EXPERIENTIA

Volume 42/No. 1 Pages 1–108 15 January 1986

Reviews

Circadian rhythms and their mechanisms

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Summary. Recent work concerning the number, site(s) and means of adjustment to the 24-h day of internal clocks is reviewed. Work on humans is considered wherever possible though much of the work involving ablation and in vitro techniques necessarily involves other species, particularly rodents. It is concluded that, though recent advances have been impressive and present techniques appear likely to continue to produce results and stimulate discussion, more attention should be directed to considering the circadian system as a whole rather than as an assemblage of individual components.
Key words. Circadian rhythms; endogenous clocks; zeitgebers; suprachiasmatic nuclei; pineal gland.

1. Introduction

Important concepts that are generally stressed early in any undergraduate physiology course are those of homeostasis and 'la fixité du milieu intérieur' of Claude Bernard; that is, a prerequisite for health is the maintenance of many physiological variables (e.g. blood pressure, blood gases, interstitial fluid composition) within a narrow range.

For example, healthy individuals make use of a whole range of mechanisms to maintain their body temperature within narrow limits in spite of climatic exigencies, and the dangers of fever during infection or hypothermia in the aged are well known. As a result of this view of the body's constancy, the diagnosis of disease often relies on ascertaining whether a particular variable falls outside a narrow range compatible with what is regarded as normal.

However, such a 'homeostatic' or 'steady-state' picture of an organism is an oversimplification, as becomes apparent if frequent measurements of a variable are taken over a protracted period of time. When this is done, it is seen that, although physiological variables remain within a narrow range, they are not constant; rather, rhythmic changes are observed. The periods (time to complete one cycle) of these rhythms encompass our every division of time. Often, however, they are synchronized to some major environmental cycle; for example, many shore-dwelling species show rhythms with a period of 12.4 h – the frequency of the tides and many mammalian and avian species show annual rhythms in reproductive function. In man, as in many other species, the commonly observed rhythms are those which oscillate once per 24 h – the length of the solar day; such rhythms are termed circadian[49].

Circadian rhythms have been documented in practically every species and at every level of organization in an organism. Indeed, it is difficult to demonstrate a body's variable which does not show such circadian variation. Figure 1 shows the circadian rhythms in a selection of physiological variables in healthy human subjects on a normal schedule of sleep, activity, etc. These variables are all under the control of homeostatic mechanisms and yet the clear circadian variation can be seen with higher daytime values than at night. Many other variables show a similar variation though several, for example, growth hormone, prolactin and cortisol, show a different pattern with higher values during the night or in the hours just after waking. A description of the many circadian rhythms which have been identified can be found in numerous reviews and books[11,12,19,24,69,73,79,82,108,109].

The aims of the present review are, first, to outline some basic concepts in the field of circadian rhythms and then to consider in more detail some recent advances made in our understanding of the physiology of the 'biological clock' controlling these rhythms. Often the latter has necessarily been performed upon species other than man and the review will concentrate upon mammals since they are believed to be most likely to act as a model for the human timing system.

2. Origin of rhythms

Since the period of most biological rhythms is identical to that of some major environmental oscillation, it might be argued that these rhythms simply reflect an animal's response to such a rhythmic environment. For example, the rhythms shown in figure 1 might be explained by the fact that we sleep, are inactive and fast at night as a result of

Experientia 42 (1986), Birkhäuser Verlag, CH-4010 Basel/Switzerland

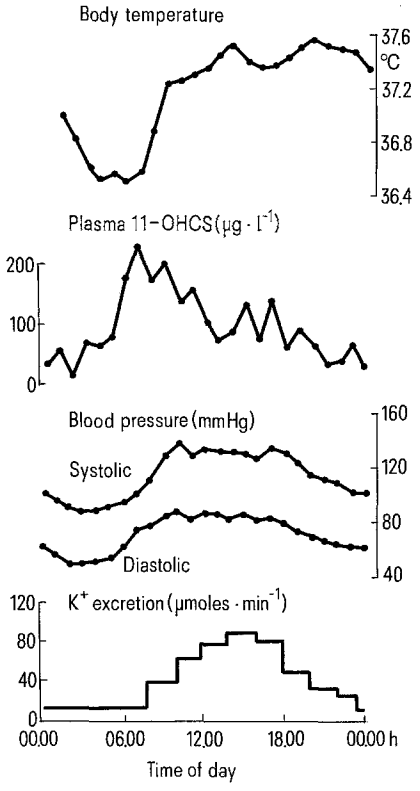

Figure 1. The normal circadian variations in deep body (rectal) temperature, plasma 11-hydroxycorticosteroids, blood pressure, and urinary excretion of potassium in a healthy subject. (From: Minors, D.S., R. Soc. Med. int. Congr. Symp. Ser. *73* (1984) 41–55, fig. 1).

which the heart rate, blood pressure, body temperature and urinary excretion of potassium are low during these hours; by contrast, during the daytime we are active and take meals leading to a rise in these variables. By such an argument, the nocturnal peak of certain hormones would also find an explanation since endocrine function is often associated with growth and repair which might take place more appropriately when the body is inactive[1,50].

However, careful inspection of the data in figure 1 shows that these explanations are not wholly appropriate. For example, it can be seen that blood pressure and body temperature continue to fall for some time after sleep onset (23.30 h) and start to rise before waking takes place (07.30 h). These observations suggest that some factor in addition to a direct influence of the sleep-wakefulness cycle is involved.

The outcome of this argument – that circadian rhythms do not wholly owe their origin to external influences impinging upon an organism – can be tested more formally by keeping an individual in constant conditions[48,74]. Thus: an individual can be kept awake for 24 h or more; the rhythmic effects of normal mealtimes can be eliminated by taking small identical snacks hourly; the individual can remain sedentary or in bed so that changes in posture are removed; and the effects of changing noise and light intensities can obviously by minimized without any difficulty. Under such constant conditions most circadian rhythms continue oscillating proving that they are not simply a passive response to our environment and normal rhythmic behaviour. An example is shown in figure 2 in which it can be seen that body temperature during constant conditions (dashed line) remains driven by some inherent or endogenous timing mechanism – a biological 'clock'.

As a result of much research effort, it is now firmly established that the majority of circadian rhythms are due, at least in part, to the possession of an endogenous oscillator. Indeed, the possession of such an oscillator is thought to allow an organism to fit better into its rhythmic environment; thus an organism can predict and 'pre-adapt' to some forthcoming event[23]. For example, a plant can raise its leaves ready for sunrise; and man can prepare his body (hormones, cardiovascular system, body temperature, renal function, etc.) for the stresses of the next day even before he has woken.

