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

I. EFFECTS OF DEUTERIUM OXIDE AND TEMPERATURE

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SUMMARY

1. The compound action potential (CAP) output from the isolated eye of *Aplysia*, in darkness at constant temperature, exhibits a circadian rhythm of CAP frequency and a circadian rhythm of CAP amplitude when recorded in culture medium for up to two weeks.

2. Deuterated culture medium lengthened the period of the CAP frequency rhythm linearly from 26·7 h in normal culture medium to 33·7 h in 50% D_2O culture medium. In 60% D_2O culture medium, the rhythm of CAP frequency was abolished after a single cycle.

3. Lowering temperature had a direct inhibitory effect on the CAP generating mechanism, so that CAP production was virtually abolished below 7 °C.

4. Lowering temperature decreased the amplitude and increased the period of the rhythm, and in eyes maintained at 9·5 °C the rhythm damped out after several cycles. Between 15 and 22·5 °C there was almost complete temperature compensation (Q_{10} 1·07).

5. It is suggested that the CAP generating cells of the eye may be similar in mechanism to central endogenous bursters in *Aplysia* and other gastropods, and that the circadian rhythm of CAP frequency and CAP burst frequency may be due to a clock-controlled change in frequency of a membrane potential oscillation in the electrotonically coupled secondary neurones.

INTRODUCTION

The tectibranch mollusc, *Aplysia californica*, possesses cerebral eyes which, when isolated at constant temperature and in darkness, produce an output of compound action potentials (CAPs) that exhibit pronounced circadian rhythms of frequency and amplitude change. The microstructure and electrophysiology of the eye, and the properties of the circadian rhythm have been examined extensively by Jacklet (1969, 1973, 1974*a*, 1976*a*). He has shown that the eye is composed of a central spheroidal lens which is surrounded by a retina containing three cell types. There are about 3600 receptor cells which interdigitate with pigmented support cells. The eye also contains about 1000 secondary cells, concentrated in clusters towards the base of the eye, where

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the axons of both receptor and secondary cells form a neuropil and then the optic nerve. The optic nerves lead to the cerebral ganglion, and it is from the severed optic nerve that the synchronized action potentials (CAPs) from the eye are recorded.

In this paper the quantitative properties of the free-running circadian rhythm of CAP frequency are described. This is followed by the results of investigations intended to characterize in detail the responses of the CAP generating mechanism and of the circadian clock to constant temperature level, contrasted with the effects of substitution of D_2O for H_2O in the culture medium. The quantitative analysis of the free-running rhythm, and of temperature effects on the rhythm and CAP generating mechanism form the basis of the experiments described in subsequent papers on the mechanism of the circadian clock or clocks, and its input/output relations. A preliminary report has been published (Benson & Jacklet, 1976).

It is important to distinguish clearly between the immediate effects of these treatments on the action-potential generating cells and the long-term effects on the circadian clock, measured in terms of changes in phase and period of the rhythm. The bursts of CAPs are the 'effectors' or 'hands of the clock' and can be perturbed independently of any effects on the underlying clock mechanism.

The rhythm of CAP frequency is particularly amenable to investigation of the effects of various external perturbations, since the entire oscillation can be measured with considerable precision. Furthermore, the eye is probably the only circadian clock that remains functional when removed from the whole organism, so that it should prove an ideal system for biochemical experimentation (Jacklet, 1977). The ultimate aim of the present series of experiments is to provide a foundation for the biochemical/ biophysical analysis of this circadian clock.

It is characteristic of circadian rhythms, even in poikilotherms, that their free-running period varies only slightly through a wide range of constant temperatures (Sweeney & Hastings, 1960). Q_{10} values are mostly between about 0·8 and 1·2, in contrast to values between 2 and 3 for many other biological processes. The Q_{10} for a circadian clock is rarely exactly 1·0, and may be less than 1·0, which suggests that circadian clocks are not temperature independent, but rather that they are temperature compensated, and may over-compensate in some conditions. This view is supported by the observation that circadian clocks can be entrained by temperature cycles and phase shifted by temperature steps (Zimmerman, Pittendrigh & Pavlidis, 1968).

