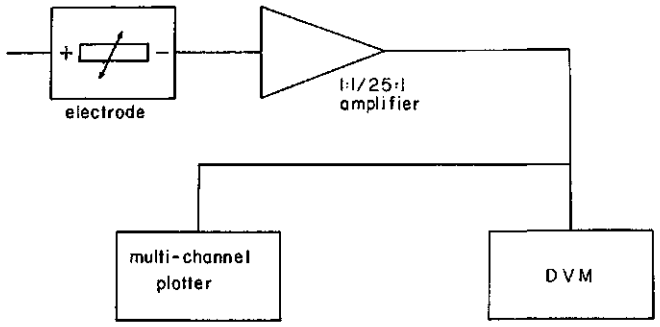

Fig. 4. Block diagram for the measuring device (Mergenhagen and Schweiger, 1971).

cells by keeping the cells in darkness before the cell nucleus is removed. If the nucleus had been removed before the dark treatment, an RNA increase could not be shown. In this experiment the increase in RNA, mainly due to chloroplast RNA synthesis, can be demonstrated in the anucleate cell. However the influence of the nucleus is shown at the same time as its presence is necessary during the dark period.

A similar limited autonomy has been found for the circadian rhythm of photosynthesis in *Acetabularia*. Although anucleate cells retain their circadian rhythmicity over weeks the cell nucleus can obviously influence the rhythm phase as shown by the following experiment (Fig. 5) (Schweiger et al., 1964b): In two cells with phases that differed by 180°, the rhizoids which contain the cell nuclei were exchanged. Under the influence of the transplanted rhizoids, the phases of the photosynthesis rhythm were then shifted within a couple of days by exactly 180°, under constant light conditions. This shift has to be expected if one assumes that the nucleus determines the phase of the cytoplasmic rhythm. This result was also obtained in a cross experiment so that

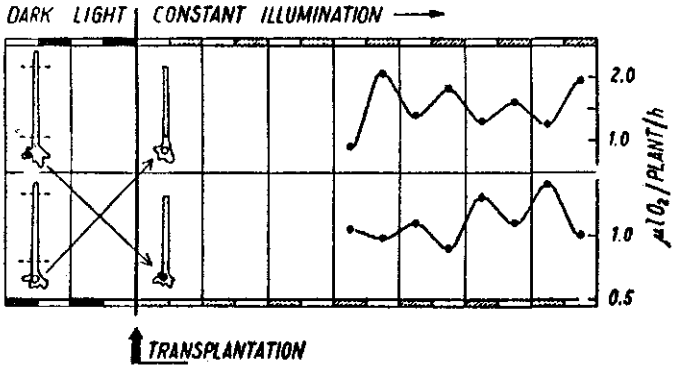

Fig. 5. Shift of the phase under the influence of the nucleus: transplantation (Schweiger et al., 1964b).

there is no possibility that the effect is due to the method or other factors which function as an exogenous 'Zeitgeber'.

The influence of the cell nucleus becomes more obvious when isolated and purified cell nuclei are transplanted (Fig. 6). In this experiment the influence of the cell nucleus on the phase of the rhythm can also be demonstrated.

In another experiment the basal and apical parts of the same cell were subjected to different illumination regimes for 14 days and then exposed to constant light (Fig. 7). This showed that the phase of the cytoplasmic rhythm is determined by the rhythm of illumination to which the nuclear area is exposed.

These results raise the question of how according the nucleus might regulate the rhythmicity in the cytoplasm and the chloroplasts. The mechanism might be similar to that which is involved in the RNA synthesis in the anucleate cell: during the dark period the nucleus may send some information to the cytoplasm which stimulates the RNA synthesis in the cytoplasm and so in the chloroplasts.

Experiments on the origin of genetic information for chloroplast proteins show how information may be transferred from the cell nucleus to the cytoplasm. One of these chloroplast proteins is supposed to be the enzyme malic dehydrogenase. During the growth of *Acetabularia* the activity of this enzyme is increased in nucleate cells and to a certain extent in anucleate cells (Schweiger et al., 1967). Different isozymes of malic dehydrogenase can be separated by gel electrophoresis. The electrophoretic pattern shows a pronounced specificity for different species of *Acetabularia*. The specific pattern for each species does not change during growth and differentiation, in nucleate or in anucleate cells; all the isozymes which contribute to the enzyme pattern behave in a similar way.

However if nuclei are exchanged between two species of *Acetabularia* a change can be seen after 28 days (Fig. 8). The enzyme pattern of the host cytoplasm disappears and a new enzyme pattern appears. This new enzyme pattern corresponds to that species from which the cell nucleus originates. That means that the enzyme pattern is transformed by the influence of the implanted nucleus. Another interesting point is that at

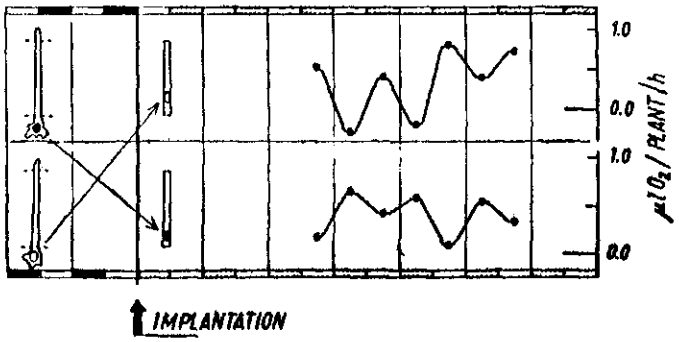

Fig. 6. Shift of the phase under the influence of the nucleus: implantation (Schweiger et al., 1964b).

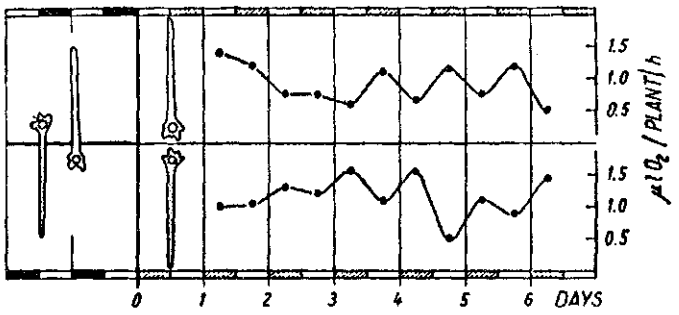

Fig. 7. Shift of the phase under the influence of the nucleus: partial illumination (Schweiger et al., 1964b).

least part of the activity of the malic dehydrogenase is situated in the chloroplast, as has been shown by several methods. The malic dehydrogenase which occurs in the chloroplasts unequivocally is coded by nuclear DNA.

In a similar way the behaviour of lactic dehydrogenase has been studied (Reuter and Schweiger, 1969). This enzyme is also determined by the cell nucleus and at least part of the enzyme activity is situated in the chloroplasts. Similar results have been obtained with membrane protein fractions from isolated chloroplasts of *Acetabularia*. They also clearly show that the cell nucleus codes for these integral constituents of the chloroplasts (Apel and Schweiger, in press).

There is also some evidence that even the proteins of the chloroplast ribosomes are determined by the cell nucleus. The following experiments were carried out (Kloppstech

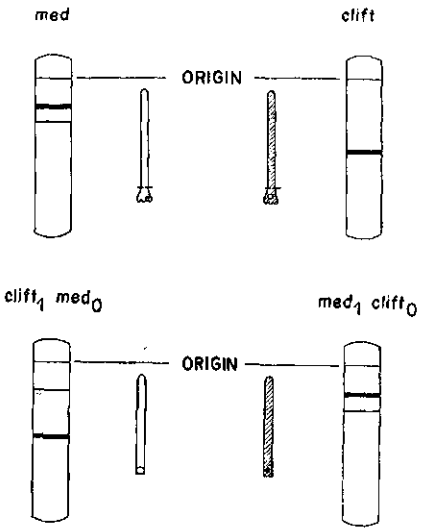

Fig. 8. MDH pattern after heterologous implantation (Schweiger et al., 1967).

and Schweiger, 1971): Ribosomes from isolated chloroplasts of *Acetabularia* were dissociated into subunits and from the 44 S ribosomal subunit proteins were prepared. The ribosomal proteins were subjected to gel electrophoresis and the protein patterns for *A. mediterranea*, *A. calyculus* and *Polyphysa cliftonii* were determined. For several bands, there were differences in the different species. If the nuclei of such species were exchanged, heterologous combinations between cell nucleus and cytoplasm were obtained.

The genetic information of these cell hybrids originates in two sources: in the implanted cell nucleus and in the organelles which are present in the cytoplasm. The latter part is expected to code at least the RNA of the chloroplasts. In such hybrid cells the heterogenous character of the genetic information is probably retained in the F_1-generation (Schweiger et al., 1969). When chloroplast DNA contributes to the coding for the chloroplast proteins it is expected that this information is retained in the F_1-generation. However, in the F_1-generation the species specific properties of the proteins of the 44 S subunit from chloroplast ribosomes had changed into those typical for the species from which the nucleus, that was implanted into the cytoplasm of the P-generation cell, originates. Therefore it is very probable that the genetic information for the ribosomal proteins of the chloroplasts also originates in the cell nucleus.

Thus for some chloroplast proteins such as malic dehydrogenase and lactic dehydrogenase, for membrane proteins and for ribosomal proteins, the genetic information originates in the cell nucleus. For some constituents and functions of the chloroplast which are involved in the expression of genetic information the prerequisites for autonomy are present in the organelles. However even these functions and constituents depend in some way on the nucleus. It is tempting to assume that DNA-dependent RNA synthesis of the chloroplasts is also regulated by nucleus-coded proteins. The facts that anucleate cells retain circadian rhythmicity and that the nucleus can shift the phase suggest that the circadian rhythm of photosynthetic capacity in *Acetabularia* is related to a comparable regulatory mechanism and has something to do with genetic information.

From a theoretical point of view an involvement of the transcription of the nuclear and chloroplast DNA as well as the translation on 80 S cytosol and on 70 S chloroplast ribosomes, in this regulation must be considered (Fig. 9). Some conclusions can be made about the mechanism of the circadian clock from experiments on anucleate cells

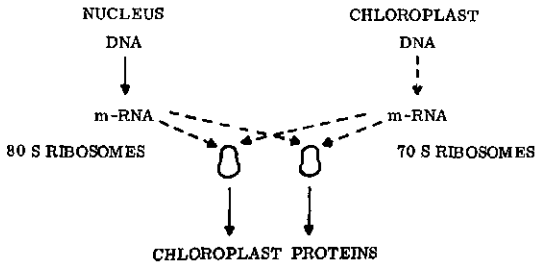

Fig. 9. Transcription and translation in *Acetabularia*.

and application of substances which specifically inhibit RNA and protein synthesis. That the circadian rhythm of the photosynthetic activity is not due exclusively to the transcription of the nuclear DNA can be seen from the persistence of the rhythm after the removal of the nucleus.

To answer the question whether the circadian rhythm is related to one of the four synthetic capabilities under consideration, several antibiotics have been used. In experiments on *Acetabularia* (Sweeney et al., 1967) as well as on *Gonyaulax* (Karakashian and Hastings, 1963) chloramphenicol never had a phase shifting effect or a periodicity repriming action. On the contrary, in *Gonyaulax* the amplitudes were increased under the influence of chloramphenicol. No inhibitory effect could be detected in anucleate cells. Since chloramphenicol is known specifically to inhibit protein synthesis on 70 S ribosomes it can be excluded that the translation on 70 S ribosomes of the chloroplasts plays an essential part in the circadian periodicity.

In a similar way the possibility was ruled out that translation on 80 S ribosomal is involved in the generation of circadian oscillations. Puromycin, a potent inhibitor of protein synthesis on 70 S and 80 S ribosomes, had no effect on the rhythmicity even in anucleate cells (Sweeney et al., 1967). The lack of an inhibitory effect of puromycin indicates that translation on 80 S or 70 S is not directly related to the circadian rhythm of photosynthesis in *Acetabularia*.

To find out whether transcription plays a role in circadian rhythms, the effect of actinomycin was studied. Actinomycin is a potent inhibitor of transcription of nuclear and extranuclear DNA. In nucleate cells of *Gonyaulax*, actinomycin substantially reduced the amplitudes so that the rhythm seemed to be discontinued (Fig. 10)

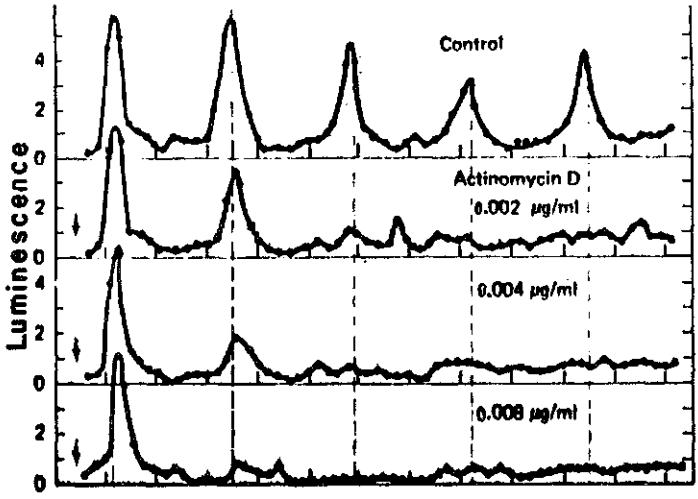

Fig. 10. Influence of actinomycin D on a circadian rhythm in *Gonyaulax* (Karakashian and Hastings, 1963).