2.1 Exogenous and endogenous components

In addition to showing the body temperature rhythm during constant conditions, figure 2 also shows the rhythm in the same subjects during conventional nychthemeral conditions (solid line) when the subjects slept and ate at normal times. Comparison of the two rhythms shows that they are not identical. This implies that, although rhythms may be driven by an endogenous oscillator, the normal rhythmic environment and our rhythmic habits are not without effect. These external influences are referred to as exogenous or masking influences[9,10] so that, as shown in figure 2, an overt rhythm is the sum of endogenous and exogenous components. Under normal circumstances, the exogenous and endogenous influences are in phase with one another so that, for example, the daytime peak in body temperature results from the body clock accentuated by diurnal activity. These general principles apply to all variables within the body as a result of which there normally exists a reliable relationship between a variable and the external environment and between different rhythms in a given individual.

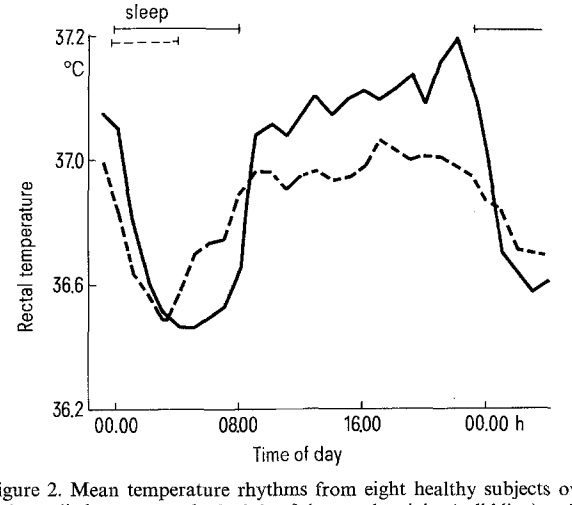

Figure 2. Mean temperature rhythms from eight healthy subjects over 28 h, studied on a normal schedule of sleep and activity (solid line) and in the same subjects woken at 04.00 h and spending the subsequent 24 h in constant routine conditions (dashed line). (From: Minors, D.S., and Waterhouse, J.M., Circadian Rhythms and the Human, fig. 2.2. Wright P.S.G. Bristol 1981).

2.1.1 The exogenous component

The nature of the rhythmic influence responsible for the exogenous component of circadian rhythms depends upon the variable under investigation. For example, the masking effect for the plasma insulin rhythm and those of various gastrointestinal hormones is mainly the result of variation in food intake; in the case of urine flow, it is the daily variation in drinking (and hence water intake) and posture; and for melatonin secretion from the pineal gland it will be light intensity. In man, however, the main exogenous influence is the normal rhythm of sleep and wakefulness. For example, sleep produces direct changes in most physiological variables – it raises growth hormone secretion, lowers cortisol secretion[106] and causes body temperature and urinary potassium loss to fall[68]. As a result, sleep is said to 'mask' the effects of the internal clock. This masking effect of sleep on the body temperature can be deduced from figure 2 by comparing the body temperature when the subjects remained awake with that when they slept at a normal time. This indicates that sleep depresses body temperature by about 0.2°C (see also Aschoff[12]).

2.1.2 The endogenous component

The endogenous component of a rhythm can be deduced by removing the exogenous component by keeping subjects awake and in constant conditions as in the experiment shown in figure 2. However, since the duration of such experiments is limited (as the effects of sleep deprivation intervene) and the characteristics of the endogenous component are difficult to define accurately in short experiments, another technique for investigating the endogenous component is required.

More information about the internal clock controlling rhythms has been derived from the 'free-running' experiment. In this experimental design, subjects, usually individually, are isolated from all cues as to the time of day in specially-constructed Isolation Units[69, 108, 109]. The subject is allowed to take meals and to sleep, etc., whenever he wishes and the times of these are monitored by the experimenter. It is assumed that, with such an experimental design, the absence of external time cues will result in the sleep-wakefulness cycle and other rhythms being determined by endogenous influences alone. Results from such experiments are often complex but the general finding is that circadian rhythms continue oscillations. This is strong evidence for a self-sustaining oscillator controlling these rhythms.

A second important result which has been found in these experiments is that, although rhythms continue oscillating, they are no longer synchronized to the solar day – their periods deviate from an exact 24 h – such rhythms are then said to be 'free-running'[20]. In other words, the inherent period of the biological clock controlling circadian rhythms is not exactly 24 h. It is for this reason that these rhythms are described as *circa* dian – the period is of *about* a day. By far the largest series of such experiments in humans has been performed by Wever[108] in which he has found a mean period of rhythms of 25.0 h in 152 subjects. Further, in any individual, the free-running period is remarkably stable. Thus, Wever[108, 111] has investigated the extent to which the period of the internal clock can be altered by external factors. Some of the factors he has considered are physical workload, lighting intensity, the presence or absence of alternating magnetic fields and single subjects versus groups of subjects. Each of these conditions produces some change in the average free-running period, but the effects are small and the general conclusion is that the internal clock is remarkably stable and resistant to such perturbations.

However, two kinds of 'anomaly' have been found in free-running experiments, especially those of considerable duration (3+ weeks). One of these is 'spontaneous internal desynchronization', where two rhythms in the same individual continue oscillating, but with different periods; the other is an increasing tendency for the sleep-activity cycle to become irregular and it has been postulated that, in time, all subjects would show this partial 'breakdown' of circadian rhythmicity[106]. The significance of these anomalies will be described in section 3.

2.2 Entrainment and zeitgebers

Although the inherent period of the endogenous clock controlling circadian rhythms found in free-running experiments is greater than 24 h in humans, it is found that, when a rhythm is measured in an individual living in normal nychthemeral conditions, its period is exactly 24 h. It follows that under normal conditions the endogenous clock must be adjusted to run with a period of 24 h. This adjustment of the endogenous clock is termed 'synchronization' or entrainment. Thus, although not a totally appropriate analogy, the biological clock has been likened to a watch which runs slow and which must be adjusted (entrained) to register correct time. Of course, such entrainment of the endogenous clock is a necessity for, otherwise, there would be a continued mismatching of our internal and external time and the internal clock would be of no use.

Entrainment of the internal clock is believed to be achieved by rhythmic changes in the external environment called synchronizers or zeitgebers (German; Zeit, time; geber, giver)[8, 49]. Thus, the alternation of light and dark is believed to be one of the most potent influences responsible for entraining the internal clock of most mammals and the rhythmic buffeting caused by high tides entrains the behavioural activity of shore-dwelling creatures to a lunar day.