Spontaneous activity in molluscan central endogenous bursting neurones which may be similar to the secondary neurones in the eyes of *Aplysia* has been shown to be temperature sensitive. Bursting is reduced or stopped when the temperature is reduced below 8–12 °C in cultured bursting neurones such as R15 in *Aplysia* (Barker & Gainer, 1975), LP12 and LP13 in *Tritonia* (Anderson, 1976), and cell 11 in *Otala* (Barker & Gainer, 1975). When the normally non-bursting cell R2 of *Aplysia* is cultured at 26–28 °C, bursting occurs. The bursting activity of neurones in the CNS of *Aplysia* depends on the presence of a negative resistance characteristic in the current–voltage curve, this characteristic being present only within particular temperature ranges (Wilson & Wachtel, 1974).

The effects of deuterium oxide (D_2O) have been measured in several circadian systems, and in all the organisms tested the addition of D_2O to the culture medium or drinking water resulted in an increase in free-running period, or in an increase in

the phase lag of the entrained rhythm behind the entraining environmental cycle. It has been suggested that the effect of D_2O is equivalent to diminishing the apparent temperature of a biological system (Lwoff & Lwoff, 1961), although not all biological reactions are slowed down by the presence of D_2O (Schimmel, 1975). The temperature equivalence hypothesis has been tested for circadian systems and rejected by McDaniel, Salzman & Hastings (1974) in *Gonyaulax*, and Caldarola & Pittendrigh (1974) in *Leucophaea*.

Chen, von Baumgarten & Harth (1973) tested the effects of 50% D_2O on a number of endogenously bursting neurones in *Aplysia*. They found that spontaneous action-potential production was suppressed in most cells, and that the slow membrane-potential oscillation was absent. Irregular spiking reappeared in some cells after 70–200 min in 50% D_2O, and the addition of 10 mM glucose shortened the silent period by half and also increased the regularity of firing.

The results for the eye rhythm presented here support the suggestion that the effects of reduced temperature are not the same as those of D_2O treatment. Both the circadian clock and the CAP generating mechanisms appear to be affected in different ways by cooling and D_2O.

MATERIALS AND METHODS

Aplysia californica were obtained from Pacific Biomarine (Venice, Ca.) and kept in LD 13:11 in Instant Ocean tanks at 15 °C, prior to experimentation. The animals were opened ventrally, and after the eyes had been dissected free, the optic nerves were severed at the cerebral ganglion. The eyes were placed in a recording chamber and the optic nerves were drawn into tubing electrodes (PE10). The plexiglass recording chamber contained 125 ml of culture medium, and was covered with a lid to reduce evaporation. The holes by which the tubing electrodes passed through the lid allowed oxygen exchange between the small volume of air contained inside the chamber, and the outside. Electrical activity in the tubing electrodes was led off via silver chloride/silver wire, amplified, and recorded on a Grass polygraph. The recording chamber was housed in a light-proof box to maintain constant darkness or controlled light exposure. Temperature control was provided by the circulation of water at constant temperature from a water bath through a glass loop inside the recording chamber, and temperature was measured by a thermistor probe suspended in the culture medium. Normal recording temperature was 15 °C.

The culture medium consisted of 90% artificial seawater (ASW) and 10% nutrient mixture. ASW composition in mmol/l was: NaCl, 425; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 22; $MgSO_4$, 26; $NaHCO_3$, 2·5. Twenty-five ml of nutrient mixture contained: 2·5 ml MEM vitamin solution, 2·5 gm of dextrose, 5 ml of MEM amino acids, 2·5 ml of non-essential amino acids, 2·5 ml of L-glutamine (200 mM), 2·5 ml of penicillin–streptomycin solution, 6·7 ml of 7·5% $NaHCO_3$, 2·7 ml of 0·5 N-NaOH and ASW. The final culture medium was filtered (0·22 μm Millipore) and the pH was adjusted to 7·8. For D_2O solutions, D_2O replaced distilled H_2O in the composition of ASW.

