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CIRCADIAN RHYTHM OF OUTPUT FROM NEURONES IN THE EYE OF *APLYSIA*

II. EFFECTS OF COLD PULSES ON A POPULATION OF COUPLED OSCILLATORS

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SUMMARY

1. The circadian clock that controls CAP frequency was stopped at or near its lowest phase point by long duration cold pulses of 6 °C. On return to normal recording temperature (15 °C), the rhythm was always reinitiated from this phase point.

2. Following long cold pulses, there was often a transient peak of CAP activity lasting 2–6 h. It is thought that this was an effect of rise in temperature after prolonged cooling and not an effect on the clock itself.

3. Twelve h cold pulses, spanning the rhythm peak, caused phase delays. 9 °C pulses caused small delays (e.g. 1·7 h) while large phase delays (e.g. 6·7 h) followed pulses of 5 °C. Some pulses at an intermediate temperature (8·5 °C) caused abnormal post-pulse cycles lasting several days, and resulting in very large phase delays (10–14 h).

4. The abnormal CAP frequency curves following 12 h cold pulses of 8·5 °C spanning the rhythm peak are interpreted as rhythm splits. It is postulated that part of the population of coupled oscillators comprising the circadian clock was slightly delayed by the cold pulse, while the other part was driven further towards the 'stopped' state, thus producing a large phase angle difference between the two subpopulations. These drew one another back into phase during several cycles to reform a normal circadian rhythm.

5. It is hypothesized that the circadian oscillations of the two subpopulations did not sum to produce the observed CAP frequency curve; rather the level of CAP output was controlled by whichever subpopulation was discharging at the higher frequency.

INTRODUCTION

Each eye of the mollusc *Aplysia californica* is composed of about 3600 receptor cells with interdigitating pigmented support cells, and approximately 1000 secondary cells (Jacklet, 1973*a*). The output recorded in the optic nerve is in the form of compound action potentials (CAPs), thought to be synchronized by electrotonic coupling among cells. Intracellular recordings from secondary cells (Jacklet, 1969, 1973*a*, 1976*b*) and

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the characteristic temporal distribution of the CAPs in 'bursts' suggest that these cells are similar in mechanism of membrane potential oscillation and action potential production to endogenously active neurones elsewhere in the CNS of *Aplysia*.

The frequency of output of CAPs from the isolated eye of *Aplysia*, in darkness and at constant temperature, shows a circadian rhythm. Additionally the amplitude of individual CAPs exhibits a circadian rhythm. It is probable that the change in amplitude of the CAPs through the free-running period of 26–27 h is due to a rhythmic change in the number of secondary cells contributing to the CAPs (Jacklet, 1971, 1973*a*, 1974). It has been hypothesized that the circadian rhythm of CAP frequency and CAP amplitude modulation is a result of the interaction of a population of coupled oscillators (Jacklet & Geronimo, 1971).

In this paper two sets of experiments will be described. The first involves the application of long duration pulses of low temperature (6 °C) intended to determine whether the clock can be 'stopped' by low temperature. Stopping the clock for the duration of cold pulses in other systems has been taken to mean that the oscillation moves to a stable phase point from which the rhythm is reinitiated following cessation of the cold pulse (Bünning, 1958; Njus, McMurry & Hastings, 1977; Wagner, 1963; Hesse, 1974). The purpose of this experiment is twofold. Firstly, by determining the precise phase point of the stopped state, quantitative information is obtained which is necessary for the construction of a comprehensive control systems model of the clock mechanism (Benson & Jacklet, 1977*b*). Secondly, this effect of temperature can be used to produce phase shifts of different magnitudes among the elements of a population of oscillators. This is the basis of the second set of experiments in which 12 h cold pulses have been utilized to 'split' the circadian rhythm. Such experiments support the hypothesis that the circadian clock in the eye consists of a population of coupled oscillators.

The eye rhythm of *Aplysia* is particularly useful for the kind of experiments reported here, since the shape of the entire oscillation can be monitored. In most other systems, only particular phases of the rhythm (e.g. active and resting phases in a locomotor rhythm) are measurable, or the clock is manifested only by the times of ecosion of individuals in populations of animals. It should be noted, however, that the frequency of the CAP output of the eye is directly decreased by temperature so that it is not possible to measure the shape of the circadian oscillation during temperature pulses, but only after the pulse is completed and the preparation is at normal recording temperature once more.

To test the population model, Jacklet & Geronimo (1971) and Jacklet (1973*a*, 1976*a*) reduced the number of cells by physical ablation of parts of the isolated eye. They found that progressive reduction in the number of neurones in the eye reduced the range and period of the circadian rhythm. When eyes were reduced to 200 or fewer secondary neurones, the CAP frequency became arrhythmic. Ablation was carried out, after removal of the lens, from the distal end of the eye. It was concluded that the neurones located near the base of the eye, where the majority of secondary neurones are concentrated, were extremely important in the production of the rhythm.

Sener (1972) performed similar experiments, and the results and interpretation of these experiments were published by Strumwasser (1973). Sener's conclusions conflicted with those of Jacklet; he considered that the period did not change with degree

of ablation, and that the circadian rhythm was still clear in extremely reduced eyes. There were several differences between the two experimental approaches. Jacklet recorded activity in culture medium for many days after ablation while Sener used short records from eyes maintained in filtered sea water. Jacklet's estimate of cell numbers at various stages of ablation was based on counts of nuclei, whereas Sener sometimes used an indirect method involving mass of protein contained in the ablated eye. Secondly, period measurements were made by periodogram and other techniques from individual records by Jacklet, but Sener used lumped data which tended to blur differences and made no actual measurement of the period of the rhythm. Finally, Jacklet reduced eyes so that they contained no receptor cells at all, as judged by histological examination and lack of a light response. These eyes showed spontaneous CAP production in DD, but the circadian rhythm was absent. Sener did not report on any eyes that were cut to this extreme.