There is a limited range of periodicities to which any zeitgeber can entrain the internal clock. If this range is exceeded, then the internal clock free-runs. This range is determined by the comparative strengths of the zeitgeber in question and the internal clock; the more does the comparison favour the zeitgeber, the wider is the range of entrainment and vice versa[69, 82, 108]. With zeitgebers of the strength normally found, the internal clock can be entrained to periods between about 23 and 26 h. The nature of zeitgebers in humans together with the means by which they might entrain the internal clock will be considered in section 3.5. However, it is worth noting at this stage that the likely zeitgebers for man (light/dark, feeding/fasting, social contacts/absence, sleep/activity) are the very same influences that are responsible for the exogenous component of rhythms. In other words, external rhythmicities

exert effects upon circadian rhythms by two means: by producing an exogenous component and by entraining the internal clock.

3. Recent research on the clock and zeitgeber

With the realization that circadian rhythms were controlled by endogenous timing mechanisms, the search was instigated for the location of the internal clock. As one might expect, the search for such a central controlling mechanism concentrated upon the brain area in general and upon the simpler brains of insects in particular. (For recent accounts, see[16,17,35,54,66,67,84,85,90,116].)

It was not until 1967, however, with the many exhaustive studies of Richter[88] that the first major clue as to the clock's siting in mammals was provided. Subsequent work has also involved finding pathways by which external zeitgebers can entrain the internal clock and then attempting to understand the mechanisms responsible for the clock. Since the work has often involved surgical intervention, one is forced into the dangerous expedient of extrapolating results from other species if one wishes to understand the circadian system of man. These advances in our understanding of the processes in mammals derive mainly from experiments upon rodents and the squirrel monkey. (For recent accounts, see[75,77,78,80,81,91,103,104,110].)

3.1 The site of the clock – I

Some of the earliest work in this field[88,90] indicated that there was a loss of the circadian rhythm in locomotor activity in hamsters after ablation of the suprachiasmatic nuclei (SCN) of the hypothalamus but not after ablation of other areas of the brain. At about the same time, the uptake into the SCN of the antimetabolite 2-deoxyglucose was found to show circadian rhythmicity[95]. More recent work has tended to confirm that the SCN does indeed act as a circadian pacemaker. For example:

a) steroid and mitotic rhythms have been altered by bilateral SCN lesions in mice[94];

b) infusion of 'anti-suprachiasmatic nucleus γ-globulin' into rats (the antibody was raised in rabbits) lead to a loss of drinking and locomotor activity[83], and;

c) rhythmic changes in C^{14} 2-deoxyglucose uptake into the SCN have been demonstrated in a wider range of species and have shown appropriate shifts of timing when the animals have been placed in changed lighting conditions[36,96].

There are two difficulties of interpretation when these results are considered. First, loss of circadian rhythmicity must not be taken to imply complete arrhythmicity. Instead, the development of ultradian components (generally with periods of 4–12 h in length) was often seen and this has given rise to the concept that the SCN might act as a coordinator of a population of ultradian components which originate elsewhere[3] – a concept that will be discussed more fully in section 3.2.1. The second difficulty of interpretation is that these ablation experiments might show no more than the SCN is part of the pathway through which clock-like activity must pass. To take a simple analogy: cutting the nerves to a muscle prevents voluntary movement, but this does not prove that movement *originates* in the motor units of the spinal cord.

It was to refute this second objection that experiments were carried out upon 'hypothalamic islands'[29,52]. In these experiments, groups of hypothalamic cells including the SCN were isolated surgically and left in situ. Such 'islands' continued to show circadian rhythmicity, unlike areas outside the islands. However, there was still the possibility that such areas were not the origin of such rhythmicity but rather were receptive to humoral influences reaching them via the cerebrospinal fluid and/or brain extracellular fluid from adjacent areas of the brain. Such a possibility can be eliminated only by in vitro measurements upon hypothalamic slices. Such preparations show evidence of rhythmicity, the phasing of which is related to the timing of the light-dark cycle in which the animal was placed[46,47,97]. Thus, the neural activity of the slices is highest at a time corresponding to the mid-light phase of the animal from which they had been taken. Even though, to date, such preparations have been maintained in a viable state for only a comparatively short period of time (up to 30 h) as far as the requirements of circadian rhythm research are concerned, they nevertheless indicate strongly that the SCN contains an autonomous circadian oscillator. Such paired structures exist in primates and man also[63,64], even though they are less clearly defined histologically and their existence has occasionally been questioned in man[82].

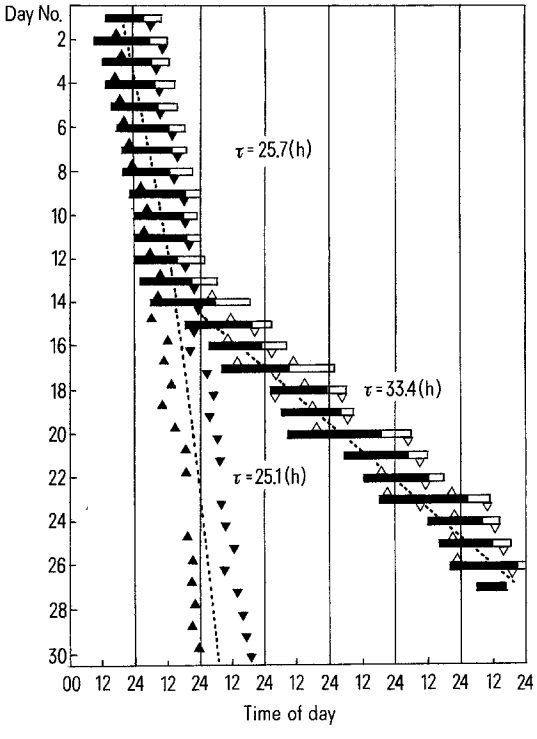

Figure 3. Times of activity, solid bars, and sleep, open bars, in a single subject in isolation who underwent spontaneous internal desynchronization on day 15. ▲, time of maximum rectal temperature, ▼, time of minimum. During desynchronization these values are also plotted as open symbols to show their relationship to the sleep-activity cycle. Time axis is extended to show 7 days to facilitate demonstration of the two periodicities. Dotted lines indicate the mean phase of the variables at different stages of the experiment. (From: Wever, R.A., Int. J. Chronobiol. 3 (1975) 19–55, fig. 7).