(Karakashian and Hastings, 1962). In nucleate *Acetabularia* cells a similar effect has been described (VandenDriessche and Bonotto, 1969; Mergenhagen and Schweiger, 1971). After a lag phase of about seven days, after the addition of actinomycin to the medium, the rhythmicity of the oxygen production is essentially lost (Fig. 11). In contrast to nucleate cells, actinomycin increases the amplitude in anucleate cells (Fig. 12) (Schweiger et al., 1964c; Sweeney et al., 1967; Vanden Driessche and Bonotto, 1969; Mergenhagen and Schweiger, 1971). An inhibition or suspension of the circadian rhythm has never been observed. The results obtained with actinomycin are supported by experiments with rifampicin which also has no inhibitory effect on the periodicity of nucleate and anucleate cells (Vanden Driessche et al., 1970). This antibiotic specifically inhibits the transcription of extranuclear DNA especially of chloroplast DNA in eucaryonts. From the results with actinomycin and with rifampicin it can be seen that the circadian rhythm does not depend on transcription of nuclear and extranuclear DNA.

Therefore in anucleate cells neither translation nor transcription play an essential part in circadian periodicity. Since in the absence of the nucleus the expression of

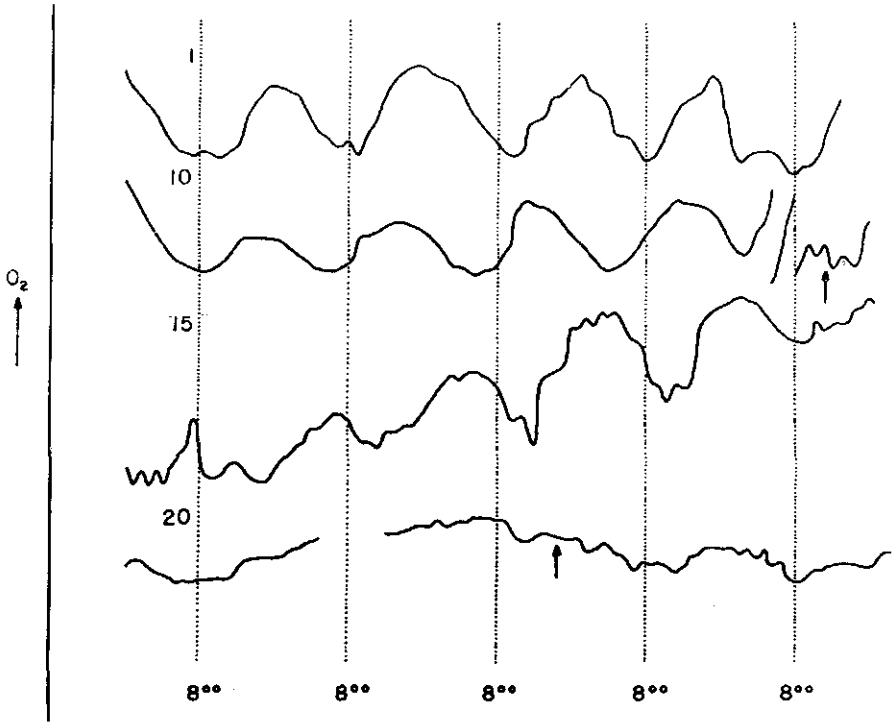

Fig. 11. Influence of actinomycin D on nucleate cells of *Acetabularia* (Mergenhagen and Schweiger, 1971).

genetic information can be excluded as a basic mechanism of rhythmicity, interest will be focused on the simplified system of anucleate cells. However it is quite difficult to understand how actinomycin affects nucleate cells. It is too simple to assume that only the suspended supply with mRNA from the nucleus is responsible for the disappearance of periodicity in the presence of actinomycin. Then a similar effect should result from the removal of the nucleus. As has been shown this is not so. On the contrary, the amplitude in the anucleate cell is increased by the addition of actinomycin. This increase seems to indicate that the transcription of extranuclear DNA, predominantly in the chloroplasts, exhibits a desynchronizing effect.

Since the transcription and the translation level in the chloroplasts can be excluded as regulatory mechanisms for the circadian rhythm in anucleate cells, one has to look for other ways of regulation which might result in circadian oscillations (Table 2). Regulation by change of enzyme concentration has been ruled out by experiments which have shown that enzyme activity can change without a change in enzyme concentration since neither transcription nor translation play a major part in circadian rhythmicity. Regulatory mechanisms in which the point of attack in the 'active sites'

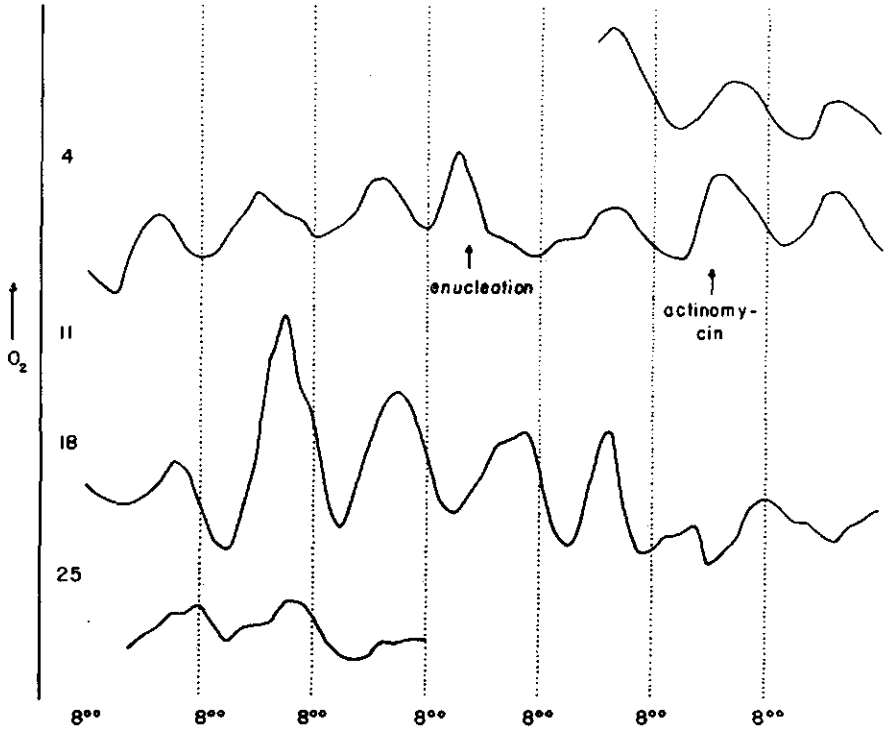

Fig. 12. Influence of actinomycin D on anucleate cells of *Acetabularia* (Mergenhagen and Schweiger, 1971).

Table 2. Control of enzyme action.

1. Change in enzyme concentration		
	a. Synthesis:	increased (induction)
		decreased (repression)
		transcription
		translation
	b. Degradation	(degrading enzymes)
		increased (induction)
		decreased (repression)
2. Change in enzyme activity without change in enzyme concentration		
	a. Active sites	(competitive inhibition)
		product inhibition
		substrate inhibition
		other inhibitors
	b. Non-active sites	allosteric regulation
		non-competitive inhibition
		inhibition by antibodies

can also be excluded. Enzyme changes occur before product and substrate inhibition.

For metabolic oscillations the regulation at the 'non-active sites', the allosteric regulation seems to be of special importance. The non-competitive inhibition and also the inhibition by antibodies can be neglected since at present there is hardly anything known about such processes. However, from a theoretical point of view regulation through the change of free and bound antibodies against key enzymes must be considered.

Allosteric regulation means that a chain of enzyme-catalysed reactions is involved. Usually the enzyme, that is allosterically regulated, is positioned at the starting point of the reaction chain, while the effector which attacks the allosteric protein, represents the reaction product of one of the final steps of the reaction chain. In accordance with this postulate, the effector is not usually related structurally to the substrates or products of the allosterically regulated protein. So far nothing is known about interrelations between allosteric regulations and circadian rhythms. However there is much information on the role of allosteric regulations in the higher-frequency oscillations in cells and in cell-free systems (Hess and Boiteux, 1971).

In some cells, such as yeast cells, damped oscillations can be produced under different conditions (Hess and Boiteux, 1968). Similar observations have been made in ascites cells (Ibsen and Schiller, 1967). The oscillating systems are not dependent on the integrity of the cells since even undamped oscillations have been produced in cell-free extracts from yeast cells, for example (Fig. 13) (Hess, 1968). Most of the studies have been carried out on glycolysis. Different parameters have been measured such as the fluorescence of the reduced nicotinamide adenine dinucleotide (NADH) and pH.

The network of the different metabolites and enzymes has been compared with an electronic network (Chance et al., 1967). Such systems have been thoroughly analysed

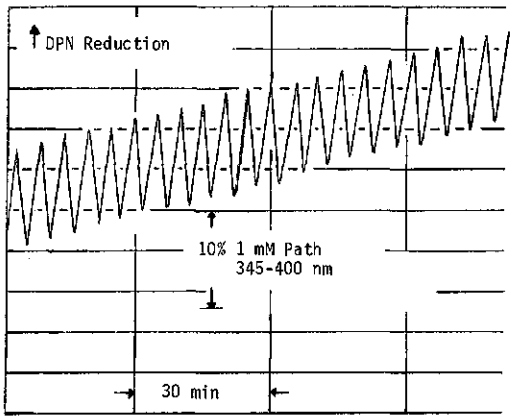

Fig. 13. Undamped oscillations in yeast extracts (Pye and Chance, 1966).

mathematically (Sel'kov, 1968), and today models can be constructed so that the length of the periods as well as the amplitudes can be varied.

Nevertheless one should not overlook that glycolysis is only one of many reaction chains which together comprise the metabolism of a cell. It is tempting to assume that certain 'key' metabolites that are common members of various metabolic chains and occur there at the same time, are instrumental in 'synchronizing' the pathways. Moreover, such a system might interconnect the metabolism of the cytoplasm and the cell nucleus.

There are several examples of substances that are released from the cell nucleus into the cytoplasm influencing chloroplasts (Schweiger and Bremer, 1961). This has also been indicated by the experiment in which a shift of phase was produced by nuclear implantation (Schweiger et al., 1964b). Information must also pass from the cytoplasm to the cell nucleus (Hämmerling, 1963). Otherwise it would be difficult to understand how the cell nucleus can receive the information for the rhythm when the clock is reset. So it is postulated a priori that the 'Zeitgeber' effect is the same in anucleate and in nucleate cells. For the circadian rhythm of the photosynthetic activity in *Acetabularia* cells it has been shown that it is possible to suspend the pathway between cell nucleus and cytoplasm by removing the nucleus. Under these circumstances the 'autonomy' of the circadian rhythm of the chloroplasts is demonstrated.

As multienzyme systems oscillate by allosteric effects, the question arises whether these high-frequency oscillations have anything to do with circadian rhythms. Although there is no actual evidence it seems to be theoretically possible.

Relationships between high- and low-frequency oscillations, that means multiplication and demultiplication of rhythms, have been detected in many biological systems. By comparing the different frequencies found for oscillating functions, it has been shown that the ratios between the frequencies of different oscillations are whole numbers. For instance, the ratios between period lengths of blood pressure waves and of respiration are 2:1 or 3:1 or 4:1 under different experimental conditions (Fig. 14) (Hildebrandt, 1967).

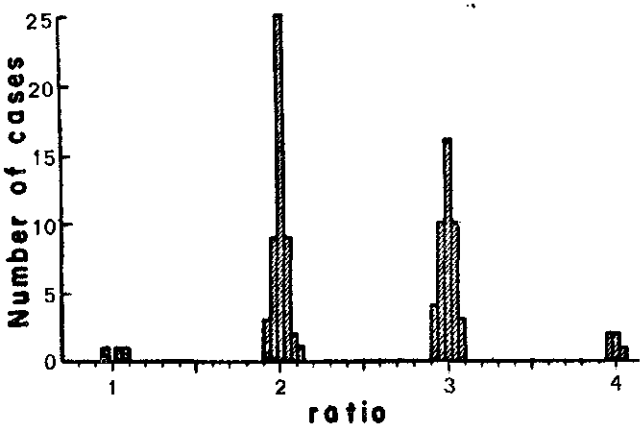

Fig. 14. Ratios of period length of blood pressure waves and of respiration under different experimental conditions (Hildebrandt, 1967).

Therefore, it can be assumed that higher-frequency rhythms can be coupled with lower-frequency ones. A theory of coupling has been thoroughly discussed by Pavlidis (1969). Such coupling might mean that biological systems are able to count events. In other words, if the intervals between events remain constant, biological systems can measure time by interval counting. However it is not yet known whether individual cells also have such a counting mechanism. Assuming a general validity of this principle of measuring time, higher-frequency oscillations should be present in all biological systems which display circadian rhythms. In *Acetabularia* and many other organisms this is true for a whole series of parameters such as for the activity of malic dehydrogenase (Fig. 15), the activity of lactic dehydrogenase, for the concentration of ATP and for other metabolites (van Klitzing, 1969). However these oscillations seem to be composite since the period lengths vary. Another interesting example is the anucleate reticulocyte. In this cell, oscillations have been found for the incorporation of ^{14}C-labelled leucine in haemoglobin (Tepper et al., 1968). The period length was in the range of 100 sec.

From the results of the different types of experiments that have been discussed a working hypothesis can be developed to gain more insight into the molecular mechanism of the biological clock (Fig. 16). In anucleate cells, one may assume that the basic oscillations are due to multienzyme systems. In addition to the multiplicity of metabolic chains (Hastings, 1970) the cell possesses a number of complex structures (e.g. ribosomes in the cytosol and in organelles) which might behave similarly. All these contributors to cell metabolism have to be synchronized even in a single cell, otherwise no rhythmicity could be observed. The high-frequency oscillations in the range of less than seconds and minutes are transformed to lower-frequency oscillations in the circadian range. The circadian organization develops on the basis of high-frequency oscillations possibly because of evolutionary adaptation to the exogenous 'Zeitgeber' day-night change.