The conclusions to be drawn from experiments involving reduction of the cell population size of the eye are that the secondary neurones are crucial to the production of the CAPs and the circadian rhythm. Considerable ablation can be carried out causing a reduction in the range and period of the rhythm, until a critical stage of reduction (200 secondary neurones) is reached. Beyond this stage, the members of the population appear to oscillate independently, possibly with short periods, resulting in arrhythmicity, or, in some cases, infradian frequencies of output. Such very reduced eyes remain spontaneously active for several days with an average CAP frequency, similar to that for whole eyes, but with the amplitude of individual CAPs greatly reduced.

Rhythm splitting or 'frequency doubling' (Pavlidis, 1973) is the phenomenon in which a circadian rhythm divides into two or more components which, at least for some time, show distinctly different frequencies. At some later time, the several components may shift back into phase to form a normal unimodal rhythm. Pittendrigh (1960) and Pittendrigh & Daan (1976*b*) have reported a number of cases in nocturnal rodents in which rhythms of locomotor activity split spontaneously into two components after prolonged constant illumination. As these components moved out of phase, their periods were, of course, different. In most cases, a fixed phase angle difference between the split components of the rhythm, usually close to 180°, was adopted for many cycles of the rhythm, during which time the periods of the separate components were identical.

Rhythm splitting may be considered evidence for the existence of several oscillators, possibly of circadian frequency, which, though normally in phase and producing a unimodal circadian output, can be forced out of phase with one another by an external disturbance (Pavlidis, 1973).

MATERIALS AND METHODS

Eyes were dissected from *Aplysia californica* kept in LD 13:11 at 15 °C prior to experimentation. Isolated eyes were then maintained in constant darkness (DD) at 15 °C in nutrient culture medium. Activity was recorded from the optic nerves with tubing electrodes. Details of culture medium composition and recording methods are given by Benson & Jacklet (1977*a*).

In most cases, at least two full cycles of the rhythm took place in normal experi-

mental conditions of DD at 15 °C before a perturbation was applied. This allowed any post-dissection transients to subside, and a precise measurement of phase and amplitude of the rhythm to be made.

The temperature of the culture medium was controlled by water circulated through glass coils connected to a controlled temperature bath. Temperature was monitored in the experimental chamber by a thermistor probe. Rate of change of temperature at the beginning and the end of low temperature pulses was approximately 1 °C per 5 min.

RESULTS

Effect of long duration cold pulses

A series of experiments was carried out to determine whether cooling the isolated eye could stop the circadian clock, as was suggested by the considerable damping of the circadian rhythm that occurred when the eye was maintained at 9·5 °C (Benson & Jacklet, 1977*a*).

These results are the basis for the analysis of the effects of 12-h cold pulses on the rhythm, as described below. Circadian clocks characteristically stop at a particular phase point which is independent of the time at which the cold pulse begins (Bünning, 1973). In other words, when the system is returned to normal temperature, the clock oscillation always starts again from the same phase point, irrespective of the phase point at which cooling began and of the duration of the cold pulse. Several hours may be required for the clock oscillation to move to this phase point, so that it is important for the cold pulse to be sufficiently long for the oscillation to reach the stable phase point at which it is said to be 'stopped'.

Fig. 1 illustrates a control and four records from experiments in which cold pulses of 6 °C lasting more than one normal clock period (26 h) were applied to isolated eyes. During the cold pulses, CAP activity ceased, because the CAP generating mechanism is temperature sensitive and very few CAPs are produced below 8 °C. The various cold pulses were initiated at phase points in the rising and falling phase of the rhythm, and were ended during similar projected phases, as can be seen by comparison with the control. The phase of the post-pulse rhythm bore a constant phase relation with the end of the cold pulse, but not with either the phase at which the cold pulse began or that at which it ended. In other words, the centroid of the first normal cycle of the reinitiated rhythm always occurred between 32 and 36 h after the end of the cold pulse. This indicates that the clock stopped during the cold pulse and was always reinitiated from the same phase point when the temperature was raised from 6 to 15 °C (Table 1). There was usually a rapid increase in CAP frequency at the end of the cold pulse, sometimes giving a small peak of 4–6 h duration, followed by normal oscillations of the circadian rhythm. This short duration peak was probably a transient effect of rapid warming after prolonged cooling on the CAP generating mechanism of the pacemaker cells of the eye. Such transient peaks sometimes followed even short or low intensity cold pulses which left the clock oscillation unaffected.

By extrapolating the reinitiated rhythm back through any short duration peak immediately following the return to 15 °C, it can be seen that in most cases the clock oscillation came to rest near its lowermost phase point during the cold pulse, and that it always began again after a cold pulse from this phase point. The data given in