In summary, therefore, the SCN are believed to play a most important part when the concept of a circadian clock is considered. However, other results indicate that the position as so far described is an over-simplification; in brief, more than one clock seems to exist.

3.2 How many clocks?

Most data relevant to this question in humans have necessarily been obtained by indirect approaches to the problem. By contrast, in animals, the techniques that have been used have often involved surgery or sacrifice of the animal.

3.2.1 Results from mammals excluding man

Evidence that more that one clock exists in mammalian species has come from the following types of evidence:
a) Partial or total SCN ablation does not always lead to an *immediate* loss of circadian rhythmicity. A variety of changes occurs:
1) In some cases, rhythms show ultradian components[90] and the decline of circadian rhythmicity has been attributed to the increasing asynchrony between a population of ultradian oscillators, this population normally being synchronized by the SCN[3,4].
2) In other cases, 'splitting' of rhythms occurs (indeed, 'splitting' can occur spontaneously when animals are kept in constant light). In this condition, two peaks are seen during the course of each cycle (generally it is activity that is measured). These peaks are often in antiphase to each other, that is, 12 h apart when the animals are following a 24-h day or separated by half the circadian period length when the animals are free-running[82].
3) When many rhythms in a single animal are studied, it is found that there is loss of some rhythms, but others continue. Thus, in the case of the squirrel monkey, SCN ablation has led to the loss of rhythmicity in drinking behaviour but not in body temperature[40].
These assessments of rhythmicity were made in conditions of constant light; this is an important point, for, in a nychthemeral environment (that is, with an alternation of light and dark), the drinking rhythm also persisted with a period of 24 h. This is likely to be a direct effect upon behaviour that is caused by the environment, and is another example of 'masking'[6,41,42]. Even so, the demonstration that only some rhythms disappear following ablation of the SCN and that those that remain are not being driven by external rhythmicities has been regarded as strong evidence for the presence of more than one clock[82]. Further, since SCN ablation results in the loss of the activity and feeding rhythms rather than that of temperature, the 'activity' oscillator has been postulated to be in the SCN. Moore-Ede and his colleagues have then developed this concept of two clocks – strong (temperature) oscillator and weak (activity) oscillator – in an attempt to account also for some of the result observed after ablation of the SCN and for rhythmicity in isolated tissues and organs[78,82]. They have postulated a network of rhythmic components normally dominated by two 'pacemakers' (or clocks) connected to numerous 'secondary oscillators'. These latter elements are not self-sustaining or autonomous but are normally driven by the pacemakers; when the pacemakers are removed, these secondary oscillators show damped rhythmicity, that is, oscillations that die away. Normally, they propose, the SCN coordinates these elements; after partial or complete ablation, this coordination is lost and so any activity from a remaining pacemaker or from secondary oscillators will persist at least temporarily. Subsequently, as synchrony begins to be lost, ultradian components, 'splitting', etc. will begin to be manifest; when the process of desynchronization is complete, then overt rhythmicity will be lost.
b) If more than one clock exists, then more than one zeitgeber is probably important to an animal. Put differently, zeitgebers would be predicted to affect the different clocks to different extents. This idea has been pursued rigorously by Moore-Ede and his colleagues using the squirrel monkey as a model[81,98,99]. They have compared the circadian rhythms of feeding, temperature and urinary excretion after manipulation of some or all of the possible zeitgebers. Manipulations of the zeitgebers have included phase shifts and changes in period. For exam-

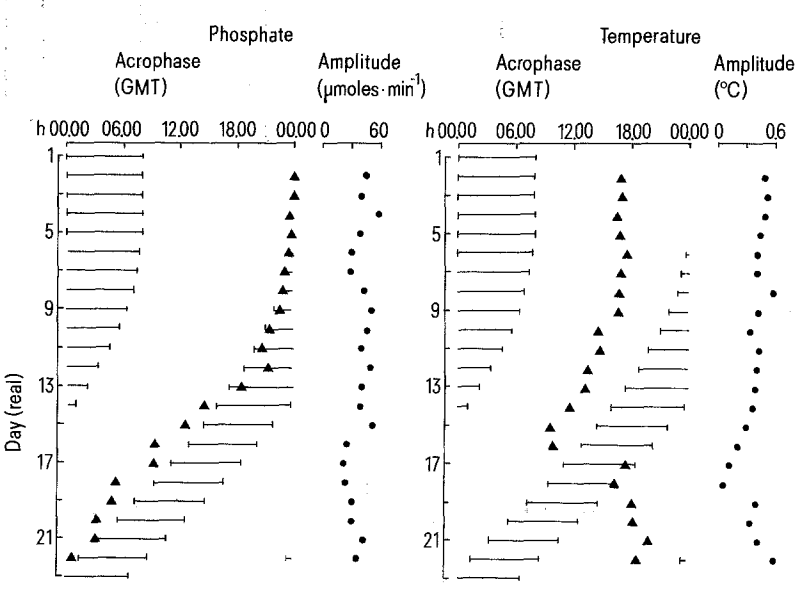

Figure 4. Time of sleep, ⊢⊣, and acrophases, ▲, and amplitudes, ●, of cosine curves fitted to successive 24 h of data of a subject placed on an imposed sleep-awake schedule of decreasing period. All times are shown as real time. Left: urinary phosphate excretion; right: rectal temperature. (From: Folkard, S., Minors, D. S., and Waterhouse, J. M., J. PHysiol. *357* (1984) 341–356, fig. 3).

ple, they have considered the effect of shifting the light/dark but not the eating/fasting regimen and they have investigated the simultaneous presence of a 3 h/21 h eating/fasting regimen and a 11½/11½ h light/dark regimen. Reliably, the urinary excretory rhythms followed the eating/fasting cycles, whilst the temperature followed the light/dark regimen. These results accord with a hypothesis in which different clocks are adjusted by their individual zeitgebers or set of zeitgebers, a hypothesis that will be considered further in section 3.5.3.

3.2.2 Results from man

For obvious reasons, partial ablation of the SCN in man has not been performed and so the evidence that more than one internal clock exists in humans is derived mainly from studies upon different forms of desynchronization[38]. The phase stability that normally exists between different variables has already been mentioned (section 2.2); under certain circumstances, however, the rhythmic structure of an individual can change so that some rhythms can show a period distinct from that of others. As a result, any phase relationship between these groups of rhythms is completely lost; this phenomenon is called internal desynchronization. It can occur in three different ways, as described in the following sections.