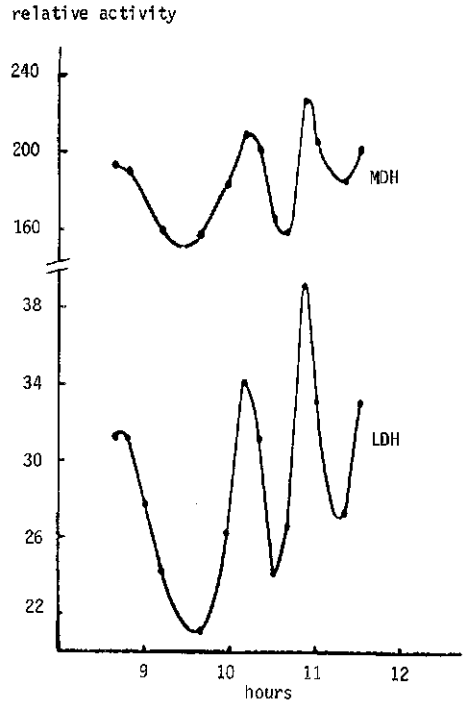

Fig. 15. Oscillations of MDH and LDH activity in nucleate *Acetabularia* cells (von Klitzing, 1969).

Metabolites probably produce the synchronizing effect within the cell. Since diffusion and translocation play an essential part in intracellular synchronization the synchronizing substances should be in the low molecular weight range. Whatever the nature of the synchronizing system is, it should be a ratio rather than absolute concentrations. One could suggest the relations ADP/ATP or NAD/NADH or glutamine/glutamic acid. In the cytoplasm such a system may oscillate independently of the cell nucleus. With such ratios as synchronizers in the anucleate fragment the phase can be reset by exogenous 'Zeitgeber'. One difficulty of this model is that constant frequencies have to be postulated for the oscillating multienzyme systems, which means that most probably the ratio of the activities of the different enzymes should be constant over long periods. However this seems doubtful, especially under the aspect

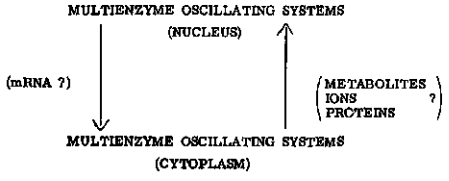

Fig. 16. Circadian organisation of the cell.

of differentiation. To overcome this difficulty one has to postulate that either the ratios of activities are changed without changing the lengths of the periods or, if the length of the period of single metabolic chains is changed, that, nevertheless, there is a compensatory effect of the co-operation of the different oscillating systems resulting in circadian rhythms.

In nucleate cells there is a close relationship between cytoplasm and cell nucleus. The synchronizing effect of the cytoplasm on the nucleus might be achieved by some metabolites, for instance proteins or nucleotides or ions. The influence of the cell nucleus on the oscillating cytoplasm can be explained by assuming that the release of RNA probably mRNA from the nucleus results in a change of enzyme activity in the cytoplasm. However this could only be an additional regulatory mechanism of the nucleus since the basic mechanism of circadian organism works in the cytoplasm even in the absence of the nucleus. The transport of metabolites from the nucleus to the cytoplasm may also be involved. From the effect of the implanted nucleus on the circadian rhythm the nucleus itself must represent an oscillating system.

Another biochemical model for circadian rhythms has been proposed by Ehret and Trucco (1967). This most stimulating and interesting model and the chronon concept can not be applied to *Acetabularia* without difficulties. The model starts from the assumption that time measurement is connected with transcription. A polycistronic element whose transcription needs about 24 hours is called the chronon. According to Ehret, chronons can be found in the genome of the cell nucleus and in the genome of the organelles. As has been shown for *Acetabularia* the anucleate cell can oscillate even in the presence of actinomycin. An inhibitory effect of actinomycin should however be expected in anucleate cells if the chronon is contained in the organelles.

Circadian rhythms represent only a special case which shows that multicellular and unicellular organisms as well as individual cells can measure time. This ability is also found in many other rhythms. Whatever the advantage in evolutionary selection for the oscillating biofunctions, the importance of time measurement in living systems is not restricted to periodic phenomena. Of still greater importance is the time measurement or temporal organization for the differentiation. An important question in this connection is whether time measurement in rhythmic and in differentiation processes is based on the same principles. An answer to this question must rely on further elucidation of the basic molecular mechanisms. Studies on individual single cells and on cell fragments promise to be helpful in achieving this goal.

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Biological clocks in animal orientation and in other functions

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Abstract

The adaptive significance of biological clocks, and the mechanisms of these clocks, are discussed in a variety of functions. Emphasis is laid on clocks involved in orientation by celestial bodies, and in pupal eclosion of insects; but clock mechanisms in reproductive isolation, in 'time memory', and in pattern and phase of the rhythm of locomotor activity are also dealt with. The paper is focussed on circadian clocks, but biological rhythms with other period length are mentioned when appropriate. It is shown that in nearly all instances the clocks are endogeneous and persist in constant conditions, with period lengths slightly deviating from those of the environmental cycles. Periodic stimuli from the environment entrain the endogeneous rhythms. In many cases such rhythms can be considered as selfsustained oscillations. The question why such oscillations were evolved is discussed.

Introduction

> 'The study of adaptation is not an optional preoccupation with fascinating fragments of natural history; it is the core of biological study.'
>
> Pittendrigh (1958, p. 395)

In a great variety of organisms it has been found that many functions show periodicities corresponding to those of the environmental cycles. These biological cycles are often not caused directly by environmental changes, but are based on endogenous periodicities which are synchronized with the external period. It is beyond the scope of this paper to discuss the adaptive value and the ecological significance of biological clocks in detail, or to describe all the behavioural, physiological, and anatomical adaptations that have been developed to place certain functions at a specific phase of the environmental cycle, and to exploit the temporal ecological niches. Therefore, I shall deal with only a few functions here, because I think that they reflect general principles. The paper will be focussed on the circadian clock. However, when appropriate, biological cycles with other cycle lengths will be mentioned.

Biological clocks in celestial orientation

Historical remarks

Celestial orientation in animals was discovered 60 years ago. In 1911, by some simple but ingeneous experiments, Santschi showed that ants can use the sun to find their way back to the nest. When he shielded the ants from the direct light of the sun, and reflected its rays with a mirror, the animals predictably altered their course. In following years a similar mode of orientation has been found in many other insects as well as in other groups. It has been shown that animals are able to keep a straight course by using the sun or an artificial light source as the directional cue. Since the light source provided a reference direction as does the needle of a compass, this type of orientation was termed 'light compass reaction'.

If an ant uses the sun to find the direction back to its nest, we must assume that it does so by remembering the angle to the sun on the way out, and following the angle corresponding to the opposite direction on return. Since the sun moves in the sky in the course of time, such an explanation would only suffice if the return followed the departure fairly rapidly. If hours elapsed between the two, reliance on the angle to the sun would be misleading. This difficulty could be overcome if the animals were able to make allowance for the time passed, and to compensate for the sun's motion accordingly. Such a possibility was already mentioned by Santschi (1913). It seemed implausible at that time, however, and later experiments with ants (Brun, 1914) and with bees (Wolf, 1927) seemed to provide contradictory evidence. The hypothesis was therefore discarded, and not considered again until 35 years later when Gustav Kramer's work on orientation in birds, and the work of Karl von Frisch on the orientation of bees, provided new insight and new directions for research.

Sun compass orientation in birds

Birds kept in captivity may show 'Zugunruhe' or migratory restlessness during the migration season. In 1949 Kramer observed that starlings (*Sturnus vulgaris*) kept in an outdoor aviary showed 'Zugunruhe' oriented in the normal migratory direction in most cases. The birds also exhibited this type of oriented behaviour in a small circular cage where the sight of landmarks was excluded. When the sky was clear the birds were oriented, but not when it was heavily overcast. By Santschi-type experiments, using mirrors, Kramer (1950) conclusively demonstrated that the sun was the orienting stimulus. Since the same direction was followed at different times of day, Kramer (1950, 1952, 1953) concluded that the birds were able to compensate for the sun's movement.

In further experiments starlings were trained to find food in given compass directions (Kramer and von St. Paul, 1950). With this method all the previous findings were confirmed (Kramer, 1952, 1953). In particular, the compensation for the sun's change in position during the day was demonstrated very reliably. Experiments with a starling that was trained between 7 and 8 a.m., and tested at other times of day are shown in

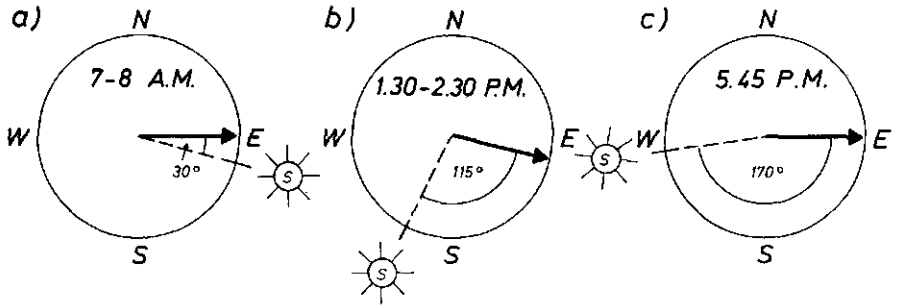

Fig. 1. Mean direction of choices of a starling trained to the east in the morning (a), and tested at other times of day (b and c). Note change of angle with the sun corresponding to time of day. (After Kramer 1952.)

Fig. 1. The bird looks for food in the same compass direction at all times, rather than keeping the same angle with the sun. Further confirmation for the capacity to compensate for the sun's movement was provided by experiments in which a fixed artificial light source was substituted for the sun. (Kramer, 1952, *1953*).

On the basis of these findings the question arose as to which clock mechanism was responsible for the compensation of the sun's motion. At that time, ideas on some unknown cosmic factor able to give information on the time of day, were still prominent and such speculations were supported by the spectacular observations of S. Takata (1949) and M. Takata (1951) on a biologically effective component of solar radiation that could be observed even indoors. (These observations were never confirmed.) In 1953 I showed under experimental conditions that the clock underlying the compensation of the sun's motion was independent of local time. By a shift of the light-dark regime I was able to shift the clock underlying sun compass orientation (Hoffmann, 1953, 1954). Fig. 2 shows the theoretical expectation from such an experiment, and Fig. 3 gives the experimental results. As can be seen, after an exposure to a light-dark regime out of phase with local time the direction chosen by the birds was

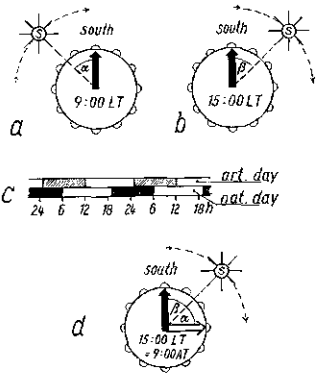

Fig. 2. Expected change of direction of a starling trained to look for food in the south, if its clock has been shifted 6 h backwards by exposing the bird to an artificial light-dark cycle 6 h behind the natural day (as indicated in c). The expected angle to the sun before (a. b) and after (d) shifting is indicated. S = sun; LT = local time; AT = artificial time. Solid arrow = training direction; open arrow = direction expected after shift of clock. The black and hatched areas in 'c' indicate darkness. (From Hoffmann, 1954.)

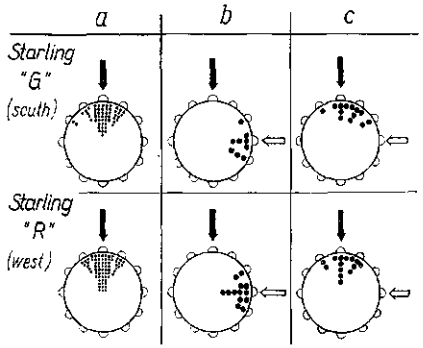

Fig. 3. Change of direction in two starlings orienting by the sun. a: Critical tests during training in natural day; b: after 12–18 days in artificial day, 6 hours behind local time; c: 8–17 days after return into the natural day (outside aviary). Note change of direction in b, and return to original direction in c. The large circle symbolizes the training apparatus, the small semicircles on its circumference represent the 12 feeders (which were empty in these tests). Each dot represents one choice. Black arrows give the original training direction, open arrows the direction expected after shift of clock. (After Hoffmann, 1953, 1954.)

predictably altered. This experiment has since been repeated by many investigators, not only with birds but also with many other animal groups (cf. Fig. 4, 7, 9). The results were nearly always the same: The clock underlying sun compass orientation could be reset by a shift of the light regime, as indicated by the change in the direction taken. Further experiments with starlings also showed that the clock is not abruptly reset by an abrupt shift of the light cycle, but is gradually drawn into phase with the cycle (Fig. 4). Similar findings result from clock-shift experiments in other functions, for instance in the circadian rhythm of locomotor activity (cf. Hoffmann, 1963).

A further set of experiments has demonstrated that the clock underlying sun compass orientation may free-run in constant conditions (Hoffmann, 1960). Starlings were kept, first in an artificial light-dark regime corresponding to that of the natural day, then in constant light, and finally again in the light cycle corresponding to external conditions (Fig. 5). During the whole time the locomotor activity of the birds was recorded. As Fig. 5 indicates, the rhythm of locomotor activity showed a period shorter than 24 hours, thereby deviating in phase more and more from the solar day. When orientation under such conditions was tested (Fig. 5b), it was found that the direction chosen deviated from the original training direction but corresponded with the direction expected according to the phase difference between the rhythm of locomotor activity and

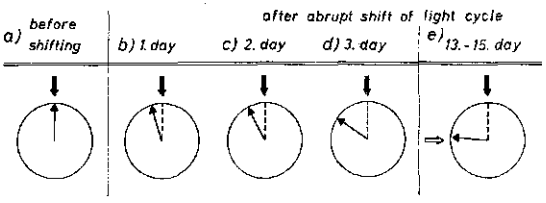

Fig. 4. Change of direction in starlings after abrupt shift of light cycle 6 h forward. Mean directions before (a), during (b–d), and after (e) the internal clock was shifted by the shift of the light cycle are indicated by the centrifugal arrows. Further explanation see Fig. 3. Experiments with 3 birds combined. (After Hoffmann, 1954.)