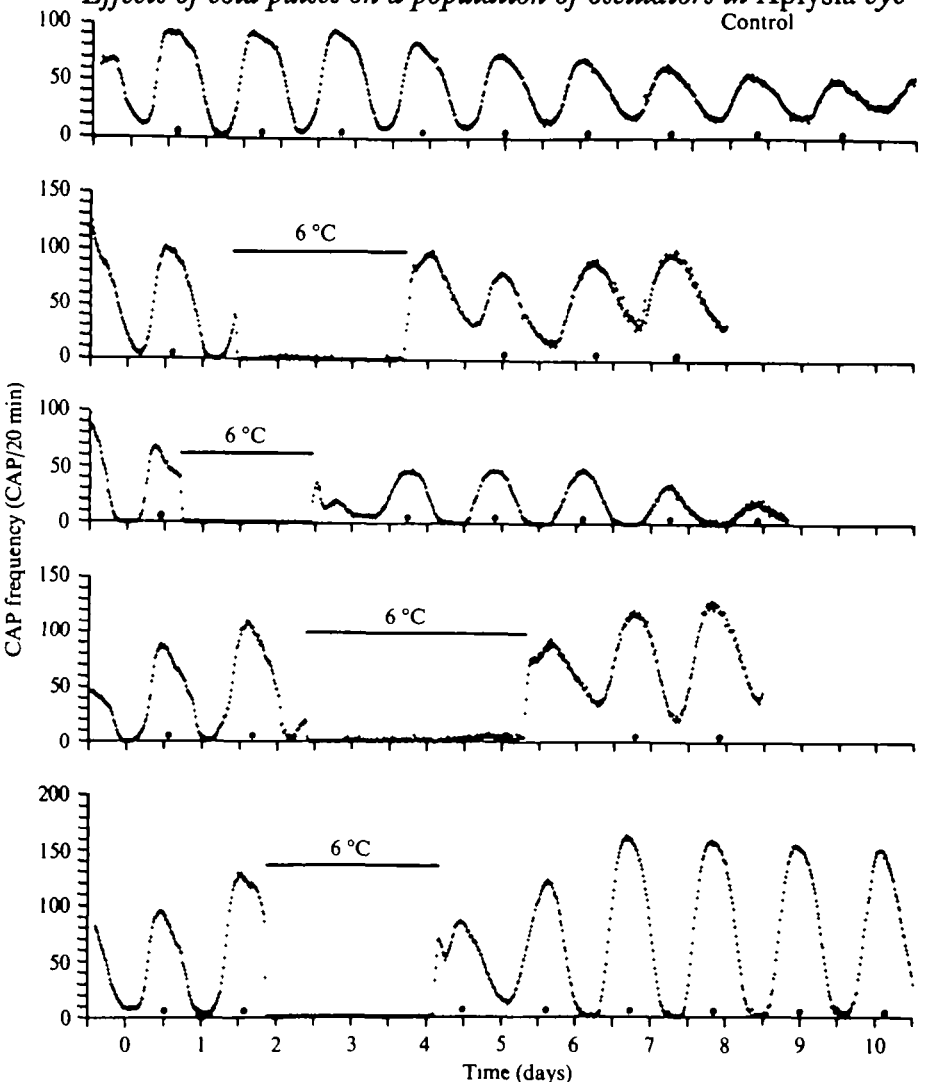

Fig. 1. Clock-stopping effects of long cold pulses. Typical examples of the effects on the rhythm of maintaining the eye at 6 °C for long periods. Whether the cold pulse begins during the rising or falling phase, or ends during the projected rising or falling phase, the centroid point of the first complete post-pulse cycle is always approximately the same number of h after the cessation of the cold pulse.

Table 1 suggest that the 'stopped' state is 2–5 h after the middle of the zero CAP frequency region. When the abnormally rapid increase in CAP frequency after long cold pulses is taken into account, the stable 'stopped' phase point is seen to be near the base of the rising phase, or in a few cases part way into the rising phase.

Effects of 12 *hour cold pulses*

The influence of 12 h pulses of temperatures just outside the compensatory range (13–22 °C), and considerably below it was measured as a preliminary to testing for rhythm-splitting effects of low temperature.

The action of a 12-h cold pulse of 5 °C applied symmetrically spanning the rhythm

Table 1. *Effects of* 6 °C *cold pulses on the rhythm*

(All phase measurements are in hours after the centroid point (phase 0·0 h).)

Record in Fig. 1	Phase of cold pulse onset	Projected phase of cold pulse cessation	Pulse duration (h)	Hours after pulse cessation to centroid of first post-pulse experimental cycle	Hours after pulse cessation to centroid of first control cycle
2	20·0	20·7	55·0	32·3	32·0
3	6·0	21·3	42·0	34·3	35·7
	6·0	21·0	42·0	34·3	30·3
4	17·3	9·0	71·0	35·3	17·3
	15·3	7·7	71·0	36·3	18·7
	16·7	9·3	71·0	36·3	17·0
	19·3	10·3	71·0	35·3	16·0
5	6·3	7·3	54·0	35·3	19·3
	6·7	7·7	54·0	34·3	19·0

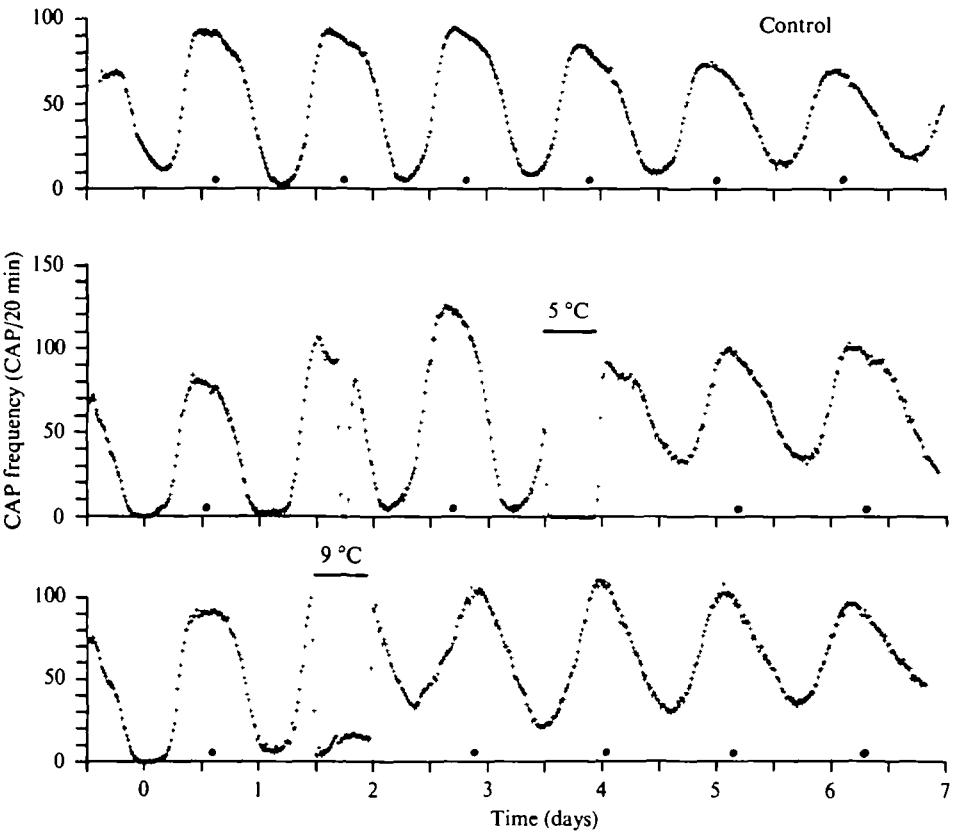

Fig. 2. Phase-shifting effects of 12-h cold pulses spanning the rhythm peak. In the second record, a 5 °C cold pulse of 12-h duration was applied spanning the rhythm peak, causing a phase delay of 6·7 h. A 9 °C pulse of the same duration applied at a similar phase caused a phase delay of only 1·7 h, as illustrated in the third record. Numerical data for these records and others are given in Table 2. A brief temperature pulse applied during the second cycle in the second record did not perturb the circadian rhythm. Control as in Fig. 1.