3.2.2.1 Spontaneous internal desynchronization

This has been observed most frequently by Wever[108] who has found it to occur in about one third of his subjects during free-running experiments. An example is shown in figure 3. It can be seen from figure 3 that for the first 14 days of the experiment, the subject's rhythms remain synchronized with one another as is usually the case. However, on day 15 spontaneous internal desynchronization takes place so that, from this day on, the temperature rhythm becomes desynchronized from the rhythm of sleep/wakefulness, the former continuing with a period of 25.1 h and the latter with a period of about 33 h. Other rhythms in the same individual gather into two groups; one group largely following the temperature rhythm and the other largely that of activity.

It is difficult to explain this type of result other than by assuming the presence of two oscillators with different free-running periods. Wever[108] has called these the 'temperature' and 'activity' oscillators or Type I and Type II oscillators; recently he has shown[114] that there is a small sex-difference when the free-running period of the 'activity' oscillator is considered, but not in the case of the 'temperature' oscillator. Moore-Ede and his colleagues[82] describe the oscillators as 'X' and 'Y' instead of Type I and Type II respectively. Since the free-running period of the individual before internal desynchronization took place (when it is assumed that the two oscillators are coupled) is 25.1 h, much closer to the 'temperature' than the 'activity' oscillator, it is inferred that the 'temperature' oscillator is stronger. When the results during internal desynchronization are considered in more detail then usually most rhythms in the individual show evidence for the presence of both periodicities. Thus, for example, for temperature there is a weak effect of the 'activity' oscillator and, for activity, one of the 'temperature' oscillator. This interaction between the two oscillators has been investigated during internal desynchronisation by considering the times of going to sleep, the duration of sleep and the phase of the temperature rhythm[26,117,120]. Thus, it is found that sleep onset occurred most frequently at about the time of minimum temperature though there was sleep onset at any phase of the temperature rhythm. Furthermore, the length of sleep does not depend upon prior wakefulness but rather upon the phase of the temperature rhythm. Thus, there is a tendency to wake on the rising phase of the temperature rhythm as a result of which, sleeps commencing after the temperature minimum tend to be short (less than 4 h) and those commencing before the temperature minimum, longer (more than 12 h).

3.2.2.2 Forced internal desynchronization

This condition can be produced by requiring subjects to live 'days' with a length that is outside the range of entrainment of the 'temperature' oscillator but is considered to be within the range of entrainment of the weaker 'activity' oscillator. This possibility has, so far, been tested in only one subject[71]. In this experiment, the subject was placed, unknown to her, on a 21-h 'day'. During this time, her sleep-activity cycle, mealtimes and most urinary constituents followed the 21-day whereas the rhythm of deep body temperature was not entrained but 'free-ran' with a period close to 25 h. After 4 days, when local and real time were 12 h out of phase, the clock was stopped and the subject was required to continue her regular regimen but *estimating time as well as she could.* The results showed that the temperature rhythm continued to show a period of about 25 h but her times of retiring, waking and taking meals, together with her urinary rhythms, continued with a period of about 21 h. Under these circumstances, the simplest explanation is that the activity, feeding and urinary rhythms were controlled by an oscillator that had been entrained to the 21-h day. Interestingly, after about 4 days with no clock (when the subject had 'gained' a day), the 21- and 25-h rhythmicities came back into phase with each other. Thereafter all rhythms had a period of about 25 h suggesting that the 'temperature' oscillator had been the stronger of the two and had caught and entrained the weaker, 'activity' oscillator.

3.2.2.3 Fractional desynchronization

This technique has been developed by Wever[112]. It is based upon the concept that, for any set of zeitgebers of constant strength, the range of entrainment of a series of endogenous clocks will be inversely related to their strengths. With this protocol, subjects are required to follow sleep-activity schedules which, unknown to them, become progressively shorter (or longer). The result of this will be that, if more than one oscillator exists, then the stronger will 'break away' from the imposed zeitgeber period, that is, will no longer be entrained by the zeitgebers, sooner in the experiment. Such events can be followed by observing the behaviour of a variety of physiological and psychological rhythms which are affected by each of the oscillators to a different extent (fig. 4).

Work using this protocol has certainly indicated that different variables 'break away' from the imposed zeitgeber period at different times[37,39,112] but there is not yet any convincing evidence that different *oscillators* break away at different stages of the protocol (but see Wever[108], fig. 94). Thus, the break-away point often occurs when the peak of a rhythm, which under normal circumstances is in the daytime, approaches the sleep phase; instead of falling within sleep, however, the peak jumps from just before sleep to just after (with zeitgebers of shortening period). This result would be predicted to occur also if sleep depressed ('masked') the variable under consideration. The relative importance of this masking effect – rather than the sudden failure of zeitgebers to entrain an oscillator – in producing this 'jump' in timing of a rhythm has not been resolved[37].

3.2.2.4 Different effects of zeitgebers

The concept that, if there existed more than one clock, then the possibility that they would be affected differently by zeitgebers has already been mentioned; but in humans there is very little evidence relating to this. One study that considers this problem to some extent is by the present authors[72]. It was based upon previous work[70] which indicated that, if subjects were placed on an irregular schedule of sleep and waking, then their rhythms free-ran. However, their rhythms could be stabilized to a 24-h period if 4 h of sleep ('anchor sleep') were taken at the same time each day. The extension of this work was to investigate what effect the anchor sleep had had upon the endogenous component of the rhythms under constant routine conditions[72], that is, with the subjects awake and sedentary for 24 h in constant environmental conditions. The results indicated that two endogenous components were present, one of which had been adjusted to a 24-h period whilst the other had continued to show a period in excess of 24 h. This can be interpreted to indicate that the anchor sleep acted as a zeitgeber for one, but not the other, oscillator. If this is correct, then a further implication is that the two oscillators are of different strengths, the weaker being easier to entrain by the zeitgeber used; this aspect will be considered again in section 3.5.3.

In summary, when these results from man and other animals are considered, the case for the presence of more than one internal clock becomes a persuasive one.

3.3 Mathematical models of the circadian system

It is comparatively recent that different groups have attempted to describe the circadian system in mathematical terms[22,27,28,30,32,33,56,58,59,113,118,119]. One approach has been to consider a model using two coupled oscillators, the two oscillators possessing different strengths and driving different rhythms[56,58,59,113]. The model is then generally tested by comparing the results it can produce with experimental data. Obviously, all data should be reproducible by a fully adequate model, but the data that have been used so far come mainly from free-running experiments and experiments in which the zeitgeber period or phase has been changed. Particular effort has been made in attempting to reproduce:

1) the changes in sleep pattern which are observed in time-free experiments of long duration (see section 2.1.2);
2) the phenomenon of spontaneous internal desynchronization and the relationship between the phase of the temperature rhythm and the duration of sleep that exists in this state (see section 3.2.2.1);
3) the behaviour of the circadian system when an oscillator is near the limits of entrainment by a zeitgeber (see[82,108]).