All CAP frequency measurements were made in terms of the number of CAPs per 20 min interval. Individual periods were measured between centroid points which were usually the same whether calculated as median count (Winfree, 1971) or by the formula of Enright (1971). Average periods for several cycles were calculated using

periodogram and autocorrelation analysis. Periodograms give precise measurements of average period but often display spurious peaks (Enright, 1965), although it is possible to introduce a confidence limit for real periods (Dörrscheidt & Beck, 1975). Auto-correlations are less precise but show significant peaks only (Binkley, 1973). The combination of these two methods of analysis provides a powerful tool in circadian rhythms frequency analysis. An example of periodogram and autocorrelation analysis of the output from the eye of *Aplysia* is given in Fig. 3.

<div align="center">RESULTS</div>

<div align="center">*CAP output in constant darkness at 15 °C*</div>

Jacklet (1969) first showed that the frequency of CAP output from the isolated eye of *Aplysia* varies rhythmically with a free-running period of 24 h or less when the eye is cultured in ASW, and about 27 h in culture medium. He also observed that the eye output shows a circadian rhythm of CAP amplitude modulation as well as a circadian rhythm of CAP frequency (Jacklet, 1974b).

Improved techniques have made it possible to obtain routinely continuous records of 11 cycles of the circadian rhythm in culture medium. The use of data from experiments in which the CAP frequency did not reach zero at the lowest point in each cycle has allowed the details in shape of the amplitude modulation rhythm to be determined completely.

Fig. 1 shows a typical recording of CAP output from an isolated eye. As the frequency of CAPs increased under the control of the circadian clock, they tended to occur in groups or 'bursts', with a more or less constant number of CAPs per burst over any given 20 min interval. As the frequency of bursts increased, the number of CAPs per burst also increased and there was a concurrent increase in the amplitude of individual CAPs. At very high CAP frequencies (not shown in Fig. 1), CAPs were no longer grouped in bursts, but occurred at regular, short intervals.

The circadian rhythms of CAP amplitude change and CAP frequency recorded from an isolated eye in constant darkness at 15 °C are represented in Fig. 2. The amplitude rhythm was determined by choosing 'by eye' an average CAP for each 20 min interval, and plotting its amplitude against an arbitrary scale (upper curve in Fig. 2). The wave-form of this amplitude rhythm is distinctly biphasic. A short duration peak occurs initially during the rising phase of the CAP frequency rhythm, but fades out after a few cycles. The broad peak of amplitude modulation occurs during the falling phase of the CAP frequency rhythm with a sharp cut-off at the end of the falling phase.

The number of CAPs per 20 min interval varies from 2 to 93 in the CAP frequency record illustrated by the lower curve in Fig. 2, with an average circadian period of 26·7 h, as shown by the periodogram and autocorrelation in Fig. 3. Centroid points, calculated by the methods described above, are marked in the CAP frequency record in Fig. 2, and the mean and standard deviations of the intercentroid periods for four records are plotted in Fig. 4. After a single transient cycle, the rhythm free-ran, slowly damping out as shown in Fig. 2, and with a general increase in period (Fig. 4), during the course of each experiment.