Table 2. *Phase shifting effects of* 12 *h cold pulses spanning the rhythm peak*

(All phase measurements are in h after the centroid point (phase 0·0 h).)

Record in Fig. 2	Phase of cold pulse onset	Pulse duration (h)	Temperature (°C)	Phase shift (h)
3	22·3	12·0	10·5	+1·3
	22·0	12·0	10·5	+0·3
	21·0	12·0	9·0	−1·7
	22·3	12·0	7·0	−1·7
	22·3	12·0	7·0	−1·7
	22·0	12·0	7·0	−1·0
2	19·3	12·0	5·0	−6·7
	19·3	12·0	5·0	−6·7

peak is shown in the second record of Fig. 2. As expected, CAP activity ceased completely for the duration of the cold pulse, and a small transient increase in CAP frequency of about 3 h duration followed the end of the cold pulse. Extrapolation of the subsequent circadian oscillations back through the transient peak shows that the clock moved towards but not completely to its lowermost phase point. The phase delay caused by this 5 °C cold pulse was 6·7 h.

The third record in Fig. 2 shows the effect of a 12-h cold pulse of 9 °C applied during almost the same phase as the 5 °C pulse. For the duration of the pulse, CAP frequency was reduced. At the end of the cold pulse, the CAP frequency returned to a high level, and the rhythm continued without a significant change in the shape of the transient cycle. There was a 1·7 h delay and the average period of all subsequent cycles was increased by about 1 h in comparison with the control.

These results are typical of the effects of most 12-h cold pulses applied across the peak of the rhythm. Pulses of 9·0 °C and above have small or zero delaying effects on the phase of subsequent cycles of the rhythm; pulses of 5 °C or less cause large phase delays or stop the clock almost completely. In many experiments, the period of the rhythm after the cold pulse was consistently longer than the period of controls by 1 or 2 h. Table 2 contains data for 12 h cold pulses applied spanning the rhythm peak. Even temperatures as low as 7 °C caused only small phase shifts, although the temperature sensitivity varied somewhat from eye to eye.

Rhythm splitting by 12*-h cold pulses*

If the circadian clock in the eye of *Aplysia* consisted of a single temperature-compensated oscillator, there would be a transition between small or zero delays, and large delays or clock stopping, in response to 12-h cold pulses applied across the activity peak. The non-linear nature of temperature compensation of period length, with almost complete compensation in the 13–22 °C range, and increasingly poor compensation below 13 °C, has already been demonstrated (Benson & Jacklet, 1977*a*). The transition temperature might vary somewhat from eye to eye, but there would be a definite temperature for any given eye at which the temperature compensation mechanism of the eye clock would break down, and the oscillation would be driven towards the stable phase point. On the other hand, if the clock were a population of coupled oscillators, and the individual oscillators had slightly differing temperature sensitivities, particularly with regard to the threshold at which temperature

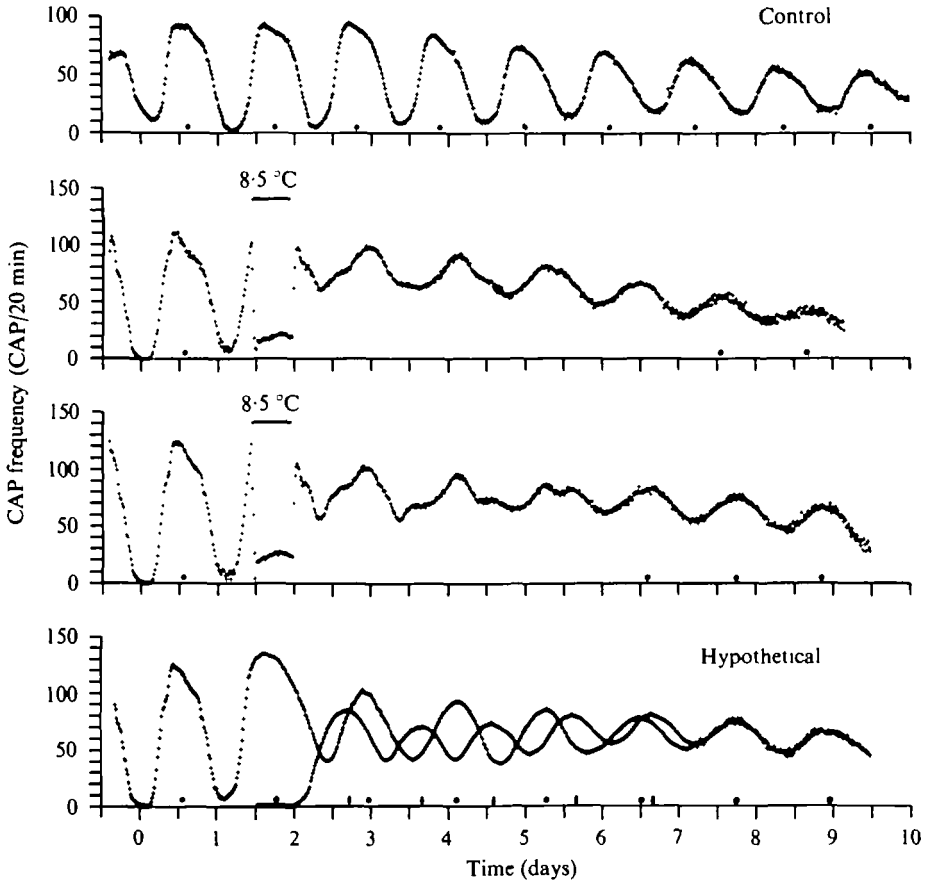

Fig. 3. Rhythm splitting effect of 12-h cold pulses. The second and third records show the effects of 12-h cold pulses of 8·5 °C applied spanning the rhythm peak. The abnormal cycles following the cold pulses are interpreted as rhythm splits, as indicated in the fourth record which was constructed by fitting two periodic curves to the third record. See text for explanation. Control as in Fig. 1.

compensation ceased, unusual behaviour might be expected at the average transition temperature.