The models then enable the effects of changes in the basic parameters – for example, the types of oscillator (pendulum or relaxation), their amplitude, period and coupling and the response to external driving forces (zeitgebers) – to be calculated.

There can be no doubt that, by suitable manipulation of the mathematical parameters, many real data can be mimicked. However, there seem to be some problems with this approach.

1) The very versatility of these models – which appears to enable them to mimic almost any result – renders them difficult to distinguish between. Thus, models based upon different assumptions appear to replicate experimental data equally well. For example, the phenomenon of internal desynchronization can be mimicked by decreasing the coupling between oscillators, changing their amplitude or changing their free-running period.
2) The correlation between a mathematical parameter and a physiological process is not always evident. This point has been tackled in the models of Enright[32] and Carpenter and Grossberg[22].

Their models are based upon properties that could reasonably be attributed to populations of neurones. These properties include a certain degree of variation (or 'unreliability') within the members of the population. One finding is that such a model predicts that the output from a group of components that individually vary with respect to amplitude, phase, etc. can be remarkably regular[32,33]. This result might have considerable relevance to the search for a single 'clock site' and might entail a reassessment of the properties we might expect from the components of a clock. It also might be relevant when ultradian rhythmicity and 'splitting' phenomena are considered (see section 3.2.1).

3) Alternative models make use of only *one* oscillator coupled with the effects of 'interest' upon bedtime and the masking effects of sleep[30] or with a stochastic process by which a 'sleep factor' accumulates during waking and dissipates during sleep[27,28,118,119].

However, it has been pointed out that the relaxation oscillator has properties similar to that of the stochastic process and that the concept of 'interest' might itself be a reflection of another oscillator. In other words, it is not clear just how different are the 'stochastic' and 'two-oscillator' models.

It is clear, however, that many more detailed experiments are required before a distinction between any of the models can be made with confidence.

3.4 Other sites with clock-like activity

One disadvantage of the mathematical models is that they rarely require the position of their components to be known; yet the data of section 3.2 are interpreted to

indicate that a second area with clock-like properties is to be sought. Additionally or alternatively, the site of the 'secondary oscillators' (see[77,78]) is unknown. Further, if the SCN coordinates rather than initiates rhythmic activity, then it is possible that a number of extra sites is required. The resolution of such a complex problem seems far away at the moment, but, even so, the following results might act as clues in attempting to find areas with 'clock-like' properties.

1) The paired SCN are not homogenous structures and recent histological studies have described the subdivisions within the nuclei and the supposed connections between them [63,76,77]. The role of these subdivisions is not yet understood, but there is always the possibility that they represent in some way the neural requirements postulated by others, viz. a population of ultradian oscillators, etc. It is against such a background that partial ablation studies have been performed[31,63,75,76,86,89]. As yet, no clear picture has emerged, no doubt due in part to the technical difficulties involved, and further results are awaited.

2) Some interest has been shown in the ventromedial (VMH) and the lateral hypothalamic nuclei as possible sites of a clock[76]. Experiments in normal rats[51] have shown that the VMH nuclei behave rhythmically and in phase with locomotor and feeding rhythms. After SCN lesions and on a schedule of restricted feeding (that is, imposing an eating/fasting cycle), neural activity in the VMH, feeding and locomotor rhythms continued to be associated; however, when in the presence of ad libitum food, rhythmic activity in the VMH was absent. By contrast, if the VMH was sectioned, but the SCN remained intact, then rhythmicity continued both in the presence and absence of restricted feeding. These results show once again how a rhythmic environment (restricted rather than ad libitum feeding) can impose its effects upon ('mask') overt circadian rhythms; the conclusion has been drawn by Inouye[51] that the VMH is a 'secondary' oscillator driven by the SCN.

3) In some avian species, the pineal gland shows autonomous circadian rhythmicity in its production of melatonin, this in turn being caused by the activity of the enzyme N-acetylserotonin transferase; pinealectomy leads to a loss of circadian rhythmicity in, for example, starlings[15]. In chicks, portions of the pineal gland studied in vitro continue to produce melatonin rhythmically in constant dark; as is the case when the whole gland is studied in situ, light decreases melatonin production and can change the phase of the rhythm of melatonin production[55,102]. In the house sparrow[100,101] locomotor rhythms in an environment in which light and dark alternate continue normally after removal of the SCN and/or pineal gland. However, in constant dark, pinealectomy results in a locomotor rhythm which 'damps out' after a few cycles; when partial ablation of the SCN was carried out in pinealectomized birds the damped locomotor activity lessened in proportion to the loss of SCN. These results were interpreted to indicate that the SCN in the house sparrow was a secondary oscillator and that the pineal gland was a pacemaker or clock and that, once again, the masking effect of an external rhythmicity had been observed. In mammals the pineal is rhythmic also but not autonomously so, being driven by the neural input from the superior cervical ganglion[14,15].

In brief, therefore, there is no shortage of areas which might act as part of the total circadian system in the body and differences between phyla, and even species, are evident. Again, much work remains to be done to clarify the anatomical correlates of the mathematical, and even physiological, models of the circadian system.

3.5 Zeitgebers and how they act

Zeitgebers are rhythmic external changes that exert a direct influence upon, and are able to entrain, the internal clock. In assessing whether a particular external rhythm is a zeitgeber, it is important to distinguish its acting as a masking effect from its acting as a zeitgeber. For example, if some stressful influence were given on a circadian basis, say at mid-day each day, it would undoubtedly change many physiological variables in a regular manner. However, this would be a masking influence unless it could be shown that such treatment changed the phase of the internal clock. The problem tends to be more complex than this, however. First, a masking influence might affect some other variable which, *in turn,* acts as a zeitgeber. Thus a rhythmic input of noise might not act as a zeitgeber itself but it might cause a change in behaviour (drinking, eating, etc.) that did act as a zeitgeber. Related to this problem is the observation that, in some primates, light acts both as a masking influence and as a zeitgeber[5]. Having established which external rhythms act as zeitgebers, it then becomes necessary to establish the pathway by which the external rhythm reaches the clock and the mechanism by which it exerts its effect.

3.5.1 Identification of zeitgebers

In principle, the ability or otherwise of an external rhythmic influence to act as a zeitgeber can be established in animals as follows:

1) Establish a free-running, circadian rhythm in, say, activity patterns by placing the animal in a constant environment (generally continuous darkness).