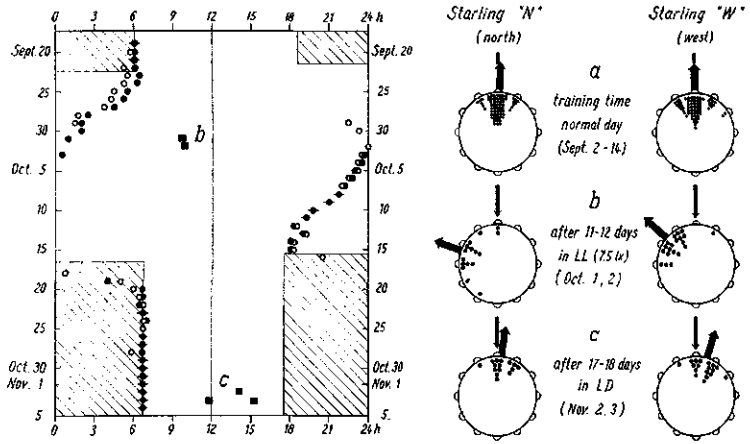

Fig. 5. Rhythm of locomotor activity (left) and direction finding (right) of two starlings kept in constant light and temperature. Note that the rhythm of activity advances about $\frac{1}{2}$ h per day in LL, and that the direction in b is changed correspondingly. Left: The hatched areas indicate darkness; onsets of activity are given by open (starling N) or solid (starling W) circles; the black squares mark the test times corresponding to b and c at the right. Right: Tests on orientation during training (a), after 10–11 days in constant conditions (b), and in artificial day synchronous with natural day (c), LL = constant light; LD = light-dark cycle corresponding to natural day; centrifugal arrows give mean direction of choices. For further explanations see Fig. 3. (From Hoffmann, 1960a.)

the natural day. When the artificial light regime with the natural day was reintroduced the rhythm of locomotor activity became resynchronized, and the birds again chose roughly the old training direction (Fig. 5c). These findings show that the clock underlying time-compensated sun orientation is endogeneous and circadian, and that the same clock mechanism underlies both orientation and the circadian rhythm of locomotor activity.

Since sun compass orientation in birds was found by observing 'Zugunruhe', one may assume that it is used as one of the orientational mechanisms in day-time migration of birds. It is also conceivable that birds use this type of orientation when foraging at larger distances from their nests. Experiments on sun orientation in starlings have been discussed here in some detail since with this species, the underlying clock mechanism was analysed earlier and in more detail than in other animals.

Sun compass orientation in other animals

In the same year that Kramer discovered time-compensated sun orientation in birds, von Frisch (1950) was able to show that the honey bee (*Apis mellifica*) is also able to compensate for the sun's motion. He trained bees to a feeding place west of the hive in the evening, moved the hive to a new site during the night, and tested their behaviour

the next morning. The majority of bees visited the feeder located to the west, i.e. in the old training direction, although they now had to fly away from the sun, instead of towards it as during training. Further experiments, including observations on the bees' dances, confirmed these findings and definitely proved the bees' capacity to compensate for the sun's motion (von Frisch, 1953, 1965).

After time-compensated sun orientation was discovered in birds and bees, it was described in an increasing number of animals, in other insects as well as in some crustaceans, spiders, fish, amphibians, reptiles and mammals. I will mention only two further examples since they provide additional information, especially on the adaptive significance of this type of orientation.

Talitrus saltator is a little sand hopper that lives on the shores of the European seas near the high water mark. It is mainly active at night and spends the day buried in the wet sand. If an animal is dug up during the day and placed in dry surroundings, it will flee in a direction perpendicular to the coastline from which it originated and will dig itself into the sand as soon as it reaches wet ground. If it is thrown into the water, it will swim in the direction of land. In a series of experiments similar to those Kramer carried out with birds, Pardi and Papi (1953) showed that *Talitrus* uses sun compass orientation. Pardi and Grassi (1955) demonstrated that the direction taken can be predictably changed by exposing the sandhoppers to a shifted light regime. The capacity to orient persists in constant conditions and is independent of local time, as was shown by Papi (1955). He collected sand hoppers near Pisa in Italy, and shipped them to Argentina. From the time of capture until they were exposed to the sun in Rosario de Santa Fé in Argentina the animals were kept in constant dark. As shown in Fig. 6, the sand hoppers chose the same angle to the sun as did animals that were collected at the same site and tested at Pisa at the same hour of Universal Time. These findings mean that the shipped animals had their clocks still running on Italian time in Argentina, after 14 days in constant dark, and that they were not influenced by the difference in the direction of the sun's movement, which is clockwise in Italy but counter-clockwise in Argentina.

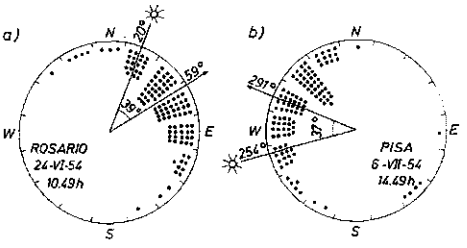

Fig. 6. Escape directions of sand hoppers (*Talitrus saltator*) collected from a population near Pisa, Italy. a: Animals tested at Rosario, Argentina, collected on 10 June. b: Animals tested near place of collection at the same Universal Time (i.e. 14.49 MGT) at which experiment a was performed. Note that the angle with the sun is the same in both cases. Dates and the official local times for the experiments at the two places are given. Each dot represents one position of one animal. The arrows give the mean escape direction. (From Papi, 1955.)

The adaptive value of the escape behaviour and its orientational mechanism in *Talitrus* is obvious. If an animal is, by some accident, transported inland, or if it drifts into deeper water, it will be able to reach the shoreline, irrespective of the special local conditions. Correspondingly, a similar escape reaction has been observed in several other littoral or riparian arthropod species.

In the southern cricket frog, *Acris gryllus*, Ferguson et al. (1965) described a comparable escape mechanism. The animals live along the margins of fairly large bodies of permanent water. If a frog is thrown into the water, it swims in a direction perpendicular to the home shoreline. This reaction may be a means of avoiding fish predators in shallow water. In day-time the animals use sun compass orientation. When frogs were placed in enclosures on other shores, the escape reaction was oriented towards the new shore line within 7 days. Fig. 7 shows the result of experiments with animals tested in an aquatic arena of 3 m diameter from which only the sky was visible (Taylor and Ferguson, 1970). Fig. 7 (I) shows the performance of (A) normal animals caught immediately before the experiment, (B) animals exposed to an illumination regime 6 hours in advance of local time for 7 days prior to the experiment and (C) animals trained to a new shore line for 7 days prior to the experiment. As can be seen, in all cases the frogs orient in the direction that was expected according to the treatment. Fig. 7 (II–IV) shows that orientation, learning a new shore-line, and shifting the orientational clock, was still possible after the eyes of the animals had been removed. Only when the pineal and the brain were shielded by opaque Teflon in addition to blinding did the orientation disappear (Fig. 7(V)).

Entrainment by light cycles of the rhythm of locomotor activity in eyeless animals has already been described in other vertebrates: in a salamander (Adler, 1969), in

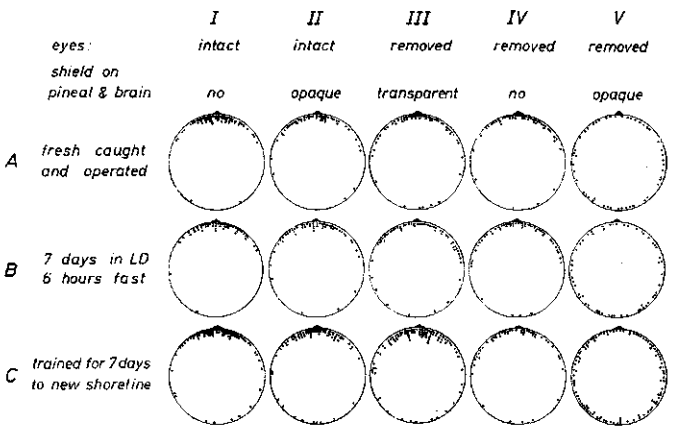

Fig. 7. Escape directions of frogs (*Acris gryllus*). The treatment prior to the experiment is indicated (cf. text). Pointers refer to direction expected according to pretreatment; black dots represent positions of frogs after testing. Note that all frogs (I–IV), except those in which the eyes had been removed and the pineal region and brain had been shielded (V), were oriented according to expectation. (After Taylor and Ferguson, 1970.)

lizards (Hoffmann, 1970; Underwood and Menaker, 1970) and in a bird (Menaker, 1968). The findings of Taylor and Ferguson (1970) in *Acris gryllus* indicate that extraretinal receptors may convey information, not only on the illumination schedule, but also on the direction of the light source.

Summarizing the findings on sun compass orientation it can be stated that this type of orientation is widespread in the animal kingdom. Its use has been demonstrated in a variety of functions. The basic components are: information on the direction of the sun, an adequate knowledge of the suns's path across the sky, and information on the time of day. The latter information, in nearly all cases, is derived from an internal circadian clock, which can be readily adjusted to local time by the external light schedule.

Moon compass orientation

In 1923 Santschi reported observations which indicated that by night some ants use the moon to maintain their direction. This was definitely proved by Jander in 1957. However, no attempt was made to investigate whether this included the ability to compensate for the moon's changing position in the sky. Yet in some crustaceans such a capacity has been reported.

Papi and Pardi (1953, 1959; Papi, 1960) found that sand hoppers *Talitrus saltator* can find the direction perpendicular to the shoreline, not only by using the sun, but also by using the moon at night. They obtained oriented escape reactions on moonlit nights between the first and last quarter. In moonless nights no orientation was observed. By mirror experiments, and by substituting an artificial light source for the moon, they conclusively showed that the moon is the guiding stimulus. The animals were able to orient, not only if they were caught immediately prior to the experiment, but also if they were collected the day before, or even at the time of the preceding new moon, and kept in constant dark until the experiment (Fig. 8).

On the basis of these findings Papi (1960) postulated that the animals possess, in addition to the circadian clock underlying sun compass orientation, an endogeneous lunar clock running on lunar-day time. Such an assumption seems feasible since tidal and bitidal rhythms that continue in constant conditions have been repeatedly reported (cf. Enright, 1963; Neumann, 1969). In an isopod Enright (1970) reported a free-running tidal rhythm of locomotor activity in constant conditions which persisted for more than four months. Another possibility for correct lunar orientation would exist if the animals were able to take the shape of the moon into consideration (cf. Hoffmann, 1965), and thereby determine the position of the sun beneath the horizon.

In the sand hopper *Orchestia cornuta*, Enright (1961) also found time-compensated lunar orientation. However, the animals compensated for the moon's motion only if they had seen sunset and/or moonrise on the night of observation. Animals collected the morning before and kept in constant dark until the time of the experiment, maintained a relatively constant angle with the moon, regardless of the moon's position in the sky, or its shape. On the basis of these observations Enright suggested an hour-glass type of clock, which is set in motion by sunset and/or moonrise. Moon orientation was

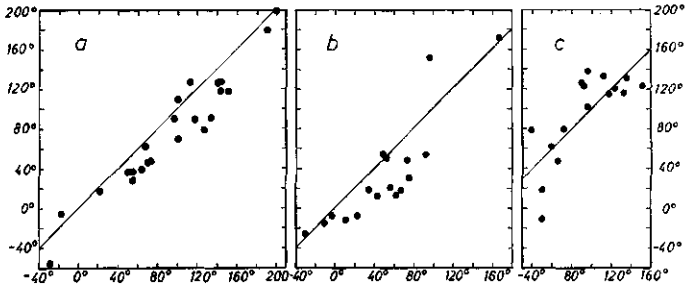

Fig. 8. Orientation by the moon in *Talitrus saltator*. a: sand hoppers collected immediately before experimentation; b: sand hoppers collected on day before the experiment, and kept in constant dark until that time; c: sand hoppers collected at the preceding new moon, and kept in darkness until the time of the experiment, 11 to 20 days later. Abscissa: expected angle with the moon; ordinate: observed angle with the moon. Each dot represents the mean direction of one experiment, consisting of between 100 and 400 single observations. (From Hoffmann 1965, after Papi and Pardi, 1953, 1959, 1963.)

also reported in other amphipods, in isopods, in spiders, and indications for orientation by the moon have been reported in some amphibians and birds. With regard to an underlying clock mechanism little information can be gained from these findings.

In general, it can be stated that direction finding with the moon has been shown in several instances. Information on the clock mechanism compensating for the moon's motion in the sky is scarce, and crucial experiments still have to be done. It may well be that the orientational mechanisms differ in different species.

Star compass orientation

With the discovery of sun compass orientation in birds a mechanism was found that would allow day-migrating birds to find compass directions. However, the majority of birds migrate at night. In 1955 Sauer and Sauer (1955, F. Sauer, 1957) discovered that some European warblers showed oriented Zugunruhe under the starry sky in a Kramer-type cage. The directions taken corresponded to the migratory direction normal for the season, and for the species. The birds were also oriented under the artificial sky of a planetarium. These findings indicated that patterns of fixed stars are the guiding stimuli. Several of these observations have been confirmed in other bird species by other investigators (cf. Hoffmann, 1965; and below).