Cold pulses of 8·5 °C symmetrically spanning the CAP activity peak were applied to eyes in DD. As would be expected, some rhythms showed the small or zero phase shifts characteristic of eyes subjected to pulses of 9·0 °C or above, although none showed large delays or complete stopping of the clock. However, five eyes exhibited CAP output frequencies such as those illustrated in the second and third records in Fig. 3. If the irregular oscillations of CAP output frequency are treated as single cycles of the rhythm with abnormal shapes, the intercentroid periods of the first 2 or 3 cycles average 5 h longer than the normal period of 26–27 h, with abnormally long periods persisting to Day 7. After that time, the rhythm returned to normal cycle shape and period. However, these outputs can be treated plausibly as the resultant of two separate oscillators or subpopulations of oscillators which cycled at slightly different but nevertheless circadian periods. They began at the end of the cold pulse approximately 12[illegible] out of phase, but as a consequence of their different periods (one slightly longer th[illegible]

mal, the other shorter), they slowly shifted through an increased phase angle difference until they were once more in phase. The fourth record in Fig. 3 illustrates two curves fitted to the experimental record above it, and Fig. 4 is an enlarged illustration of the lower two records in Fig. 3, which shows more clearly the fit between the measured data and the hypothetical curves. It is hypothesized that one subpopulation of oscillators continued the prepulse rhythm. Although slowed during the cold pulse, it continued to oscillate with a small phase delay at a period initially slightly longer than the normal circadian period, reverting to the normal period in 4 cycles. The post-pulse periods in h of this subpopulation were 27·3, 28·0, 29·7, 29·7 and 29·0, where 29·0 h is a normal period for the rhythm after 8–9 days (cf. Fig. 3 of Benson & Jacklet, 1977*a*). The other curve represents a second subpopulation of oscillators which was stopped or driven close to the lowermost phase point during the cold pulse, and began to oscillate from that point at the end of the cold pulse. The large delay made this subpopulation approximately 120° out of phase with the other, and it oscillated at a shorter than normal period, increasing in period until it came into phase with the other subpopulation. The post-pulse periods, in h, in this case were 22·7, 22·3, 25·7, 24·0, 26·0 and 29·0. The circadian rhythm then continued with normal period. Similar curves with slightly different phase relations and amplitude ratios can be fitted to the second record in Fig. 3. The re-established rhythms, beginning on Day 7, show nett phase shifts of 10 h delay in the second record, and 14 h delay in the third record. An important feature of this hypothesis is that the oscillations of the separate subpopulations did not sum. Instead, it is postulated that the rhythm level (i.e. the measured CAP frequency) was controlled by whichever subpopulation was firing at the higher rate. During the split cycles of the rhythm the amplitudes of individual CAPs were very uneven, but when the normal rhythm was re-established, CAP amplitude returned to the smoothly modulated rhythm characteristic of a free-running rhythm.

DISCUSSION

Cooling the eye of *Aplysia* resulted in an immediate decrease in CAP frequency, with eventual cessation of CAP production at about 8 °C. Such behaviour is characteristic of bursting pacemaker cells, and is reflected in the loss of the negative resistance characteristic or negative slope region in the steady-state, voltage clamp current-voltage curve, which is essential for bursting pacemaker activity (Wilson & Wachtel, 1974; Barker & Gainer, 1975*a*, 1975*b*). It is thought that the secondary cells in the eye are electrotonically coupled pacemaker neurones, with properties similar to those elsewhere in the CNS of *Aplysia* (Benson & Jacklet, 1977*a*). The decrease in CAP frequency during cold pulses most probably reflects the action of temperature on the mechanism of discharge of pacemaker neurones in the eye rather than on the clock mechanism itself.

The short duration peak of CAP production which sometimes followed cold pulses was probably a transient effect of rapid temperature increase after prolonged cooling on the secondary neurone CAP generating mechanism. A transient increase in CAP frequency occurred in about 50% of the eyes of *Aplysia* subjected to cold pulses, most often after prolonged cooling, but occasionally after short pulses which caused only small phase shifts of the circadian clock. General observation suggested that rate of

temperature change at the end of a cold pulse governed the presence or absence of a transient peak. Slower cooling appeared to produce larger peaks. Carpenter (1967) reported transient frequency changes during temperature change in both bursting and non-bursting neurones in *Aplysia*. Anderson (1976) found that a transient period of hyperpolarization and absence of spikes lasting about 1 h, followed a temperature change from −3 to 11·5 °C applied to bursting pacemakers in *Tritonia*. Salanki, Vadasz & Vero (1973) consider that the rate of temperature change affected the transient discharge frequencies of bursting neurones in *Helix*, but that a steady rate of discharge, dependent on ambient temperature, occurred after about 15 min.

By cooling the eye to 6 °C for periods of more than a day, it was possible to 'stop' the clock by driving it to a stable point near the lowermost phase of the rhythm. Slight discrepancies seen in some records can be accounted for in terms of residual activity in the clock mechanism at 6 °C. It will be postulated in a later paper (Benson & Jacklet, 1977*b*) that the rhythm of CAP frequency is in phase with the clock oscillation, and therefore that this phase point is also near the lowest point on the clock oscillation. The rhythm was always reinitiated immediately from the stable phase point. In many cases one or two cycles of increasing amplitude occurred before a steady-state amplitude was reached. Since the effects of warming after prolonged cooling on the CAP generating mechanism appear to be confined to the transient peak which is only a few hours in duration, the amplitude changes in the first 2 or 3 days after the end of the cold pulse probably reflect changes in the amplitude of the clock oscillation.