2) Impose the putative zeitgeber upon this environment and observe whether or not the rhythm is entrained to this cycle or continues to free-run. The zeitgeber is normally imposed as a regular 'present/absent' schedule with a period of 24 h, for example, '16 h light/8 h dark'.

3) Remove the putative zeitgeber. The entrained rhythm should free-run again *starting from the entrained position.* This is an important condition since it distinguishes entrainment from masking.

Thus, if the imposition of external rhythmicity had acted only as a masking influence, then its removal would uncover the fact that the internal clock had free-run throughout its presence since the rhythm would appear to jump from the 'masked' position to the position determined by the internal clock. This method had been used with considerable success in the chair-acclimatized squirrel monkey[98].

An alternative method has been to change either the period or the phase of a putative zeitgeber whilst leaving all other external influences unchanged or to change different zeitgebers in different ways[57,81,98]. In such experi-

ments any shift of rhythm appropriate to the imposed shift in the putative zeitgeber has then to be distinguished from being a direct 'masking' effect.

Both these methods investigate the potential of an external rhythm to act as a zeitgeber in an 'isolationist' manner. That is, they see if a single rhythm *by itself* can prevent circadian rhythms from free-running (First method) or if a single rhythm can change a circadian rhythm *in spite of* other rhythmic influences remaining unchanged (second method). However, since, under normal circumstances, many rhythmic influences are present in the environment, it might be a *combination* of some of these that acts as a zeitgeber; if this were the case, then it would necessitate some change in the protocol for these experiments.

Finally, it should be noted (see[81] and sections 2.2, 3.2.2.4 and 3.5.3) that, if more than one internal clock exists, then more than one set of zeitgebers might be required, each with its own strength relative to the clock it adjusts.

3.5.2 Zeitgebers in humans

The identification of zeitgebers in humans is important, for a knowledge of them would enable advice to be given when adjustment to new schedules was required (for example, following a time-zone transition or starting nightwork). Possible zeitgebers in humans are mealtimes, the light/dark cycle and social influences.

a) Mealtimes

The role of mealtimes as a zeitgeber has been reviewed recently by Reinberg[87]. The main evidence in favour of them acting as a zeitgeber in humans has been the studies of Goetz et al.[43] and Graeber et al.[44,45]. They showed that taking a single meal at breakfast or dinner produced changes in certain circadian rhythms, e.g. insulin, glucagon. However, this result is likely to be a direct 'masking' effect of the mealtimes through their effects on plasma glucose levels; other circadian rhythms not affected as directly by food intake, e.g. plasma cortisol and deep body temperature, had their phasing affected far less. Reinberg[87] believes that mealtimes must be, at the most, a weak zeitgeber – a conclusion in agreement with that of the authors. Thus, in human subjects living on irregular sleep-wake schedules, circadian rhythms free-ran even though, in these experiments, meals were taken at the same clock hour each day[70]. Even if, in man, the role of mealtimes as a zeitgeber is small, it is interesting to note that there is a relationship between the dietary intake of amino acids and their concentration in the plasma in both man[65] and rats[53]. Further, there is competition between uptake of amino acids into the brain across the blood-brain barrier and this, in turn, will affect the relative rate of synthesis of 5-hydroxytryptamine, and catecholamines[34]. That is, plausible links between food intake and the synthesis of neurotransmitters that could modify activity in neurones comprising a clock can be made.

b) Light-dark cycles and social influences

Aschoff and Wever[11,108] have advanced the view that the alternation of light and dark is a weaker zeitgeber than are rhythmic cues provided by social influences. They based this view on the observation that subjects in time-free environments were not entrained by a light/dark schedule alone, but only when gong strokes (to summon the subject to perform tasks) were regularly superimposed. It was argued that the subjects perceived these strokes as social contacts from the experimenters. One problem in interpreting these experiments is that subjects were free to use accessory lighting during the imposed 'night'. Thus, there was no real need to make use of the rhythmic information provided by the light/dark cycle alone. However, when the gong strokes were added, since they were more frequent in the 'day' than during the 'night', it would have been inconvenient to have ignored the information they gave. Of course, the protocol does not enable us to say if social influences (gong strokes) alone act as a zeitgeber.

However, for individuals living in society there are considerable pressures to comply with arrangements that discourage activity at night and encourage it in the daytime. The importance of such 'social compliance' can be seen even in free-running experiments performed upon groups of subjects[7,13]. Thus the members of the group show free-running periods which are practically indistinguishable. It seems reasonable to interpret this result as showing mutual entrainment resulting from social zeitgebers. Others[25,82] have argued that the light/dark cycle can act as a zeitgeber in humans. Thus, they have shown that humans under free-running conditions become entrained to an imposed light/dark cycle when no auxilliary lighting was allowed during the dark phase. In the absence of this light/dark cycle, rhythms free-ran even though the experimenters maintained that the subjects could have produced their own social zeitgebers by talking to the experimenters if and when they wished. In these experiments the effects of the light/dark cycle as an entraining agent seem clear enough since, when the putative zeitgeber was removed, rhythms began to free-run from an appropriate phase (see section 3.5.1); but the protocol is such that it would have been inconvenient (if not impracticable) not to comply and so the combined actions of entrainment and masking seem likely[5]. Further the social influences in these experiments seem not to have been rhythmic (because the opportunity to talk was present *all* the time); this would not seem to be the ideal test for a zeitgeber.

More recently, Wever and his group[115] have used an imposed light/dark cycle and shown that the sleep-activity cycle and temperature rhythm in some subjects can adjust to a 'day' length of up to 29 h provided that the light strength was high enough (4000 lux). With weaker light intensities (1000 lux) adjustment was limited to day lengths of about 27 h. Undoubtedly, sleep becomes difficult in the bright light but the result was not attributed to 'masking' alone since there were changes in amplitude and phasing of the temperature rhythm which, the authors maintained, indicated entrainment.

