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# The Biological Clock

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One of the interesting things that biologists have learned in recent years is that almost every creature and plant carries a built-in clock. This timepiece directs creatures to wake and sleep, to eat and drink, and even directs their sexual activities in some instances. Many living organisms carry out activities at a fixed time of the day: Birds sing at dawn, bats fly at night, many flowers open and close at regular times.

Plants and animals not only know the time of day; they are aware of seasonal changes, and they can measure the length of the daily light period as well. Trees know by the length of the daily light period when to start growing and when to stay dormant in order to stay alive.

Biologists have become aware of this phenomenon during the past 30 years or so, and they are now making many studies to determine how time influences the growing patterns of plants and animals, and how organisms measure time.

These studies have shown that creatures appear to act as though they possess not only timers, but also clocks which may run for days in the absence of any external time signal. The rate of many biological processes changes in a rhythmic manner — for example, the luminescence of Gonyaulax (one of the organisms responsible for the luminescent display sometimes observed in the ocean when the water is disturbed). The respiration rate of many plants also varies between a maximum and minimum in a regular manner, and when the rates are plotted against time the resulting graph suggests a rhythm. The maxima and minima occur at approximately the same time on successive days, so the period of the rhythm is 24 hours.

Research has shown that these organisms identify time of the day by an initial signal from the environment. Generally, the signal is a single factor or event in the environment, and with very few exceptions this signal is the light-dark or dark-light change. The organisms respond to this environmental signal after a certain period of time, and the length of that period is determined genetically — although it may depend somewhat on conditions.

All the examples that have been mentioned so far have one thing in common: The pattern of activity can be directly related to a single environmental signal, and the time interval between the signal and start of the activity is fixed. In such cases the only requirement for the time-measuring system is that it should be able to measure fixed time intervals, so the time-measuring device need not be very elaborate. In fact, an hourglass might well simulate this time-measuring element.

We can imagine how such a biological hourglass may work. The environmental signal starts some physiological process — for example, the synthesis of a hormone. When sufficient hormone has been accumulated the organism becomes active — the bird starts singing or the bat starts flying. This model can also be adapted to account for rhythmic processes.

The response of plant growth and development to the length of the light or dark period, called photoperiodism, can be explained in the same manner. Some plants (called short-day plants) can be made to flower when the length of the dark period is greater than a critical number of hours. Following the hourglass model, we can assume that something is being made by the plant in darkness which causes flowering. A minimum number of hours of darkness is required to make a sufficient amount of this substance, or to complete the synthesis of a stable product.

When the dark period is too short, the quantity made is too small, or synthesis stops at an unstable product. The experimental evidence supports the second alternative. In either case no flowering will occur.

In order to explain the absence of flowering when the night length is kept shorter than the critical one, we assume that any product made during such short nights is destroyed in the following light period.

There is ample evidence for such destruction, which will prevent the accumulation of an adequate supply of the flowering stimulus over a larger number of nights.

Is the time-measuring device of living organisms really an hourglass and can it explain all observations involving time measurement? In many organisms the measuring device appears to be more sophisticated than the simple hourglass. Take, for example, the complex performance of the honey bee. Bees are able to return to a feeding place on successive days at the precise time of the day at which they initially discovered it. Moreover, honey bees can be trained to come for food to a certain place at a fixed time of the day by offering them food at a certain hour — say, from 10 till noon — for seven days. Then an empty feeding dish is placed at the feeding spot. The trained bees continue to come to this spot between 10 and noon for many days, even though no food is offered. The bees even start coming back to the feeding spot again at the fixed time after they have been unable to fly for some days because of bad weather.

In these beautiful experiments we find that a bee can recognize any time of the day and that it does so by measuring the time elapsed since dawn. Apparently bees can also remember what happened at any given time. Although the honey bee measures time intervals, its performance is more complex than that of birds, bats, and flowers because the bee is able to look at its timing system at any moment of the day and draw conclusions about the time passed since dawn. In the time-measuring device of the other creatures, some activity always occurred at a fixed time after a signal. The time-measuring instrument of the bee has to be more refined than an hourglass unless the bee can accurately estimate how much sand has run through its hourglass at any one time.