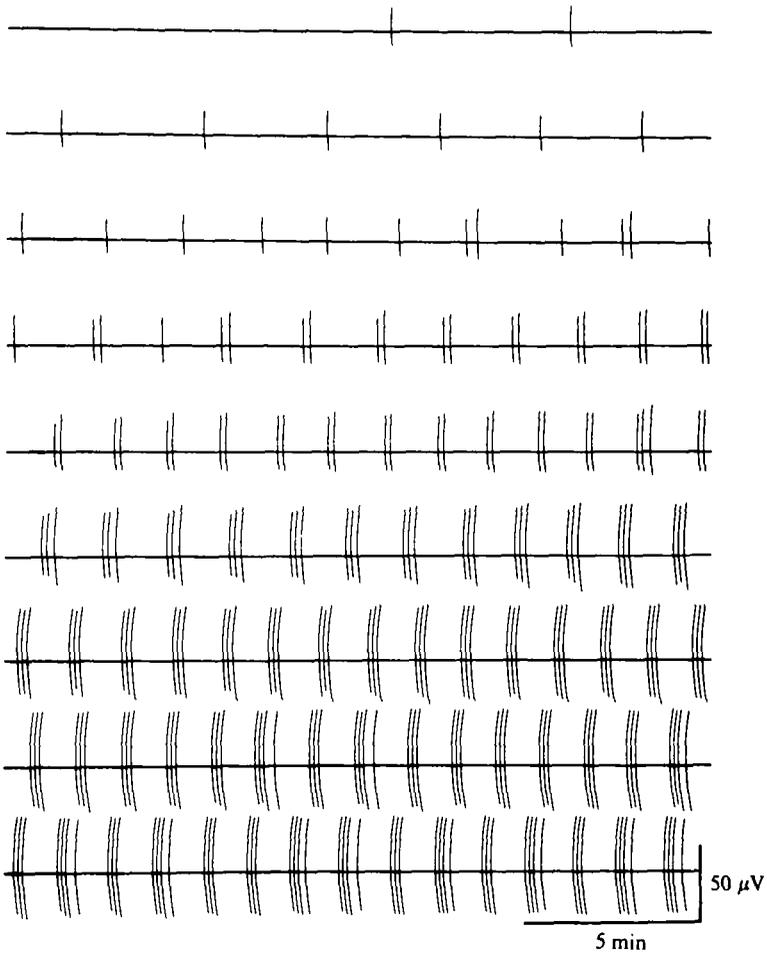It has been confirmed that there is a clear circadian rhythm of burst frequency

Fig. 1. Continuous recording of CAPs generated by an isolated eye in culture medium in darkness at 15 °C. The amplitude of the CAPs, the frequency of the CAPs, the frequency of the bursts of CAPs, and the number of CAPs per burst change with time under the control of a circadian clock.
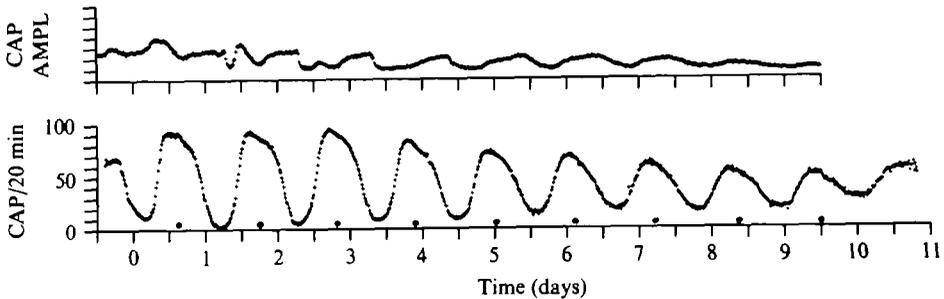
Fig. 2. Circadian rhythms of CAP amplitude and frequency recorded in constant conditions. The upper record shows CAP amplitude in arbitrary units plotted against time, and the lower record is the CAP frequency for the same eye plotted as number of CAPs per 20 min interval, on the same time scale. The centroid point is indicated for each cycle of the frequency rhythm.
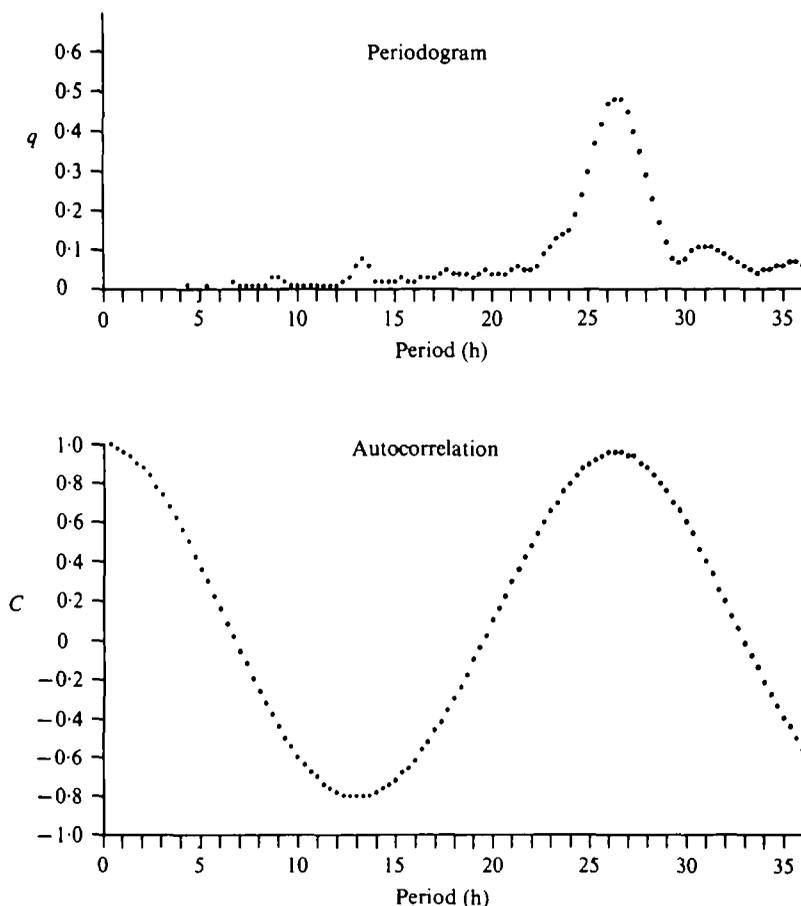
Fig. 3. Periodogram and autocorrelation analysis of the free-running circadian rhythm of CAP frequency illustrated in Fig. 2. q is a measure of the similarity between the observed record and a reference value that depends on the trial period being tested. C is the correlation coefficient giving the degree of correlation between the observed record and values from the observed record at various trial periods. Numerical values of q and C were calculated to determine periods precisely.