Essentially no time mechanism is necessary to find compass directions with the help of stars. We all can do so without knowing the time of night, or the season, if we have some knowledge of star configurations. Even without such knowledge we might be able to determine the centre of rotation of the stars, and thus the celestial pole. However, Sauer (1957, 1960) found correct orientation under the stationary planetarium sky, but only when the planetarium was adjusted to local time and season. If he projected a sky that differed from that to be seen at the hour and season of the experiment, he observed

gross deviations or disorientation in many cases. Sauer interpreted these findings as showing that the birds are able to compensate for the longitudinal displacement that was simulated by the time-shifted star patterns. It has been pointed out that these interpretations far exceed the evidence, and that there are several inconsistencies (Wallraff, 1960a, b; Hoffmann, 1965). Nevertheless, these deviations might imply that a mechanism of compensation for time is involved in stellar orientation.

In the indigo bunting, (*Passerina cyanea*), Emlen (1967 a,b) was able to partly confirm Sauer's findings. He observed correct orientation under the natural sky as well as in a stationary planetarium. However, if he exposed the birds to star configurations 3, 6 or 12 hours out of phase with local time, there was no marked difference in the direction chosen under the sky adjusted to local time and season. Deviations similar to those reported by Sauer were found only in 3 out of 28 such experiments, and might be due to other factors. Emlen concluced that 'experimental evidence suggesting a bicoordinate navigational ability in the indigo bunting is entirely lacking' (cf. p. 187). Wallraff (1969), working with mallards (*Anas platyrhynchos*) trained to directions under planetarium skies, also could not detect any indication of a compensation for time.

A direct attempt to test whether a clock mechanism is involved in stellar orientation was made by Matthews (1963). Bellrose (1958) has shown in Illinois that mallards caught at their wintering grounds and released some distance from the place of capture, turned northward regardless of the release point's distance or direction. Matthews confirmed these findings for Slimbridge in England. Here the birds turned toward the northwest. Since no function for this behaviour could be suggested, Matthews called it 'nonsense orientation'. As was already shown by Bellrose the ducks were well oriented by day or night under a clear sky, but not under a heavily overcast sky. When Matthews released such mallards after they had been exposed to a light schedule advanced, or delayed, for 6 hours relative to the natural day, the direction taken by the ducks was predictably altered under the sun, but not under the stars (Fig. 9). These findings

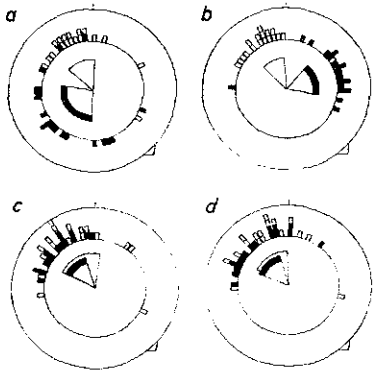

Fig. 9. Initial orientation of Mallards released under clear sky. The birds were followed with binoculars until out of sight. The bearings at which each bird was lost to sight is given by a 5° block. White blocks represent untreated controls, black blocks birds which had been exposed to an artificial day, advanced (a and c) or delayed (b and e) for 6 hours relative to local day. The central fans summarize the mean deviations of the two groups from their medians. The arrowheads at the outer circle give the home direction. a and b: releases at day time under the sun; c and d: releases at night time under the starlit sky. Note that initial orientation of birds whose clock had been shifted differs from the controls, only under the sun (a and b), but not under the stars (c and d). (From Matthews, 1963, two figures combined.)

suggest that in this case stellar orientation is not based on a clock mechanism and they concur with Emlen's results (for further discussion cf. Hoffmann, 1965)

The observations on a single bobolink (*Dolichonyx oryzivorus*) by Hamilton (1962) give some indications for the participation of a clock in stellar orientation, but the data are too few and the situation too complex for a definite conclusion. Rabøl (1970) found no difference in initial orientation between normal birds that had been exposed to a shifted light schedule, when the birds were displaced, and their nocturnal activity recorded in Kramer-type cages. Orientation under moonless clear skies was also reported in amphibians (Ferguson et al., 1965) but these experiments supply no information on the involvment of a clock mechanism.

In general it can be said that the evidence for the participation of a clock in direction finding by stars is still rather equivocal. From the astronomical situation, there is no need to postulate a clock mechanism. With regard to true bi-coordinate navigation by stars it can be stated that, though such a possibility cannot be entirely ruled out, no evidence so far necessitates such an assumption.

One clock mechanism, however, has recently been shown to be involved in orientation by stars. It concerns the direction taken rather than the mechanism of orientation. Fig. 10 shows experiments by Emlen (1969) with indigo buntings. By photoperiodic treatment, the physiological state of a group of buntings was advanced so that they were in 'fall' condition while normal birds were in 'spring' condition. Both groups were tested under the spring sky in the planetarium. A clear cleavage of the direction taken was found, corresponding to the physiological condition of the birds. No indications for a 'conflict of antagonistic migrating drives' after 'confrontation with the artificial starry sky of the contrary migration period', as Sauer (1957) had reported, could be detected in these experiments.

Direction finding by the earth's magnetic field

During the last 13 years Merkel, Fromme and Wiltschko (Merkel et al., 1964, Merkel and Fromme, 1958; Fromme, 1961; Merkel and Wiltschko, 1965; Wiltschko, 1968, 1971) have accumulated evidence that European robins (*Erithacus rubecula*) are able to

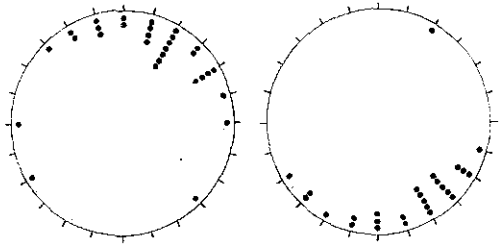

Fig. 10. Orientation of Indigo Buntings, tested in spring under a spring planetarium sky. Left: birds in spring migratory condition (normal); right: birds in fall migratory condition (by photoperiodic treatment). Each dot gives the mean direction of a bird in one night of testing. North is at the top. Note difference in directions taken by the two groups. (After Emlen, 1969.)

find the migratory direction in the absence of celestial cues, and that they use the earth's magnetic field for orientation. There is now so much evidence, including predictable changes in the direction taken after manipulation of the magnetic field that doubt seems no longer justifiable. In initial orientation of pigeons (*Columba livia*) influences of interference with the magnetic field have also recently been reported (Keeton, 1971).

In the past many people have tried to show orientation in closed rooms, or the influence of interferences with the magnetic field on orientation, but nearly all such attempts have failed. The last attempt reported by Emlen (1970) also had entirely negative results. Perdeck (1963) and Wallraff (1966) attempted to find oriented 'Zugunruhe' in European robins after excluding celestial cues in Kramer-type cages of their own construction, but failed to get oriented behaviour. However, in cages borrowed from Merkel, Wallraff (pers. comm.) recently recorded oriented migratory behaviour in this species. It must also be pointed out that in practically all cases in which direction finding by celestial cues was shown, random orientation was reported when these stimuli were not available. This holds for experiments under a heavily overcast natural sky, as well as for planetarium experiments when the stars were not projected; it holds for experiments in which animals were trained to compass directions, as in the case of trained starlings (see Fig. 11), as well as for observations or recordings of spontaneous migratory behaviour.

How can these conflicting findings be reconciled? No answer is readily apparent. Nothing is known so far about the site and the physiology of reception of the stimuli derived from the magnetic field. One explanation for the discrepancies might be that direction finding by the magnetic field is a complicated and inaccurate process, especially under the restricted conditions of an orientational cage, and that for some reason,

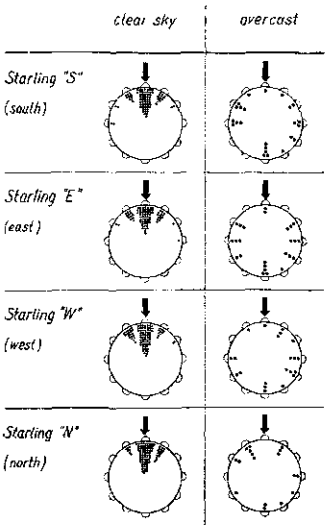

Fig. 11. Choices of four trained starlings under clear sky (left) and under heavy overcast (right). Each bird had been trained to one of the four cardinal compass directions. Further explanations see Fig. 3. (Original.)

as yet unknown, the cage constructed by Merkel is more suitable for picking up such weak stimuli than any other cage that has been constructed so far. This is admittedly a rather vague arguement, but support for it can be gained from the fact that Wallraff (1966) found no orientation in cages of his own construction, but could observe oriented behaviour in Merkel's cage, using the same bird species in both instances. It must also be stressed that in the experiments reported by Merkel and associates, the orientation was rather inaccurate and showed a large degree of scatter. Their reports are based on an extensive amount of data, much more than was accumulated in most experiments on orientation by celestial cues. In the latter case usually only a few experiments were performed under conditions in which celestial stimuli were excluded. Experiments with homing pigeons have also shown that whenever celestial cues for direction finding are available, they are used (Graue, 1963; Keeton, 1969).

Orientation by magnetic field has also been claimed in a number of other animals (cf. Emlen, 1970).

Bicoordinate navigation by sun?

Up to this point a relatively simple type of orientation has been considered, the finding of compass directions. Extensive experiments with homing pigeons as well as with wild bird species have shown that birds are also capable of true bicoordinate navigation (Kramer, 1961; Matthews, 1968; Schmidt-Koenig, 1965; Wallraff, 1967). Birds taken from the loft or nesting site and released at unfamiliar sites, may often turn homeward regardless of the release point's position relative to home.

When direction finding by celestial bodies had been discovered, it was thought that birds might also use celestial stimuli for bicoordinate navigation. Sauer (1957) assumed that birds can use stars for navigation. The evidence for this claim has already been discussed (see p. 183f). A complete hypothesis for navigation by the sun was proposed by Matthews (1953, 1968). It is not appropriate to discuss here all the evidence for and against this hypothesis. Only some findings will be mentioned. Some early experiments, in which birds were prevented from seeing the sun for several days prior to release during the time of equinox, seemed to strongly support the hypothesis (Matthews 1953). However, these findings could not be repeated by other investigators (cf. Hoffmann 1958, Keeton 1970). Clock-shift experiments by Matthews (1955) suggested that displacements could be simulated thereby. However extensive experimentation by Schmidt-Koenig (1961, 1965) and others, and more recently by Keeton (1969), show that the sun is only used as a compass in initial orientation of displaced birds. Deviations similar to those I reported (1953, 1954) in starlings were found (cf. Fig. 2 and 3). While in the majority of cases impairment of initial orientation or random departures were reported under heavily overcast skies, in several cases good orientation was found (Schmidt-Koenig, 1965; Wallraff, 1966; Keeton, 1969). Keeton (1971) recently reported that magnets may interfere with initial orientation of released homing pigeons, especially under overcast conditions.

The mechanism of navigation in birds is still unknown. The evidence so far suggests that sun compass orientation, and possibly orientation by the earth's magnetic field,

are parts of the navigational system, though neither of them seems to be indispensable in the presence of the other. In view of the many difficulties that enter into the critical evaluation of experiments on bird navigation, such as the role of motivation, learning and previous experience, influences of known and unknown landmarks and the possibility of 'nonsense' orientation and in view of the conflicting results reported by different researchers for similar experiments, the possibility of navigation by the sun cannot be rigidly excluded, although this becomes increasingly unlikely. To date no one has suggested a coherent and independent hypothesis that is testable. The clock mechanism to be postulated for bicoordinate sun navigation would be drastically different from the circadian clock which has been shown to underly sun compass orientation. It should possess a much higher accuracy and it should be resistent to a shift of the external cycle of day and night. (For further discussion of the clock required cf. Hoffmann, 1965.)

Function and mechanism of clock-controlled pupal eclosion in insects

General remarks

In many insects emergence of the imago from the pupa is restricted to certain times of day or night. Such a rhythm of eclosion seems to be the rule rather than the exception in most groups of holometabolic insects (for a review cf. Remmert, 1962). The classical example is *Drosophila*, since this genus has been extensively used for experimental work on the physiological mechanism of the circadian clock.

While the phenomenon is obvious, the adaptive value of eclosion rhythms has only been demonstrated in a limited number of cases. In *Drosophila*, Pittendrigh (1958) has shown that eclosion is phased close to the part of day with the highest relative humidity and that it is closer to this time in *D. pseudoobscura*, which occupies dryer, warmer areas and habitats than in *D. persimilis*, which is restricted to cooler and moister surroundings. Phasing emergence into the moister part of the day is important since at eclosion the flies are very susceptible to desiccation. Its wings may fail to expand properly in low humidity. However, this is not the universal function of eclosion rhythms. Many insects emerge from the pupa at some other time (cf. Remmert, 1962).

In a number of insects, adult life is extremely short; it lasts only a few hours. For these species it is of utmost importance that the sexes are concentrated for pairing and reproduction. Remmert (1962) suggested for such animals a mechanism of concentration by the concerted action of several biological clocks, which restrict reproduction in the populations to short distinct times. The most famous example for such a 'Ballungsmechanismus' is the swarming of the Palolo worm (Caspers 1951). An even more fascinating instance is the semilunar rhythm of emergence in the midge *Clunio marinus*. The timing mechanism of this behaviour was recently unravelled by Neumann (1963, 1966a, b) in a series of elegant experiments. Since it serves to illustrate two of the main functions of biological clocks, choosing the optimum phase of the abiotic cycle for a vital function and synchronizing the sexes for reproduction, it will be discussed in some detail.