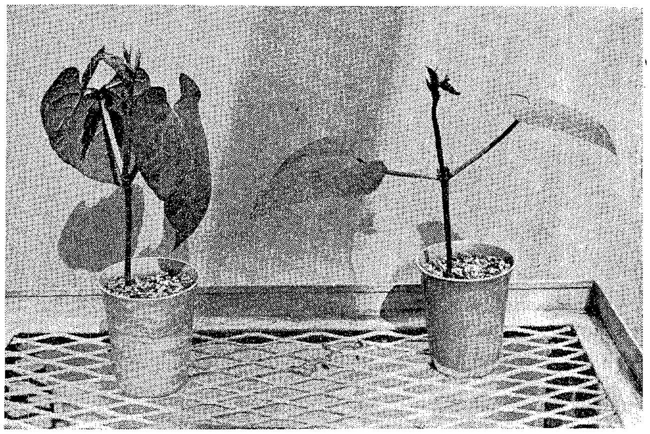
A second group of phenomena requires a similar timing mechanism. During the 1950's some remarkable cases of celestial navigation were discovered in birds and arthropods. These animals use the sun as direction-giver. In order to compensate for the shift in position of the direction-giver during the day, they make use of an internal timing system. The following experiment demonstrates this very elegantly: Two starlings, trained to look for food in a given direction, are placed in a room and subjected to a light-dark regime which is displaced by six hours in comparison with the natural regime. Thus the birds receive artificial light from six hours after sunrise until six hours after sunset, and they remain in darkness for the remainder of the 24 hours. Temperature conditions correspond to the new light regime.

After approximately two weeks of such treatment the birds are tested in the original environment and their behavior appears to be changed. A bird trained to look for food in a westerly direction now looks for food in a northerly direction. It seems as if the starling applies a wrong correction factor to the position of the sun and overcorrects by 90°. As the position of the sun shifts 90° in six hours, the logical explanation is that the timing of the starling is off by six hours. In other words, due to this abnormal treatment, the synchronization between the internal time-measuring device of the bird and the sun has been upset: The timing system of the bird is slow.

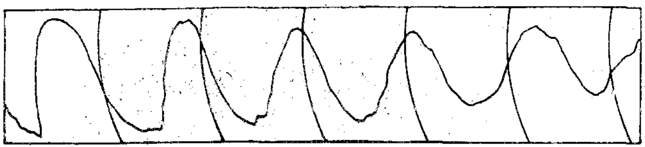
This experiment demonstrates that the starling is able to refer to its time-measuring device continuously and can tell at any moment the exact time elapsed since dawn, just as the bee does. It uses that information for its orientation in space. The same is true in other cases of celestial navigation which have been investigated so far. In all these cases it has been shown that the timing is very accurate.

A third group of phenomena also bears on the nature of time-measurement in living organisms. Many activities continue on a rigid time schedule in the absence of obvious signals from the environment. We will use the movement of bean leaves as an example, but there are many others to choose from. Bean leaves have a day and a night position. The position of the leaf changes in a regular manner between the two extremes shown in the photograph below. Normally the time interval between two peaks is 24 hours because the dark-light change is the signal which induces the leaf to start opening or rising.

What happens when bean plants are transferred to an environment which does not provide any obvious signals? Such an environment can be achieved by maintaining the temperature at a constant value and keeping the plants in continuous light or in continuous darkness. In the absence of obvious cues the rhythmic leaf movement continues for many days and the peaks are 27 hours apart. Rhythms which persist in the absence of environmental signals are called endogenous rhythms. Such rhythms have been known for many years.



*The leaves of the bean plant have a night (left) and a day position (right). The normal time interval between the two positions is 24 hours.*



*The rhythmic movement of bean leaves continues, with a period of 27 hours, in a signal-less environment. The peaks in this record, which covers 6 days, correspond to the night position.*

The earliest reports originate from Zinn (1759) and extensive studies were carried out in the last quarter of the 19th century. The persistence of rhythms in a cueless environment tells us that the time measuring continues under such conditions. It is not restarted every day by an environmental signal. Moreover, the time-measuring device apparently is cyclic in nature. In the case of the bean leaves, one cycle lasts 27 hours and any given leaf position recurs every 27 hours.

The study of the emergence of adult fruit flies (*Drosophila*) from puparia is an interesting aspect of internal rhythm. Normally the emergence occurs in bursts during the first few hours after dawn. It is inhibited during the rest of the day. The emergence pattern persists in continuous darkness and constant temperature, and the time interval between emergence peaks is 24 hours as expected. This in itself does not prove that the apparently cueless environment does not provide any signals, or that the rhythm can persist without external signals.