A second after-effect of cooling, even following cold pulses of only 12 h duration, was a small but significant steady-state period increase. Pittendrigh & Daan (1976*a*) have drawn attention to a general lability, within narrow limits, of period length which can be distinguished from day-to-day instability. Large perturbations applied to free-running clocks have often produced after-effects (Pittendrigh, 1960), particularly changes in period in the new steady-state following phase shifts. Pittendrigh and Daan showed that large phase delays were characteristically followed in the new steady-state by small increases in period, and large phase advances were followed by period decreases. These steady-state period changes, which have not been accounted for in most models for circadian rhythms (Benson, 1976), are to be distinguished from transient cycles of abnormal period which constitute the phase shifting process itself. In the *Aplysia* CAP rhythm, phase delays caused by cold pulses were completed in a single transient cycle, but the subsequent steady-state period, as measured between centroids, often increased by 1–1·5 h in comparison with controls.

The phase delaying action of 12-h cold pulses spanning the activity peak of the rhythm was intensity dependent. Cold pulses of 9 °C or above, at which temperatures the clock is known to compensate (Benson & Jacklet, 1977*a*) caused small delays of no more than 3 h. Low temperature pulses (5 °C) of the same duration produced major delays of magnitude approaching the duration of the cold pulse. Since these pulses began early in the rising phase of the rhythm, it is suggested that the oscillation was forced towards the stable phase point, possibly reaching it and stopping, in less than 12 h during sufficiently intense pulses. The reinitiated rhythm began from this point at the end of the cold pulse, thus being delayed by approximately the duration of the pulse. Clearly it must take some time for the clock oscillation to fall to its lowest level, depending on how far into the rising phase the cold pulse begins. This will

influence the magnitude of the delay; the further the oscillation falls towards the lowest point, the larger the delay. Benson & Jacklet (1977*b*) described the action of cold pulses applied at different phases of the rhythm.

8·5 °C is thought to be at or near the average temperature at which compensation for temperature change breaks down in the eye clock, although there is variation in temperature sensitivity of the clock from one eye to another. In five experiments, pulses at this temperature, applied spanning the activity peak, caused abnormal cycle shapes in the first few cycles after the cold pulse. One possible explanation for this could be that these cycles were transients with long periods and altered shapes, which led ultimately to phase delays of between 10 and 14 h. However, the periods involved range up to 31 h, which is far outside the normal variation of period length (Benson & Jacklet, 1977*a*). Furthermore, 12 h cold pulses of greater intensity applied at the same phase produced phase delays of up to 8 h with a single transient and a maximum period increase in subsequent cycles of 1 h in comparison with controls.

A more likely explanation for abnormal cycles of the rhythm following cold pulses of 8·5 °C is in terms of a rhythm split, in which a population of oscillators was divided into two subpopulations which oscillated out of phase with one another for several cycles. Jacklet & Geronimo (1971) have provided evidence that a population of oscillators, probably located individually in secondary neurones, make up the circadian clock in the eye of *Aplysia*. If these individual oscillators differed in their temperature sensitivities or were somewhat out of phase, it would be reasonable to suggest that during a cold pulse of 8·5 °C, part of the population was below the threshold of compensation and moved partly or completely to the stable 'stopped' state, while the remainder of the population was phase delayed. This phase delay would have been due to the temperature effect which is illustrated in Fig. 2, but also partly due to the coupling among the members of the whole population. It is hypothesized that at the end of the cold pulse, the two subpopulations were approximately 120° out of phase. Although they were oscillating as separate subpopulations in terms of their phasing, it is assumed that inter-individual coupling remained at its normal level once the cold pulse was terminated. Thus the two subpopulations tended to draw one another back into phase. In the cases shown in Fig. 3, and detailed in Fig. 4, the subpopulations must have been almost equal in size, since the deviations of their periods above and below normal circadian period were about equal. If one subpopulation were larger than the other, it would dominate phase and period control, drawing the smaller subpopulation towards its own phase and period. After 4 to 5 cycles, the two subpopulations merged to produce a rhythmic output normal in shape and period, but with a considerable phase shift in relation to the control.

The data presented in Fig. 3 do not prove that there were more than two oscillators in the population. Differences in cycle amplitude are not necessarily related to the number of oscillators comprising a population. However, differences in the relative contributions to phase control by the subpopulations hypothesized for other records of rhythm splitting strongly suggest that large numbers of oscillators were involved, and that they can be split into unequal subpopulations. The size of subpopulations may also vary during the resynchronizing process as individual oscillators shift from one to the other.

A crucial assumption of the proposed interaction of coupled subpopulations is that