(Fig. 5) in phase with the CAP frequency and amplitude rhythms. As the frequency of bursts increased, the number of CAPs per burst also increased so that the amplitude of the CAP frequency rhythm is much greater than that of the burst rhythm, as illustrated in Fig. 5.

Effect of deuterium oxide on the CAP frequency rhythm

The CAPs recorded from eyes cultured in medium containing various concentrations of D_2O were indistinguishable from those of eyes in the normal medium (Fig. 6), in terms of CAP shape, CAP amplitude, and frequencies recorded, but regularity of firing and burst formation was generally less in D_2O culture medium.

A series of records of CAP frequency for eyes in control conditions, and in 15, 30, 40 and 50% D_2O culture medium is shown in Fig. 7. The centroid points are indicated to show the increase in period with increase in D_2O concentration. The influence of D_2O on the period appears to take full effect within 36 h, since the slight changes in
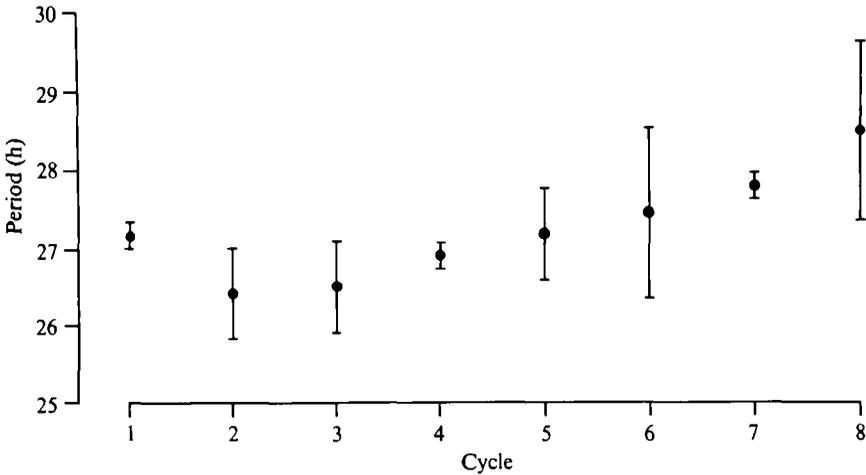
Fig. 4. Change in period during the course of a free-run. The means and standard deviations of the intercentroid periods for four free-running rhythms are plotted against the cycle from which each period measurement was taken. After a single post-dissection transient, the periods of the rhythms show a general increase of about 2 h over 7 cycles.
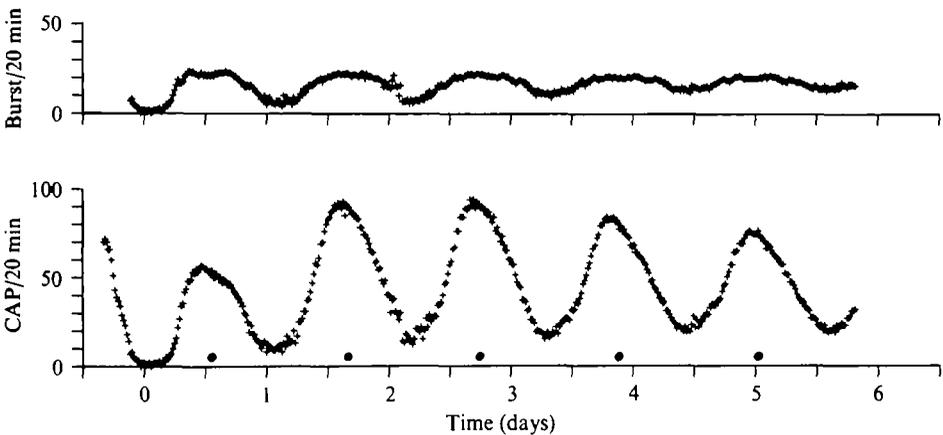