The probability that an external signal is received in continuous darkness and constant temperature is practically eliminated by the following experiment: It is possible to displace the rhythm in relation to the solar periodicity by using the environmental variable which normally functions as a signal. In the case of the emergence in *Drosophila* the signal is the dark-light change. One can subject a culture of *Drosophila* to a dark-light change at any time of the day — for example, midnight — by using artificial light. After transfer of such a culture to continuous darkness it will show a persistent rhythm, and the location of the emergence peak is determined by the experimentally established "dawn," which, in the example, is at midnight. Rhythms established in this manner will persist, even though they are completely out of phase with solar periodicity.

Cultures can be raised from eggs in continuous darkness and constant temperature. Such cultures have never experienced a 24-hour periodicity and the emergence is evenly distributed over the 24 hours. Yet, a single, unrepeated dark-light change starts off rhythmic emergence with 24 hours between peaks. The signal can be given by shining light for four hours on cultures which have been raised in darkness. This treatment offers two potential signals: a dark-light and a light-dark change.

Although it is known that a light-dark change is not effective in *Drosophila*, it would be more elegant to offer a single change. The experiment has been carried out by transferring cultures which have been raised in continuous darkness to continuous light. In this case the cultures receive one dark-light change. Immediately after the transfer such cultures, which did not show a rhythm before, start the rhythmic emergence with a period of 24 hours.

These striking observations strongly suggest that the clock is internal and that it has the inherent ability to measure off cycles of approximately 24 hours. It can do this without regular, external signals. What is the nature of the action of a single signal? Does the signal start the time measurement, or does it synchronize time measurement already in progress in the individuals? The problem cannot be resolved using the emergence of *Drosophila*, because the periodicity cannot be measured as a function of the individual. It is probable that a signal turns an aperiodic culture into a periodic one by synchronizing the time measurement in the individuals. However, this has not been proven rigorously.

The existence of persistent rhythms, the evidence from experiments with bees, and the role of time measurement in celestial navigation suggest that the biological time-measuring system is more complex than an hourglass. Rather we have to conclude that the time-measuring apparatus is cyclic in nature and that it can be continuously consulted. Therefore, animals and plants possess a real, and very good, clock.

Many other examples of accurate time measuring, besides the ones which have been discussed here, have been found, and in the last 5 or 10 years the word "clock" has become more and more common in biological literature. The biological clock is very accurate. This can be investigated in an environment without obvious signals. The period found in such environment is called the "free running" period. In many cases the free running period is different from 24 hours, and this is an additional indication that the environment really lacks cues as far as the clock is concerned. The free running period is very constant in any one individual and its length is determined genetically. It is maintained accurately for many days. In general the free running period is relatively close to 24 hours, although for the bean this period is 27 hours.

In their natural environment plants and animals are exposed to alternating periods of light and dark, the combined lengths of which are 24 hours. It does not matter very much that the free running period is different from 24 hours because every day the biological clock is synchronized with the outside world by some change in the environment. Light-dark changes or dark-light changes are most effective in this respect. However, synchronization is possible only to a limited extent.

This can be illustrated by two types of experiments which have been carried out at the Institute. It has been shown here that the growth of some plants, particularly tomatoes, is sensitive to the light regime. Tomato plants require a daily dark period for optimal development, and leaf injury appears very quickly when tomato plants are grown in continuous light and constant temperature. Obviously, such plants with small, yellow leaves will grow poorly. So alternating light and dark periods are the requirement for good and normal development of the tomato plant.

On the other hand, the combined length of the light and dark period, which we call "cycle length," must be relatively close to 24 hours; otherwise, again, leaf injury and reduced growth result. This suggests that tomato plants have an internal 24-hour periodicity which is involved in the growth process, and that for optimal growth the endogenous periodicity has to be properly in register with the external periodicity.