Clunio marinus is a small midge of the family Chironomidae which has invaded the intertidal zones. The animal is distributed in Europe from temperate to arctic latitudes. On the Atlantic coasts of France and Spain the larvae live mainly in the lowest parts of the intertidal zones, which are only exposed during the times of spring low water. This means that the eggs have to be deposited during this time, since they float and would be carried away by the tides if laid on the water surface. The imagines can only reproduce for up to two hours. The female is wingless, and during emergence she is usually assisted by the male who then copulates and carries her to the larval habitat where the eggs are deposited.

For the habitat of a *Clunio* population in Normandy, France, Fig. 12A shows the change of the height of low water during the lunar month. Twice during that period, just after full and new moon, the values reach a minimum. Low water occurs twice daily, the times are shown in Fig. 12B. On the days of spring tides this is in the early morning and in the evening. Fig. 12B also shows the times of eclosion. The midges emerge only during the days of spring tides, when the lowest part of the intertidal zone is exposed, and only before or at the times of the evening low water. This suggested that the tides are not directly timing emergence.

When Neumann (1963, 1966a, b) cultivated *Clunio* in artificial light-dark cycles in the laboratory, he found that eclosion occurred at the phase of the light cycle corresponding to the time of day at which the midges emerge in their natural habitat. Fig. 13

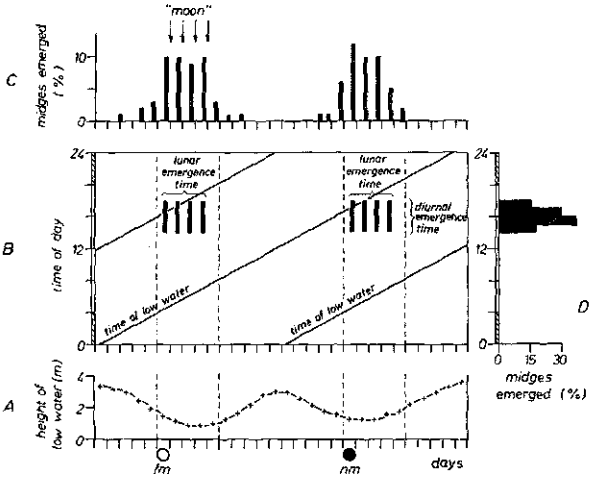

Fig. 12. *Clunio marinus* population from Normandy, France. A: Change of height of low water during lunar month. fm = full moon; nm = new moon. B: Change of time of day of low water during lunar month (schematical), and times of emergence in the field. C: Emerged imagines in light-dark cycle (16:8 h) with artificial moon-light (every 30 days 4 nights with 0.4 lux). D. Diurnal emergence time in LD 16:8 (L from 4.00 to 20.00). Note the correspondence between field observations and laboratory results. (From Neumann, 1966a.)

shows this for a population from Heligoland for three different light-dark ratios. Eclosion was restricted to the end of the light phase, corresponding well to the eclosion times found in the field for this species. Males emerged before the females, which seems common in insects with an extremely short adult life (cf. Remmert, 1962). Since the males need time to find the females, especially if the latter are wingless, the biological significance is obvious.

Neumann (1966b) could also show that the rhythm is truly circadian. By a shift of the light-dark cycle, the rhythm of eclosion could be shifted to any phase relation with the natural day. It continued in constant conditions with a period differing slightly from 24 hours. Cultures raised in constant illumination from the beginning did not emerge rhythmically. If, however, they were exposed to a single brief period of dark which did not contain any information on cycle length, rhythmic emergence was initiated. These results clearly show that the time of day of eclosion in *Clunio* is determined by a circadian clock which can be entrained by the light cycle.

While the dates of spring tides are the same at all parts of the coast, the phase of the 12.4-hour tidal cycle relative to local time may show drastic differences, even at about the same longitude. One should therefore expect equally drastic differences in the time of day of emergence between different populations. In the laboratory under identical conditions, Neumann (1966 a, b) was able to find such differences, and the same differences were also observed in the field. They are genotypic adaptations. Neumann (1966a, b) was able to crossbreed two populations which normally show no overlap in eclosion time, by shifting the light cycles in such a way that the times of emergence coincided. Fig. 14 gives the results. The F_1 showed an intermediate time of emergence; in the F_2 the maximum was still intermediate, but with much larger deviations, reaching the range of both the parental populations. These results and those of backcross

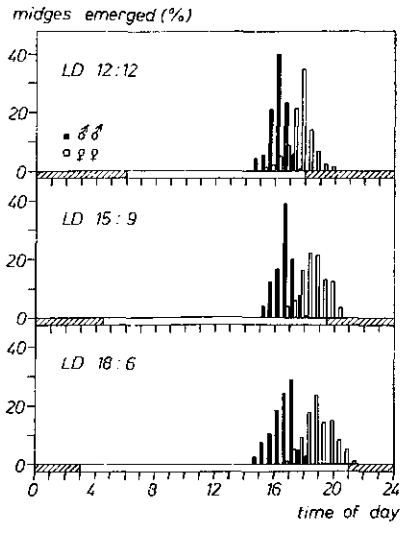

Fig. 13. Time of eclosion in *Clunio* population from Heligoland kept in three different artificial light-dark regimes. (From Neumann, 1966b.)

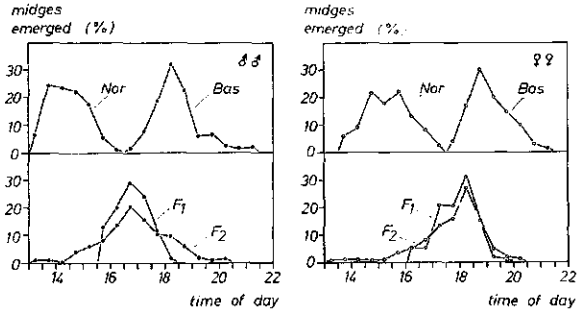

Fig. 14. Cross-breeding between two populations of *Clunio* which differ in their daily eclosion time. Above: Emergence time of a population from Normandy, France (Nor), and from the Basque Coast, Southern France (Bas). Below: Emergence times of F_1 and F_2 generations. Left: males; right: females. (After Neumann, 1966b.)

experiments indicate that the difference in time of emergence is controlled by a few genes only (Neumann, 1966b).

The question remains whether, in the different populations with the time of eclosion phased to different times of day, the whole circadian cycle is phase-shifted in relation to the daily light cycle, or whether the time of emergence is simply shifted on basically the same circadian cycle, like a rider on a time-clock. No experimental evidence is available so far. The mechanism could be even more complex, as it has been shown that an organism may possess not only one but many circadian oscillators, and that these oscillators may change phase to each other, depending on the external conditions (cf. Pittendrigh, 1960, 1965; Hoffmann 1970a, b, 1971).

The semilunar cycle of eclosion in Clunio

The findings described so far demonstrate that a circadian rhythm is involved in controlling the time of emergence in *Clunio*. However, in none of these experiments in which the animals had been exposed to a regular daily change of artificial light and dark, had there been any indication of a semilunar rhythm of emergence as it was observed in nature. Eclosion continued daily until the number of larvae and pupae was exhausted. This suggested that either the lunar light cycle, or the tidal cycle, was responsible for restricting emergence to the days around spring tides.

In experiments with artificial 'moonlight' Neumann (1966b) was able to initiate semilunar cycles of eclosion. He kept *Clunio* cultures in artificial light-dark cycles with 12 or 16 hours light per day, and in addition provided weak light of about 0.4 lux during the dark time for four or six days, at intervals of 30 days. Fig. 15 illustrates the treatment and its results. A clearcut semilunar cycle could be established, while in a control culture, without additional light at night, no such rhythm was discernible. Neumann (1966b) was also able to show that the semilunar rhythm is endogeneous. By giving only one period of artificial moonlight during the life of the larvae, he could

institute a semilunar cycle that ran on for more than three cycles, until the number of larvae and pupae was exhausted.

Fig. 12 sums up the whole picture and compares the observations in the field with the findings in the laboratory for one population. As can be seen, the behaviour in the field can be explained by the experiments. Also in the other populations examined, field observations and findings from laboratory experiments were in good agreement; in some cases the unkown times of emergence in the field could be correctly predicted from the experiments.

It should be mentioned that the semilunar cycle does not act directly on eclosion, but determines the beginning of pupation. Two to five days later the imagines emerge. Beginning of pupation is not restricted to a special time of day. Hence the two cycles influence successive developmental steps. The semilunar cycle, synchronized by moonlight, determines the beginning of pupation; the circadian cycle, synchronized by the daily light cycle, determines the time of day of emergence. Both cycles contribute in timing the important event, eclosion, to the optimum phase of the environmental cycle.

Some special cases

While Neumann (1966b) found in all populations of *Clunio* from France and Spain that artificial moonlight could initiate and entrain the semilunar (or, in one population, the lunar) cycle of emergence, he achieved only very weak synchronization in the Heligoland population with the same treatment. Heligoland is more northerly (54 °N) than the places of origin of the other populations. Here the culmination altitude of the full moon at summer solstice will reach only about 12.5 ° on average, and may be as low as 7.5 °, due to the fact that the moon's path varies in a 18.6-year cycle. Taken together with the greater brightness at night in summer, moonlight may be too weak a 'Zeitgeber' and too unreliable at northern latitudes. Nevertheless the Heligoland population shows a marked semilunar cycle of emergence in the field.

In a further set of experiments, Neumann (1968) was able to show that in this population stimuli from the tides act as 'Zeitgeber' for the semilunar cycle. In all experiments, the period of the induced semilunar cycle was correlated with the return of an equal phase relation between the tides and the daily cycle. It can be concluded that in the Heligoland population two 'Zeitgebers' may have an entraining effect on the semi-

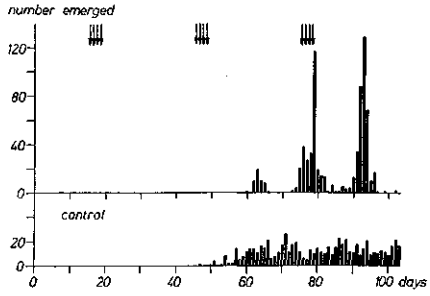

Fig. 15. Semilunar rhythm of emergence induced by artificial moonlight (4 nights with weak – 0.4 lux – light every 30 days) in a population of *Clunio* from Normandy, France. Above: experimentals; below: controls without additional illumination at night. (From Neumann, 1966b).

lunar cycle of emergence, a weak effect from the moon's light cycle, and a stronger effect from the interaction of the circadian and the tidal cycle. Different 'Zeitgeber' modalities may entrain the same biological rhythm, as has been repeatedly shown in circadian rhythms (cf. Hoffmann, 1969a). It is not yet known whether the more southerly populations, which can be fully entrained by moonlight, also react to tidal stimuli.

In a population living in Norway north of the Arctic Circle, Neumann and Honegger (1969) observed a strictly tidal cycle of eclosion. Preliminary experiments suggest that here timing of emergence is based on an interval timer, set in motion by the previous tide (Neumann, pers. comm.).

Concluding remarks

Clunio has been selected to demonstrate the function of clock-controlled emergence in insects since this animal has been best analysed so far. It also serves as an example for the fruitfulness of combining field observations with laboratory studies and shows the complexities with which the experimental ecologist is faced in this field, as well as the final rewards of carefully planned work. The differences between different populations, especially between those from northern and from more southern latitudes, also illustrate that the same function, adaptation to the temporal variations of the environment, may be achieved by different mechanisms.

Clunio also offers an extreme example for two of the main functions of clock controlled processes: the adaptation to cyclically changing surroundings, and the synchronization of the sexes. In this context it should be mentioned that lunar cycles of emergence have been reported, not only from marine insects, but also from several insect species from Lake Victoria in Africa (cf. Remmert, 1965). The functional significance of lunar cycles in such cases seems to be mainly the concentration of the sexes, since these insects are extremely short-lived and also possess a circadian cycle of emergence. However it must also be kept in mind that at least statistically, lunar cycles of, for instance, precipitation have been reported in some areas (Bradley et al., 1962; Adderly and Brown, 1962).

Biological clocks in other functions

Besides celestial orientation and eclosion in insects, many other functions have been described in which clocks are involved and in which the adaptive value of clock control can be demonstrated, or at least made plausible. A few of these functions will be briefly mentioned since they illustrate general principles (for more detailed discussions, and more examples cf. Cloudsley-Thompson, 1961, and Remmert, 1965).

Clock-controlled pheromone release

In many species of moths the males are attracted to the females over long distances by sex pheromones. It has been observed that the flight times of males and the numbers of

visiting females show a marked daily variation. Experiments have demonstrated that release of pheromone in the females, as well as reactivity in the males, can be governed by a circadian cycle entrained by the daily light cycle, which may continue in dim light (Shorey and Gaston, 1965; Bartell and Shorey, 1969; Jacklin and Yonce, 1969; Traynier, 1970; Sower et al., 1970). An example is given in Fig. 16.

The chemical constitution of the sex pheromones has been identified in a few cases only. However, by painstaking experiments with about 700 species of 27 Lepidoptera families, Priesner (1968, 1969, 1970) electrophysiologically determined the effectiveness of the sex pheromones of different species. He found that closely related monophyletic groups share the same sex attractant in the majority of cases. It is only the rare exception that speciation is accompanied by a change in sex pheromone. These findings were corroborated by behavioural observations.

This means that in related sympatric species the males would be attracted by the pheromone of the other species' females. One of the mechanisms of sexual isolation in such cases is different timing. Sympatric species with the same pheromone fly at different seasons, or at different times of day (cf. Kettlewell, 1942; Priesner, 1970; Malicky, 1970; Roelofs and Cardé, 1971). In *Rebelia*, for instance, pairs of species may occur at the same locality and during the same season, but they are effectively isolated since flight-time is at dawn in one species and at dusk in the other (Priesner, 1970). A similar example for two saturniid moth species from North America is given in Fig. 17.