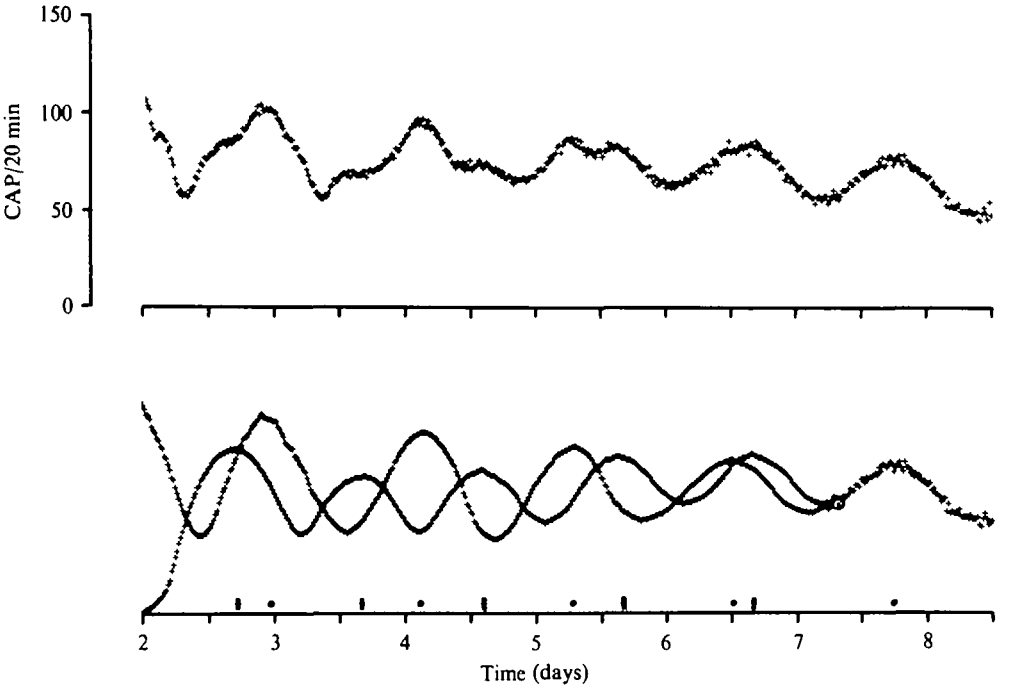

Fig. 4. Detail of rhythm splitting effect. These records are larger scale illustrations of the third and fourth records in Fig. 3, showing details of the shape of the post-pulse CAP frequency curve.

the frequency curves of their outputs did not sum to produce the measured rhythm of CAP frequency. It is postulated that the frequency of total CAP production was determined by whichever subpopulation was firing at the higher frequency. If the frequency curves of the subpopulations had summed, the observed output would have been almost arrhythmic for the first 3 to 4 days after the cold pulse. This is a consequence of the fact that two similar periodic curves tend to cancel one another out when summed at phase angle differences of close to 180°. Furthermore, the maximum CAP frequency due to the summed cycles when they were once more in phase would have been much greater than the frequency of the arrhythmic region produced by cancellation. Neither of these features is seen in the measured data.

CAPs are the result of synchronous discharge of action potentials by the secondary neurones. Synchrony is most likely induced by electrotonic coupling or electrical synapses between these neurones (Jacklet, 1973*b*). Probably there is synchrony of the slow membrane potential oscillation that underlies the activity of bursting pacemaker neurones with good coupling of spike potentials in the total population. The control of rhythmic CAP production by two subpopulations of secondary neurones whose rhythms are out of phase is hypothesized to be as follows. Secondary neurones either fire at the same time as most others or not at all. The subpopulation that has the higher firing frequency thus controls the time of firing for all neurones contributing to the CAPs. The neurones in the subpopulation with the lower frequency fire only when the controlling subpopulation fires but do not contribute to every CAP. The result of this interaction is that the measured CAP frequency follows the frequency of whichever subpopulation is firing at the higher rate.

The contribution of the separate subpopulations to each CAP would be visible only in the amplitudes of individual CAPs, since the larger the number of neurones contributing to a CAP the greater its amplitude. In fact, during the split part of the CAP rhythms, CAP amplitude was extremely irregular, supporting the suggestion of unusual variation in the number of neurones contributing to each CAP. Practical as well as theoretical problems have prevented a detailed numerical analysis of individual CAP amplitudes following a rhythm split.

Several mathematical models have been proposed for populations of coupled oscillators with many of the properties of circadian rhythms (Winfree, 1967; Pavlidis, 1969, 1971; Boon & Strackee, 1976), but very few experimental data have been accumulated to allow precise conclusions about the particular type of oscillator or coupling involved, either in mathematical or biochemical/biophysical terms. Most recently and with considerable support from experimental observations of the activity of nocturnal rodents, Pittendrigh & Daan (1976*a*) have hypothesized control of circadian rhythms by two oscillators or principal groups of oscillators, with opposite dependence of native frequency on light intensity. They suggested two stable phase relations or 'coupling modes', one corresponding to the normal unsplit rhythm, the other to the stably split system in which the two components are 180° out of phase. The split form of rhythm occurred spontaneously in nocturnal rodents kept in constant light of more than 100 lux for 40–60 days.

In the case of the eye clock of *Aplysia*, it has not been possible to determine whether the individual oscillators have circadian or shorter periods. Evidently the oscillators are strongly coupled, and there is no indication of a stable antiphase coupling mode, since the divided subpopulations rapidly drew one another back into phase. The population reduction experiments carried out by Jacklet & Geronimo (1971) resulted in a reduction in range and period, which allows the possibility of individual oscillators of circadian period.

There is much evidence that clocks of different periods may coexist in the same animal, as in circadian and circatidal clocks in marine crustacea (Palmer, 1973; Benson & Lewis, 1976), and *Aplysia* apparently has circadian clocks other than the eyes, since in a few cases removal of the eyes has left the free-running locomotor activity rhythm intact (Lickey *et al.* 1976). Rhythm splitting in rodents and birds, as well as differences in free-running period of various rhythms in humans (Wever, 1973) may reflect the presence of several clocks, each of which could be a population of coupled oscillators such as that localized in the eye of *Aplysia*. Although the clocks in the individual eyes of *Aplysia* are apparently only weakly coupled (Lickey *et al.* 1976), if the clocks of other bilateral organisms are located in the brain (Sokolove, 1975; Stetson & Watson-Whitmyre, 1976), they may be complexly coupled with the possibility of a stable antiphase coupling mode. It seems likely, therefore, that at least in some organisms, there are two levels of endogenously periodic mechanisms; populations of coupled oscillators which form circadian clocks as in the eye of *Aplysia*, and groups of these clocks, with various degrees of coupling, which control the overt rhythms in the behaviour of organisms in their natural environment.

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REFERENCES

ANDERSON, W. W. (1976). Endogenous bursting in *Tritonia* neurons at low temperature. *Brain Res.* **103**, 407–411.

BARKER, J. L. & GAINER, H. (1975*a*). Studies on bursting pacemaker potential activity in molluscan neurons. I. Membrane properties and ionic contributions. *Brain Res.* **84**, 461–477.

BARKER, J. L. & GAINER, H. (1975*b*). Studies on bursting pacemaker potential activity in molluscan neurons. II. Regulation by divalent cations. *Brain Res.* **84**, 479–500.

BENSON, J. A. (1976). Entrainment of a circadian rhythm of activity in an amphipod by skeleton photoperiods: experiments and theory. *J. interdiscipl. Cycle Res.* **8**, 37-45.