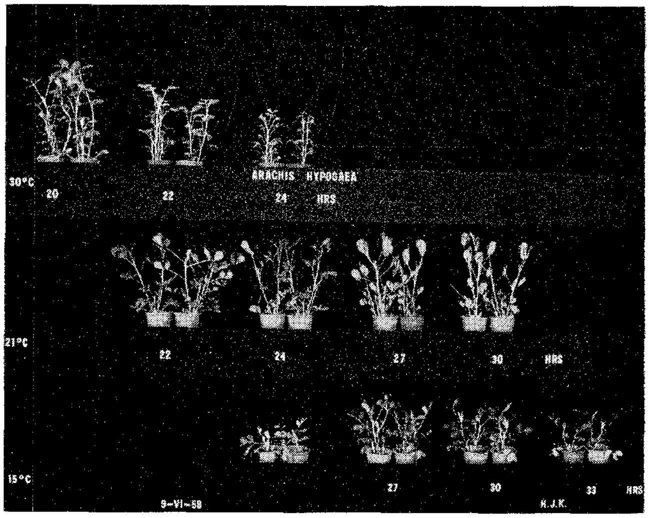
When the external periodicity differs from the internal periodicity, the plant is able to compensate for this difference to some extent, but when the two periodicities are too different, such compensation is not possible any more and injury results. For example, leaf injury results when tomato plants are grown under 18-hour or 36-hour cycles at 17°C instead of under the natural 24-hour cycle. However, growth is affected by changes in cycle length of as little as two to three hours. Such small deviations from 24 hours cause a reduction in growth. Experiments with the leaf movement of beans, where the period of internal periodicity can be measured directly, have also shown that the internal period can follow the external periodicity to a limited extent only.

## *Tomatoes, peas and peanuts*

Tomato plants are more sensitive to light-dark periodicity than other plants which have been investigated. Pea and peanut plants grow faster in continuous light than in any other regime, and they complete their life cycle more quickly. Apparently these plants do not require a daily dark period, while the tomato plant has an absolute requirement for a daily dark period. However, peas and peanuts respond to differences in the external cycle length: When an external periodicity is imposed, the cycle length has to be close to 24 hours, or else the rate of growth is reduced.

No visible symptoms of injury have been observed in peas and peanut plants grown in continuous light. Perhaps we may conclude that the bad effect of continuous light on tomato plants has nothing to do with the biological clock, because a number of plants, in which an endogenous 24-hour periodicity is definitely involved in the growth process, do not show such injurious effects. These experiments certainly indicate that plants are closely adapted to the natural 24-hour cycle.

A second type of experiment provides confirming data and points out an important property of the clock. Plants grow better at some temperatures than at others. This fact has been of considerable interest to plant physiologists at the Institute. Investigations into a possible chemical nature of the bad effect of temperature have gone on for some time and it has been found that temperature damage can be partially overcome in a few cases by supplying chemicals to the plants. Which substances are active depends on the plant and on the temperature conditions. However, another type of experiment has been carried out as well.



*The cycle length which allows best growth of peanut plants depends on temperature. At 30°C. (top row) plants grow biggest in a cycle length of 20 hours. At 21° (middle row) the optimal cycle length is 24 hours, at 15° (bottom row) 27 hours.*

Peanut plants and tomato plants are grown in artificial light at three different temperatures – for example, 15°, 21°, and 30°C. The plants are grown under four different cycle lengths at each temperature. Again, cycle length is the combined length of a light and a dark period. Every cycle consists of equal periods of light and dark. Therefore, when we say that the cycle length is 27 hours, this means that the plants are receiving 13½ hours light and 13½ hours dark during every cycle.

The results of such an experiment with peanut plants appear in the photograph above. The plants respond strikingly to cycle lengths at the high and low temperatures, and the cycle length which gives best growth is different for the three temperatures – 27 hours at 15°, 24 hours at 21°, and 20 hours at 30°. At 21° the effect is not very obvious by just looking, but when one determines the increase in dry weight of the plants during the experimental period the differences are quite clear.

Leaf injury which occurs at the high and low temperatures under a 24-hour cycle disappears when the external cycle length is adjusted by as little as three to four hours. By adjusting the outside periodicity to the optimal value for a given temperature, very often

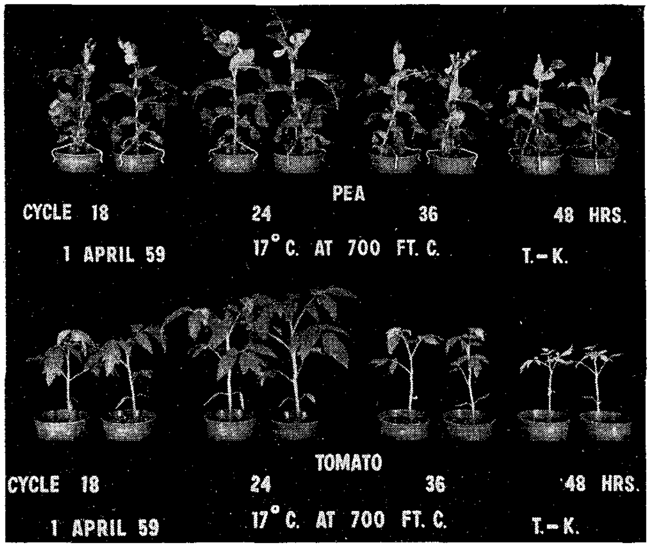
50 percent or more of the reduction in growth which would normally occur at that temperature can be prevented.