These examples show still another function of clock-controlled processes, reproductive isolation. Such use of biological clocks is probably not uncommon in many groups of animals.

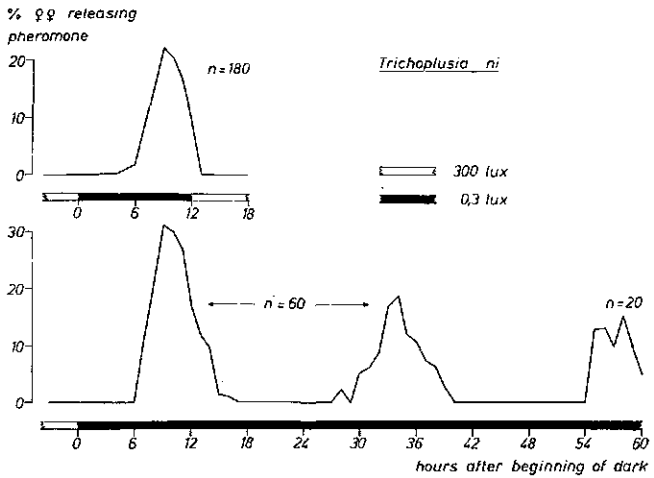

Fig. 16. Sex pheromone release in females of the Cabbage Looper Moth (*Trichoplusia ni*) in an artificial light-dark cycle (above), and in constant dim light of 0.3 lux (below). Note persistence of rhythm in constant conditions. (After Sower et al., 1970.)

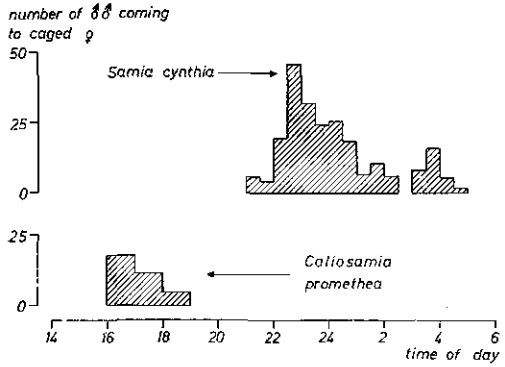

Fig. 17. Different times of day at which males visit females, an example for an isolating mechanism in two saturniid moth species from North America. (After Wilson and Bossert, 1963.)

'Time sense'

One of the first and most famous examples of the functional value of biological clocks was what has been called the 'time sense' or 'time memory' in the honey bee. In 1929 Beling showed that bees can be trained to come to feeding places at set times of day. The function of this capacity was mentioned by her. Flowers of different species may open at different times of day and may show a marked diurnal periodicity of secretion of nectar and of pollen supply, with different peak times for different plants (cf. Bünning, 1967). The correspondence between the behaviour of the bees, and the diurnal rhythm of flowers, has been analysed in detail by Kleber (1935). Bees can be readily trained within one or two days, but the behaviour is easily extinguished with lack of positive reenforcement. This is a good adaptation to the biological situation, in which one plant species will stop flowering and others will start, but it hampers physiological analysis.

Recently, however, Beier (1968) and Beier and Lindauer (1970) have developed a method to delay extinction. With this method they were able to show that the underlying clock is circadian. It can be shifted by a shift of the illumination cycle, it free-runs in constant light with a period of about 23.4 hours, and it can be entrained to light-dark cycles between 20 and 26 hours. This range of entrainment fits well into the range known from the circadian rhythm of other functions in other animals. However, in experiments combining laboratory and field conditions, Beier and Lindauer found that the sight of the position of the sun may have an additional influence.

Another recent report from this group shall be mentioned here. Medugorac (1967) and Medugorac and Lindauer (1967) found an unexpected effect of CO_2-narcosis on the mechanism involved in 'time memory'. After such narcosis, trained bees turned up at the original feeding time and also some time later, the delay of the second peak depending on the length of narcosis and on the concentration of the CO_2. Fig. 18 gives examples. While the second peak can be interpreted by the assumption that the underlying clock is stopped or slowed down by the narcosis, the persistance of the original peak is more difficult to interpret. In a series of elegant and careful experiments

Medugorac and Lindauer (1967) were able to exclude every other explanation except the assumption that two clocks are involved in this function. One of these can be stopped or delayed by the narcosis, while the other runs on unimpaired. In this context it should be mentioned that indications for several circadian clocks within the same organism are turning up in increasing numbers (cf. Hoffmann, 1970b).

Training to certain feeding times has also been successfully accomplished in birds

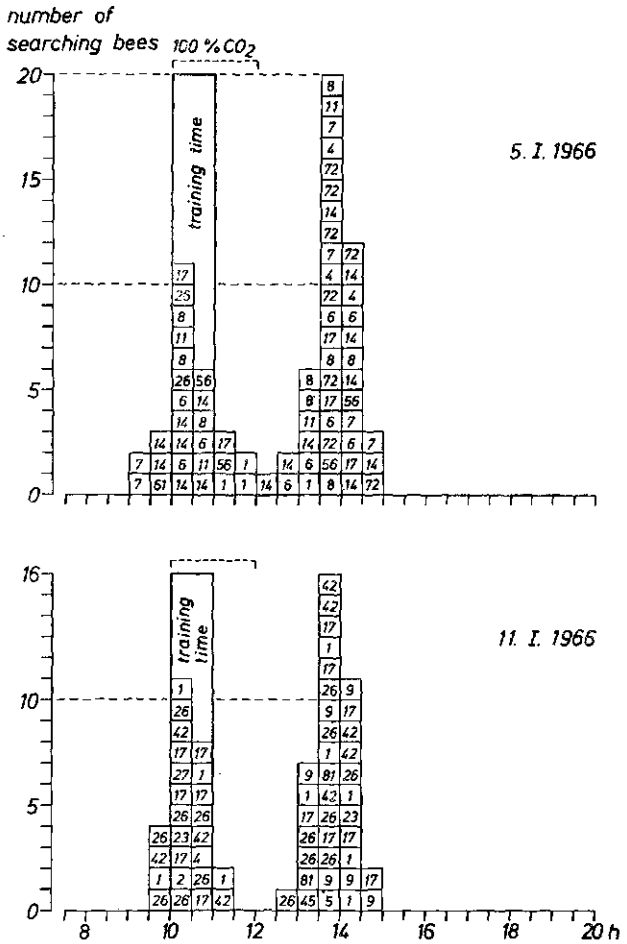

Fig. 18. Visits of bees, trained to come to a feeder at a specific time of day (bordered by heavy line), after CO_2 narcosis. The bees were kept in a bee-room, narcotized in 100 % CO_2 for 2 h beginning at their usual training time, and kept in constant illumination from narcosis until the next day, when the feeding place was constantly observed. Each visit was recorded. The bees were marked, individual bees are indicated by the numbers in the squares which represent visits. Note the two maxima of visits. (After Medugorac and Lindauer, 1967).

(Stein, 1951; Adler, 1963; 1964). Under field conditions this is probably much more common in many animals than the small number of known cases suggests. An adaptive value for such a performance could be suggested in many species, and further research in this direction might be fruitful.

Diurnalism and nocturnalism

It is well known that in many animals locomotor activity is largely or exclusively restricted to the light or the dark part of the daily cycle. In most of these animals the same distribution of activity was found in artificial light-dark cycles. The results of many experiments with many species show that the external cycle does not directly cause activity or rest, but synchronizes an endogenous circadian cycle.

Based on experiments in constant conditions as well as in artificial light cycles, Aschoff (1964) and Wever (1965) have developed a mathematical model for circadian rhythms, describing the behaviour of the circadian cycle in light-active and in dark-active animals. It is beyond the scope of this paper to discuss the model and its implications. However, it is worth noting that, especially in birds, but also in some mammals, data obtained under natural conditions were in good agreement with predictions derived from the model. In particular, the regular seasonal changes of activity time, and of the phase relation between activity rhythms and the natural light cycle, as well as their differences in different latitudes, could be described by the model (Aschoff, 1969). From this model it was suggested that the length of the light period and the duration of twilight are the effective stimuli. Aschoff et al. (1970) conclude that 'the circadian system and its mechanism of synchronization have been developed in such a way that, in addition to providing a stable phase relationship with the Zeitgeber, they match the ecological requirements', i.e. the adaptation to different lengths of the light-time.

However, scarcely any details are known about the adaptive significance of this feature of the rhythm of activity. It would be greatly desirable to test the applicability of the model in more detail and with more ecological data, particularly in closely related species with striking ecological differences.

Temporal patterns

The daily rhythm of a function observed in the field often shows not one but several peaks. Bimodal patterns are particularly common. It has often been assumed that the peaks, or the trough between them, are directly caused by environmental factors, e.g. that heat and low humidity around noon suppress activity, and thus cause the bimodality. However bimodal patterns are often observed in the laboratory in a rectangular light-dark cycle, with no change of external conditions in the middle of the light period, or even in constant conditions. Fig. 19 gives an example. Such findings show that even particular features of the pattern of activity may be preprogrammed internally, and are not necessarily caused by the direct influence of external factors and their changes (for more data see Aschoff, 1966).

The influence of external factors, however, should not be overlooked. They may

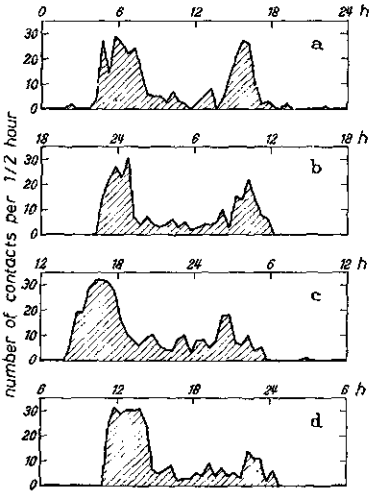

Fig. 19. Pattern of activity in two starlings kept in constant illumination of 7.5 lux. Note the bimodality in spite of the constant conditions. Since the birds free-ran under these conditions, the phase of activity time relative to normal time differs. a and d: starling N; b and c: starling W. (From Hoffmann, 1960b.)

modify the pattern, and accentuate or suppress parts of it. An example is given in Fig. 20, which shows the rhythm of locomotor activity of a wood mouse (*Apodemus sylvaticus*) in a temperature cycle. The temperature cycle does not entrain the circadian cycle of locomotor activity, but the pattern of activity is modified by the momentary conditions. High temperatures tend to suppress activity, low temperatures induce additional activity, not programmed by the internal cycle. In general one can say that the behaviour in the field is caused by the interaction of internal and external

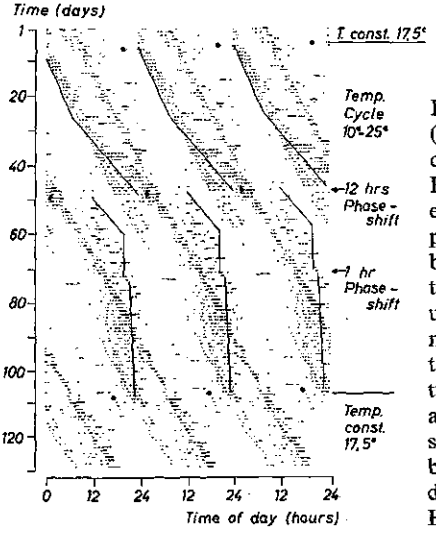

Fig. 20. Locomotor activity of a wood mouse (*Apodemus flavicollis*) in a sinusoidal temperature cycle (range 10–25°C) and constant light. Running wheel activity was registered by an event recorder, activity is indicated by vertical pen markings, successive days are plotted one below the other. To fascilitate visual following, the total record is given 3 times, each displaced upwards one day. The drawn lines connect the minima of the temperature cycle. Dots mark times at which the temperature cycle was instituted, phaseshifted abruptly, or ended. Note accentuation of activity by low temperatures, and suppression by high temperatures. Note also bimodality of activity rhythm in constant conditions (top and bottom of recording). (From Hoffmann, 1969.)

factors, a truism that is occasionally overlooked (for a more thorough discussion see Aschoff, 1966, and Enright, 1970).

Concluding considerations

A broad array of functions has been mentioned involving biological clocks, which are endogeneous, and only entrained to, but not directly caused by, the environmental cycles. In this context one point should be stressed. In none of the cases, including those in which proper timing was of vital importance for survival or reproduction, was there any indication of 'evasive unknown external factors' giving information on the time of day, or causing the diurnal cycle. In all experiments in which the animals were exposed to constant conditions for a sufficiently long time, it was found that the underlying clock could deviate from the exact period length of the geophysical cycle.

In organisms which live long enough, and in which a function may be recorded continuously without difficulty and without damaging the animal, it has been shown that a circadian or a tidal cycle may run on practically indefinitely (i.e. for the life time of the animal) in constant conditions, without a damping of the amplitude. In most cases the period under these conditions is slightly different from that of the geophysical cycle. This means that biological clocks, at least those underlying circadian or circatidal cycles, can be considered self-sustained oscillations in the technical or mathematical sense (cf. Pittendrigh, 1960; Aschoff, 1964; Wever, 1965). Predictions based on such an assumption have been experimentally verified in circadian rhythms of locomotor activity (Hoffmann, 1969b).