BENSON, J. A. & JACKLET, J. W. (1977*a*). Circadian rhythm of output from neurons in the eye of *Aplysia*. I. Effects of deuterium oxide and temperature. *J. exp. Biol.* **70**, 151–161.

BENSON, J. A. & JACKLET, J. W. (1977*b*). Circadian rhythm of output from neurons in the eye of *Aplysia*. IV. A model of the clock: differential sensitivity to light and low temperature pulses. *J. exp. Biol.* **70**, 195–211.

BENSON, J. A. & LEWIS, R. D. (1976). An analysis of the activity rhythm of the sand beach amphipod, *Talorchestia quoyana*. *J. comp. Physiol.* **105**, 339–352.

BOON, P. N. M. & STRACKEE, J. (1976). A population of coupled oscillators. *J. theor. Biol.* **57**, 491–500.

BÜNNING, E. (1958). Uber den Temperatureinfluß auf die endogene Tagesrhythmik, bei *Periplaneta americana*. *Biol. Zbl.* **77**, 141–152.

BÜNNING, E. (1973). *The Physiological Clock*. London: English Universities Press.

CARPENTER, D. O. (1967). Temperature effects on pacemaker generation, membrane potential, and critical firing threshold in *Aplysia* neurons. *J. gen. Physiol.* **50**, 1469–1484.

HESSE, M. (1974). Der einfluss niedriger temperatur auf die endogene rhythmik der produktionsfähigkeit von *Chlorella*. *Z. Pflanzenphysiol.* **71**, 428–436.

JACKLET, J. W. (1969). Electrophysiological organization of the eye of *Aplysia*. *J. gen. Physiol.* **53**, 21–42.

JACKLET, J. W. (1971). Circadian rhythm in the activity of a population of coupled oscillators. *Fedn. Proc. Am. Socs exp. Biol.* **30**, 551.

JACKLET, J. W. (1973*a*). Neuronal population interactions in a circadian rhythm of *Aplysia*. In *Neurobiology of Invertebrates* (ed. J. Salanki), pp. 363–380. Budapest: Hungarian Academy of Sciences.

JACKLET, J. W. (1973*b*). The circadian rhythm in the eye of *Aplysia*. Effects of low calcium and high magnesium. *J. comp. Physiol.* **87**, 329–338.

JACKLET, J. W. (1974). The effects of constant light and light pulses on the circadian rhythm in the eye of *Aplysia*. *J. comp. Physiol.* **90**, 33–45.

JACKLET, J. W. (1976*a*). Circadian rhythms in the nervous system of a marine gastropod, *Aplysia*. In *Biological rhythms in the marine environment* (ed. P. J. DeCoursey), pp. 17–31. Columbia, S.C.: University of South Carolina Press.

JACKLET, J. W. (1976*b*). Dye marking neurons in the eye of *Aplysia*. *Comp. Biochem. Physiol.* **55A**, 373–377.

JACKLET, J. W. & GERONIMO, J. (1971). Circadian rhythm: population of interacting neurons. *Science, N.Y.* **174**, 299–302.

LICKEY, M. E., BLOCK, G. D., HUDSON, D. J. & SMITH, J. T. (1976). Circadian oscillators and photoreceptors in the gastropod, *Aplysia*. *Photochem. Photobiol.* **23**, 253–273.

NJUS, D., MCMURRAY, L. & HASTINGS, J. W. (1977). Conditionality of circadian rhythmicity: synergistic action of light and temperature. *J. comp. Physiol.* **117**, 335-344.

PALMER, J. D. (1973). Tidal rhythms: the clock control of the rhythmic physiology of marine organisms. *Biol. Rev.* **48**, 377–418.

PAVLIDIS, T. (1969). Populations of interacting oscillators as circadian clocks. *J. theor. Biol.* **33**, 319–338.

PAVLIDIS, T. (1973). *Biological oscillators: their mathematical analysis*. 207 pp. New York: Academic Press.

PITTENDRIGH, C. S. (1960). Circadian rhythms and the circadian organization of living systems. *Cold Spring Harb. Symp. quant. Biol.* **25**, 159–184.

PITTENDRIGH, C. S. & DAAN, S. (1976*a*). A functional analysis of circadian pacemakers in nocturnal rodents. I. The stability and lability of spontaneous frequency. *J. comp. Physiol.* **106**, 223–252.

PITTENDRIGH, C. S. & DAAN, S. (1976*b*). A functional analysis of circadian pacemakers in nocturnal rodents. V. Pacemaker structure: a clock for all seasons. *J. comp. Physiol.* **106**, 333–355.

SALANKI, J., VADASZ, I. & VERO, M. (1973). Temperature dependence of the activity pattern in the Br-type cell of the snail *Helix pomatia* L. *Acta physiol. hung.* **43**, 115–124.

SENER, R. (1972). Site of circadian rhythm production in *Aplysia* eye. *The Physiologist* **15**, 262.

SOKOLOVE, P. G. (1975). Localization of the cockroach optic lobe circadian pacemaker with microlesions. *Brain Res.* **87**, 13–21.

STETSON, M. H. & WATSON-WHITMYRE, M. (1976). Nucleus suprachiasmaticus: the biological clock of the hamster? *Science, N.Y.* **191**, 197–199.

STRUMWASSER, F. (1973). Neural and hormonal factors in the temporal organization of behaviour. *The Physiologist* **16**, 9–42.

WAGNER, R. (1963). Der einfluss niedriger temperatur auf die phasenlage der endogentagesperiodischen blattbewegungen von *Phaseolus multiflorus*. *Z. Bot.* **51**, 179–204.

WEVER, R. (1973). Internal phase angle differences in human circadian rhythms: causes for changes and problems of determinations. *Int. J. Chronobiol.* **1**, 371–390.

WILSON, W. A. & WACHTEL, H. (1974). Negative resistance characteristic essential for the maintenance of slow oscillations in bursting neurons. *Science, N.Y.* **186**, 932–934.

WINFREE, A. T. (1967). Biological rhythms and the behavior of populations of coupled oscillators. *J. theor. Biol.* **16**, 15–42.