The optimal cycle length is dependent on the prevailing temperature and, interestingly enough, the amount by which the optimal cycle length changes with temperature approximates the amount by which the periods of internal rhythms change with temperature. The results support the notion that plants possess some inner, cyclical, time-measuring device, and that for optimal growth the external periodicity must be sychronized with the internal periodicity of the plant. The internal clock appears to run more slowly at lower temperatures and more rapidly at higher temperatures. At such temperatures it is not in register with an environmental 24-hour cycle, and it cannot be adequately synchronized with the environment.

Lack of synchronization between the internal and the environmental light-dark cycle is apparently the cause of at least part of the reduction in growth observed in plants grown under unfavorable temperature conditions. The experiment demonstrates that small changes in cycle length have a significant effect on plant growth and development.

Although temperature has an effect on the period of the clock, this effect is relatively small — certainly in view of the quite appreciable effects which temperature generally has in biological systems. It is quite possible that the effect of temperature on the clock is even smaller than indicated in these experiments, because it is difficult to determine the optimal cycle length exactly. In experiments where it is possible to determine the period of the clock accurately, it appears that the clock is only slightly affected by temperature. To all intents and purposes the clock is temperature-independent. This indicates a very sophisticated timepiece.

Temperature independence has been found in all internal rhythms. It also has been demonstrated in unicellular organisms, and it appears to be a basic property of the clock. This does not mean that the clock is insensitive to temperature — only that the effect of temperature on the period of the clock is small. The period may be changed for as little as 30 minutes by a change in temperature of 10°C. This is very remarkable for a biological system.



*Peas and tomatoes grow best when the environmental periodicity is close to 24 hours.*

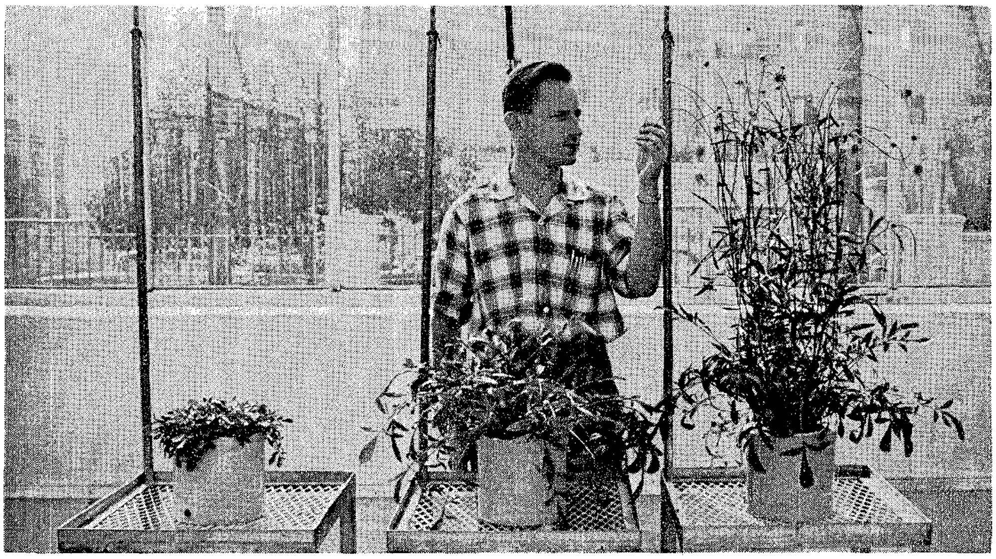
Temperature independence is a necessity for functional time-measurement by periods and the biological clock shares this property with our mechanical clocks. We have no idea how the organism achieves temperature independence. Presumably the clock is either a physical system or a temperature-compensated chemical system, component parts of which may be temperature-dependent. Although we know nothing about the mechanism of the biological clock, it is quite clear that living organisms possess a highly sophisticated timepiece.