One question remains: Why was a self-sustained oscillation evolved as the clock mechanism underlying the timing of many biological processes? It seems, that in many instances a stimulus-response type of timing would suffice. In other cases a kind of hour-glass clock would be equally adaptive, it would meet the requirements of being prepared in advance for a coming event. One could argue that sometimes the events setting this hour-glass in motion might be blurred, or not perceivable by the organism. From this point of view one could expect that a damped oscillation would be a simple and adaquate mechanism. A self-sustained oscillation, however, running on practically indefinitely, seems to have very little adaptive meaning. If an animal is prevented from perceiving the synchronizing stimuli for weeks or months, as for instance during adverse weather in a burrow, or during hibernation, its self-sustained circadian clock would be out of phase with the environmental cycle, and not give useful information on the phase of the environmental cycle.

One might speculate therefore, that what we consider an uncomplicated mechanism is not simple in the organisation of an organism. It could also be assumed that self-sustained oscillations are required, or at least facilitate intrinsic physiological processes and their regulation, and that the circadian, or circatidal, period was a secondary adaptation to the environmental cycles. Another possibility would be that self-sustained oscillations have developed by chance (for a more detailed discussion cf. Enright, 1970).

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Discussion

Participants: K. Hoffmann (Author) and E. R. Shlomi

Assuming circadian clocks are genetically controlled, is it probable that the following factors may act as phase timers throughout the individuals life:
1. The time of hatching (birth) – day or night – under given environmental conditions?
2. A certain period of training after hatching which might, then, be called 'a critical period'? (SHLOMI).
I am not sure that I quite understand the questions. If it means whether the clock, or its later phasing to the daily environmental cycle, is the result of some kind of imprinting, the answer is: No, in nearly all cases known. For instance, the locomotor activity rhythm of newly-hatched lizards, recorded in constant conditions, is about the same in animals that were incubated in environmental cycles of 18 and of 36 h period length, and does not differ from that found in animals that were incubated in constant conditions, or that were caught in the field. Comparable findings were reported in birds, in mice, and in several insects (for review cf. Hoffmann 1959). To my knowledge, the only clear case in which aberrant cycles persisted in constant conditions was found in the alga Hydrodictyon (discussion cf. Bünning, 1967). There is also no indication

that the phasing of the circadian cycle relative to the environmental cycle is influenced by the time of, or the environmental conditions during, hatching or birth. I also want to repeat that the circadian clock can be entrained to external cycles, within a certain range of entrainment, and that it can be shifted relative to the natural day by a shift of the 'Zeitgeber' cycle.

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Concluding remarks

Botanical aspects

E. C. Wassink[1]

The Programme now says 'Conclusions' and so Professor de Wilde and I will try to answer the question: 'What shall we take home from this Symposium'. It seems that my special task is to answer this question from the botanical point of view, although to separate the botanical and zoological aspects of circadian rhythms seems rather artificial since there are common features when the phenomenon is studied from both aspects.

But first of all, I would like to say how privileged we have been to hear the up-to-date view of several top specialists in the field of Biological Clocks. We are all most grateful that they accepted the invitation of the Committee to come here.

On a similar occasion Professor Bünning remarked that a symposium like this could well be entitled: 'The adaptation of organisms to the rotation of the earth'. He was impressed by the first known investigator of circadian rhythms in leaf movements in plants being an astronomer.

We do not know what the driving force is behind evolution and we do not understand the mysterious tendency, that seems inherent in all living beings, to try to escape for a short period and within restricted boundaries, from the power of the 2nd law of thermodynamics, and, for their lifetime, to strive towards realizing the improbable.

However, from the beginning, evolution of life on earth has taken place whilst the earth rotates around its own axis in 24 hours and around the sun in a year. Both plants and animals seem to have developed 'time-measuring devices', founded on this co-operation between sun and earth. For instance, the development of annual periodicity is based on processes, governed primarily by the daily duration of light, leading to photoperiodism, and to a lesser extent on certain annual temperature sequences leading to leaf emergence, vernalization and thermoperiodicity.

Bünning, especially has developed the view – and I quote here mainly from a summa-

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ry by Dr Cumming – that the general basic phenomenon is an inherent circadian rhythmicity, an endogenous free-running oscillation that can continue without periodic changes in the environment, with a periodicity of about 24 hours. Each oscillation involves two half-cycles (photophil and scotophil), each of about 12 hours with different innate biochemical and biophysical properties and different sensitivities to external controllers. It is further suggested that the endogenous free-running rhythm allows the organism to be photoperiodically induced for the transition of one developmental phase to another, e.g. from vegetative growth to flowering, and that the controlling rhythms may be entrained or rephased by light.

Cumming further describes photoperiodism as a process evolved by organisms for timing their activities daily and seasonally with circadian rhythmicity as the basic timing mechanism. Moreover, he classifies a rhythmic phenomenon as circadian when it shows at least 2 or 3 approximately 24-hour free-runnning oscillations in continuous light or darkness.

However, under natural conditions suitable for plant growth, the daily natural cycles are the main cues or synchronizers for photoperiodic timing by the organism. In the last decades, evidence has increased for the occurrence of rhythmic phenomena of various types in plants and animals exposed to prolonged periods of light or darkness. Wellknown examples are leaf movements in higher plants, and bioluminescence in certain unicellular organisms e.g. *Goniaulax*. Dr Sweeney's elegant and varied experiments with *Goniaulax* have considerably increased our knowledge of specific properties of rhythmic phenomena.

Interesting additional properties of endogenous rhythms have become known, e.g. that the time-setting requires only very low energies and that the period length has a low Q_{10}-value.

With the first property, the circadian rhythms come into line with the general characteristic of stimulus or amplifier processes, i.e. such processes in which transfer of a stimulus to a reaction site takes place, and amplification of the energy is involved, leading to the ultimate manifestation. Many of these processes show a logarithmic relationship at some stage of the energy pathway, and early investigators have already pointed out that they obey the so-called *Weber-Fechner* law.

The low Q_{10} may mean that the input of light energy acts as a limiting factor during the flash or the light period used as a time-setter, but it can also mean that there is a long pathway between the perception of the phase-setter and the effect, in which a rate-limiting diffusion or mass-flow step may be involved. This is in line with details of Dr Schweiger's suggestions.

It is worth remarking here that Pfeffer, who during this Symposium has been quoted as a pioneer in the study of rhythms in plants, also laid the foundations for a proper understanding of the basic features of stimulus and amplifier processes in general.

Professor Bünning, on several occasions, has warned against mistaking the hands for the clock, the display of photoperiodic phenomena etc. just being movements of the hands. It seems logical that people who started out studying circadian rhythms should take this attitude and look upon photoperiodism as something additional, superimposed on a general and basic time-measuring device. On the other hand I think,

students in the field of photoperiodicity may have taken advantage of the fact, discussed at length during this Symposium, that these rhythms will have undergone the time-setting effect of their experimental design, e.g. of light flashes at certain moments of a dark period, the introduction of dark periods in a continuous light treatment, or light of different spectral composition in certain successions and durations.

After a light period, the organism in the adjacent dark period shows certain sequential biophysical and biochemical changes that may well lead to changes in sensitivity towards external agents, e.g. light, and may be different for light of different spectral regions. Perhaps it should be emphasized that students of circadian rhythms will probably have to pay more attention to the study of the effects of different qualities of light.

In our laboratory, El-Hattab found differential changes in sensitivity for various spectral regions in photoperiodic experiments involving dark periods of normal duration. It has been known for a long time that the concept of critical daylength, that has played a major role in considerations about photoperiodicity, falls down completely when plants are grown and studied entirely under light of restricted spectral regions.

It is impossible in this short closing address to give justice to all the interesting and detailed facts and ideas presented at this Symposium. The time-setting effect of light was a major topic for most speakers. There have been speculations on the type and working mechanism of the 'circadian clock'. Besides the biochemical approach, a biophysical approach has been suggested, especially since the properties of membranes may play an important role. However, this biophysical approach should not be overestimated. In our laboratory, e.g., Dr Kuiper showed that biological membranes act as complicated biochemical systems of variable composition and that simple phenomena such as water uptake, may show extremely high Q_{10}-values in certain temperature regions.

There is evidence that plants and animals are able to develop a memory for a rhythm imposed at least some days before. This might well be connected with a process governed by RNA-enzyme synthesis. The brilliant talk of Dr Schweiger supports this suggestion.

As a student of plant photoperiodicity, and trying to come to a tentative conclusion, I would like to emphasize that, in any sort of 'explanation' of experimental results, we should always be well aware of the generalisation level at which we talk. Ideally, an explanation of an experiment should include the separate reaction of all plant cells. This being virtually impossible, plant physiologists, for a long time, have successfully put large sections of the system studied into, what system theory calls, 'black boxes': parts of the system that we believe do not change essentially during the experiment. These boxes provide us with some sort of an output upon a given input and we take their contents for granted as long as we keep them firmly closed. From the discussions of these days I am left with some hope that in several types of experiments on photoperiodism in plants, we may perhaps successfully keep the rhythmic system in a black box as long as we can adjust the hands of the clock to the desired position by the photoperiodic treatment itself.

Clearly, we should be aware that this hope may not be fully justified in certain types of experiments, and that interference of the rhythmic system, whatever its nature may

be, occurs especially in experiments on photoperiodism which include periods of light or darkness longer than 24 hours.

I would like to take home from this Symposium the hope that I have expressed, and the awareness of its limitations.

Zoological aspects

J. de Wilde[1]

In theory, this symposium should not be split up into a zoological and a botanical part.

As Dr Sweeney so well expressed, it is the individual cell that counts on the level of circadian rhythmicity. Circadian cycles are so prevalent among unicellular organisms, that the evolution of Metazoa, which clearly bears the character of cell colonies although of tremendous complexity, has apparently left enough freedom to the subcellular processes to retain their capacity for circadian rhythmicity. There is, of course, the problem of cellular synchronization, which in plants and animals is solved in a very different way; diffuse in plants, highly centralized in animals. In this respect, the development of a milieu interieur with homeostatic regulation has led to an even more efficient, more centralized homeostatic apparatus, culminating in the development of a brain as a centre of neuro-endocrine integration. Diurnal rhythms of activity in animals are diurnal rhythms of neurones and neurosecretory cells.

It is not surprising that, in animals, circadian clocks (Or must we say governors of circadian clocks?) are situated in the brain. As in botany, the 'black box' approach in combination with model conception has been helpful in the beginning phase, and has enabled several properties of the clocks and the modes of their entrainment by light to be described. In this respect, botanists have been favoured by the presence of phytochromes, providing a sound biochemical basis. Our knowledge of photosensitive pigments in the brain of animals is vague.

In organizing this symposium, we have tried to select several crucial topics, pertaining to the relations between circadian rhythmicity and long-term processes such as homeostasis, photoperiodism and orientation.

Let me try to summarize very briefly, in that order, what I think I have learned from the more zoological papers. Professor Wassink will subsequently comment on the botanical aspect.

First of all, our speakers have been careful not to make undue generalizations. This is understandable when one realizes that two of them are dealing with insects that as a group are overwhelmingly diverse.

We have learned that the incretion of the prothoracotropic hormone and of eclosion hormone in *Cecropia* are both subject to circadian rhythmicity. Both hormones are produced by the medial neurosecretory cells in the brain. Light entrained eclosion

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rhythms had long been a rather baffling feature in many insect species such as *Drosophila*. It had been shown that the entrained condition is carried over through several larval instars up to adult emergence. Truman has shown that here one has to deal with a clock localized in the brain, phase setting occurring by extrasensory perception of light. A dark process is obligatory, while light sets the phase, continuous light wipes out all previous experience.

This is contrary to the type of clocks governing locomotory activity cycles, which can free run in continuous light and which receive their light input by the compound eyes. But also in *Cecropia*, the onset of light stimulates emergence, this being due to the action of the compound eyes overruling the effect of the brain receptor.

It is a most intriguing idea that both types of clocks might occur more widely in the animal kingdom.

Few of us really memorize even the most important phenomena detected by Dr Lees in his well-known and very thorough work with the aphid *Megoura*. The crucial question: how cycles of light and darkness induce photoperiodic control of parthenogenetic and sexual generations, has been studied to an extent unsurpassed by any other work in this field. Thus Dr Lees disposes of a bulk of data which enables him to deal very effectively with his critics.

As the Bünning-Pittendrigh model is no more than a hypothesis; it only stands as long as it is constantly supported by evidence. The same is true for the hour-glass model.

Many of us are convinced that the data obtained by Lees are more elegantly explained by an hour-glass model, combined with an apparatus for storing photoperiodic information.

Lees painstakingly criticizes many arguments brought forward in favour of the coupled oscillator. The existence of very narrow peaks of light sensitivity during the night, with regard to photoperiodic induction, observed in many insects, bearing a strict relation to the moment of on-switch of the photophases and largely independent of the duration of short-day photophases, is still insufficiently accounted for by Lees' data. This type of evidence still leaves an important place for the Bünning-Pittendrigh concept. It seems hardly probable that such a fundamental process as photoperiodic response, induced during evolutionary times, should depend on widely different basic mechanisms. An unified concept of the oscillator and the hour glass may well be expected.

No one would ever deny the interference of circadian clocks in celestial orientation of honey bees after the experiments of von Frisch, and in birds after the pioneer work by Kramer.

While these clocks tend to compensate orientation responses for shifting celestial marking during periods where entrainment is lacking, they ensure a steady direction of movement.

Other adaptive functions of a clock-controlled activity are found in the eclosion rhythm of the Tendipedid *Clunia*, where the responses are genetically set so as to

produce local strains differing in lunar periodicity.

In *Trichuplusia ni*, and other pheromone-releasing Lepidoptera, activity rhythms bear relation to the optimum time for pheromone release with relation to physical conditions and interspecific isolation.

That a clock is involved in time memory would be surprising to a layman, but now appears to be a well-established scientific fact, at least for the honey bee.

In short, the overall importance of biological clocks in ecological adaptation of animals has now been well-established. In many ways, we wouldn't be here but for Circadian clocks!