The route by which a light/dark cycle might act as a zeitgeber has been investigated. Thus, a direct neural pathway between the retina and SCN has been found in a number of mammalian species[18,82,92], including man[93]. The position is complicated by the observation that the pineal gland shows oscillation activity (see section 3.4) and that

melatonin production is inhibited by bright light[61,62]. Indeed, Wever et al.[115] believed that changes in the concentration of melatonin were responsible for the adjustment of the sleep-wake cycle observed in their experiments (above); Lewy[60] also has expressed the view that melatonin influences activity.

c) Combination of rhythmic influences

It is the view of the present authors that attempts to find the most important *single* zeitgeber might be of limited usefulness since, under normal circumstances, more than one rhythmicity might be important[69]. A similar view, describing the importance of more than one zeitgeber in the control of the cortisol rhythm was advanced by Vernikos-Danellis and Winget[105]. The argument favouring a combination of zeitgeber influences could run: individuals go to bed since they know that they need a certain amount of sleep to prepare for the rigours of the day; domestic (e.g.shopping), social and business commitments are arranged to take place during the daytime and so it is *convenient* to arrange their waking time to coincide with daytime. Once the sleep-wakefulness cycle has been established, all other potential zeitgebers (light/dark, feeding/fasting, social mixing/social isolation, noise/quiet, etc.) will be adjusted to coincide[70]. In connection with this view, we note that Weitzman et al.[107] believe that sleep (and, by implication, other external influences) might act as a zeitgeber for the cortisol rhythm. If this approach is correct, then to assess the potency of any individual zeitgeber might be less important; instead, circumstances in which the synchrony that normally exists between different zeitgebers is changed (shift work) might be of far greater relevance to a human's well-being.

3.5.3 Which zeitgebers affect which clock?

The question has been considered whether the different zeitgebers exert their effects upon different clocks (wherever these might be) or upon a single site.

It is currently believed that the weak oscillator, which is found in the SCN (section 3.2.2.1), is influenced by the light/dark cycle. The evidence for this is (see[58,59]):

1) The existence of a direct neural input from the retina to the SCN (section 3.5.2).

2) When individuals are placed in a time-free environment, the sleep-activity rhythm delays relative to the temperature rhythm and appears to become phase-locked to it. This is believed to result from the sleep-activity rhythm no longer being entrained by an external light/dark cycle[58].

3) After changes in the external light/dark cycle, the sleep activity cycle adjusts more rapidly than the temperature rhythm[58].

In his mathematical model, Kronauer[58,59] has the zeitgebers acting upon the Y (activity) oscillator and these produce entrainment of the X (temperature) oscillator via coupling between the Y and X oscillators.

Without knowledge of the whereabouts of the X oscillator it is not possible to establish whether or not a direct or indirect influence upon the X oscillator exists. These influences need not be only from sense organs, of course, since transport of humoral substances via the cerebrospinal fluid and even the production of neurotransmitters (see section 3.5.2) might play important roles also [2,21].

The belief that the temperature rhythm cannot be adjusted directly by zeitgebers is not necessarily held by all (see Wever's comments in discussion of Kronauer[58]) and the results from Moore-Ede et al.[81] are more easy to explain if different zeitgebers affect different oscillators preferentially. For example (see section 3.2.1) in the squirrel monkey, changes in the phasing or period of an eating/fasting zeitgeber exerted more effect upon urinary than feeding and temperature rhythms whereas the opposite result was seen when changes to the light/dark zeitgeber were made[81,99]. A full understanding of these kinds of result will require a much more complete knowledge of the nexus between different circadian rhythms in addition to more data on clock sites and zeitgeber pathways.

4) Conclusion

The interest shown in circadian rhythms, together with the amount of research this has engendered, continues to increase. Techniques that have been developed in other fields (particularly tissue culture, electrophysiology and neurochemistry) are being used to discern the properties of areas of the brain believed to be potential sites for the clock(s). In addition, there has recently been developed a series of models – both mathematical and physiological – which attempts to explain the circadian system as a whole in terms of interacting oscillators. It seems likely that the rapid accumulation of data and ideas will continue.

There are two points which the authors would like to draw attention to.

The first is that the link between the two approaches is sometimes unclear. This problem is particularly acute when the sites and interactions between the different clocks are considered. Thus, we do not understand clearly the anatomical and physiological correlates either of many of the parameters in mathematical equations or of the models which propose a hierarchy of oscillator types. Reviewing the recent scientific literature in this field it sometimes appears that we begin to be in danger of letting the models become an end in themselves rather than a means to understanding the anatomy, biochemistry and physiology of the system.

The second point is that, as is the case in other fields of study, researchers have investigated the individual components of a system while tending to ignore the interaction of these to produce a single, integrated whole. Examples of this are when the identification of zeitgebers and the search for the clock site(s) are considered. Perhaps it will turn out to be as difficult to identify individual zeitgeber types and clock sites as it has been to identify (say) 'respiratory centres' or the site of 'memory'.

The problem raised by these points has a more general aspect. Rhythmicity is a phenomenon associated with the Living Kingdom in general and is found at all levels of organization, in single cells (or unicellular organisms), tissues, organs and in whole animals. In more complex animals, individual cells, tissues and organs are believed to be subservient to (or driven by) a whole complex of primary and secondary oscillators[69,82]. To integrate the circadian properties of the individual components into

the circadian system of an animal as complex as a mammal is likely to provide experimental and conceptual challenges to research workers for some time into the future.

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0014-4754/86/010001-13$1.50 + 0.20/0

Sensitivity variations in insect chemoreceptors; a review[1]

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Key words. Insects; chemoreceptor; receptor sensitivity; feeding behavior; central regulating mechanisms; peripheral regulating mechanisms; sensory behavior.

Introduction

The feeding behavior of insects, like that of other animals, depends heavily on neural input from their chemical senses. Therefore the chemoreceptors of insects have attracted much interest and the gustatory sense of blowflies, and to a lesser extent of lepidopterous larvae, are among the best studied chemoreceptor systems in the invertebrates[23, 60]. These studies have concentrated on determining receptor specificities and sensitivities, with the aim of elucidating the neural code which governs food selection behavior. Concomitant observations on the structure of chemoreceptors have revealed their microarchitecture[76]. Since there is no unequivocal evidence for the existence of efferent neural control of insect chemoreceptors, most studies on the relationships between sensory input and insect behavior assume receptor activity to be solely dependent on stimulus characteristics. A growing number of reports, however, indicate that receptor sensitivity may vary depending on developmental stage, feeding history and/or physiological state of the insect. Such peripheral neural changes and the processes which regulate them are the subject of this review.

Changes in receptor characteristics

Inconstancies in sensory input to the central nervous system (cns) under standardized stimulus conditions may be due to either changes in the accessibility of the receptors to the stimulus or to sensitivity changes in the receptors per se. The gustatory pegs on the palps of locusts exemplify sensilla which become unresponsive to chemicals after the insect has finished a meal, due to the closure of their distal orifices[9]. Some reports in the literature suggest that in flies and caterpillars also, a partial con-