## *Clocks and photoperiodism*

Apparently many — perhaps all — living organisms possess clocks. It may be worthwhile to look again at photoperiodism with this in mind. It will be very satisfying if it can be shown that the clock is responsible for every case of time measurement, including photoperiodism. A theory has been proposed which interprets photoperiodic responses as the result of an interaction between the endogenous clock and the environmental periodicity. According to this theory the dark-light change starts off a cyclic, or rhythmic, change in the physiological state of the plant. Once the rhythmic change has been initiated, its course is determined exclusively by the clock and it is not disturbed by a light-dark change, nor by brief periods of light during darkness. The change involves the sensitivity of the plant to light: The plant is supposed to pass through a phase during which light promotes flowering (and other processes affected by light) and a phase during which light inhibits flowering. During each of these phases the sensitivity to light changes quantitatively.

The explanation for the induction of flowering in short-day plants according to this model is as follows: The start of the light period (dark-light change) starts the phase during which light is promotive. After 10-12 hours the change in sensitivity, which is regulated by the biological clock, has progressed so far that light becomes inhibitory. Short-day plants will not flower when the external light period lasts so long that the plants receive light after they have changed into the state where light is inhibitory. Therefore, short-day plants will not flower when the light period is longer than 10-12 hours.

There is little direct evidence for this model, but a few observations suggest that internal rhythms may be involved in photoperiodic responses. A close correlation has been found in some plants between leaf



*Hendrik Ketellapper checks the effect of day-length on the Coreopsis plant. The plant on the left has been grown in short days (8 hours light), the one in the middle in long days (16 hours light). The plant at the right was grown in short days first, and then transfered to long days. This treatment makes the plant flower.*

movement and the sensitivity to light as measured by the flowering response. Supporters of the clock theory of photoperiodism claim that this proves that leaf movement and changes in light sensitivity are both regulated by the biological clock. Such coincidence, however, is of value only as an indication. It is not absolute proof.

Short-day plants flower when the dark period is longer than a critical number of hours. If the hourglass model is correct, one would expect that progressively longer dark periods will be better for flowering and that eventually a saturation point may be reached beyond which further increase of the dark period will have no effect. It has been found in experiments with the soybean – a short-day plant – that this is not true.

Soybeans receive an 8-hour light period, but then different groups of plants are exposed to dark periods varying from 12 to 48 hours. A 16-hour dark period causes excellent flowering, but when the length of the dark period is increased, the amount of flowering decreases and when the dark period is 36 hours, there is no flowering at all. Dark periods of 40-44 hours again cause excellent flowering. In fact, such dark periods are just as effective as 16 hours. No flowering at all is elicited by 48 hours darkness. Apparently a 24-hour periodicity is involved, and flowering is stimulated when the cycle length is 24 hours or multiples of 24 hours. Somewhat similar results have been obtained in other experiments.

At the present time it is not possible to decide between the two models for photoperiodism: the hourglass or the clock. There is no compelling reason to accept the model employing the clock. Many plant physiologists feel that by involving the clock we replace something we do not know much about by something which we understand even less. The hourglass model seems to be the simpler model and it is capable of explaining the vast majority of phenomena. Furthermore, it is quite possible that, in those cases where an endogenous rhythm seems to be involved, the involvement is at a secondary level. The merit of the model involving the biological clock is that it relates all instances of time measurement to a common cellular clock.

Time measurement plays a role in many aspects of plant and animal life in addition to the ones discussed so far. Only in the last few years has its importance been generally realized. However, the most interesting questions still remain. Where is the clock located? What does it look like? And how does it work? Unfortunately the answers to these questions are not known.

In plants, presumably every cell has its own clock, and the environmental events synchronize all these clocks so that the organism acts as a unit. In animals, many experiments indicate that the nervous system is involved and may be the site of time measurement. However, we have no idea of what the clock looks like or even what to look for. It is not known in what part of the cell it occurs, whether in the nucleus or in the protoplasm. The mechanism of the clock is not known either.

A number of model systems have been proposed and they are in the process of being tested. The model systems have a tendency to become more and more complicated and it is not clear what cellular systems could correspond to the model. The answers to these fundamental questions may not be found soon because of the difficulty of the task, both philosophically and technically. As so little is known about the clock, it offers a great challenge and the clock is now being investigated by many biologists. A large part of the work is descriptive and concerns itself with phenomena, but some investigators look for answers to basic questions. These answers will help our understanding of life and in time we may be able to make practical use of this knowledge.