Fig. 5. Circadian rhythm of burst frequency recorded from an eye in constant darkness at 15 °C. The upper record illustrates the circadian rhythm of burst frequency, where a burst can be one or more CAPs, and the lower record shows the CAP frequency rhythm for the same eye.

period during the course of each experiment are not significantly different from the changes which occur in the control.

The average periods for entire records of CAP output from individual eyes, as measured by periodogram and autocorrelation, in the various concentrations of D₂O are plotted in Fig. 8. There is a linear increase in period from about 26·7 h, in controls, to 33·7 h in 50 % D₂O.

When eyes were cultured in medium containing 60 % D₂O, the rhythm of CAP frequency was abolished after a single cycle (Fig. 9). The frequency of CAP output ▶mained at a high level so that it appears that the circadian clock was stopped at its uppermost phase point, or the CAP generating mechanism was uncoupled from clock
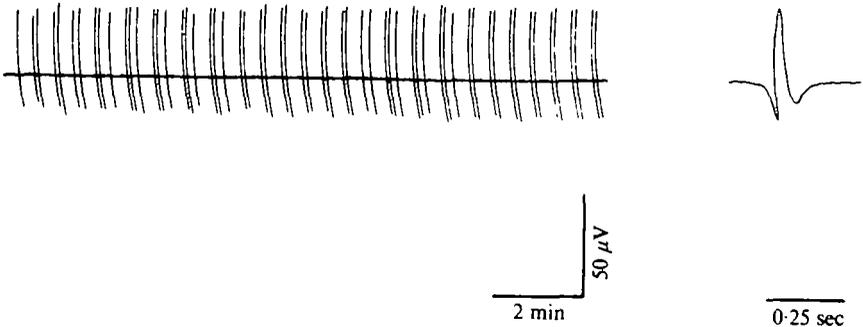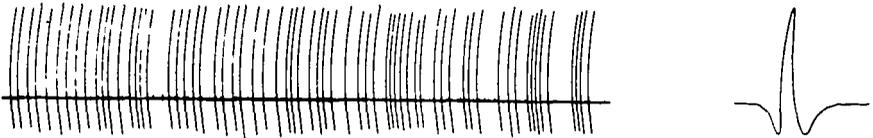
(A)   Normal medium

2 min   0·25 sec

(B)   30 % D$_2$O medium

Fig. 6. Records of CAPs from eyes in normal medium and in 30 % D$_2$O medium. Bursting is generally less distinct in 30 % D$_2$O, but CAP production is otherwise unaffected. These records are from different eyes and from different parts of the circadian cycle so that their CAP frequencies are different.

control. There was a slight decline in the frequency of CAP output during the remainder of the recording period, but this decline was not much greater than the decline in peak CAP frequency counts seen in controls. A similar set of results was obtained from eyes cultured in 70 % D$_2$O. Individual CAPs appeared normal in all D$_2$O experiments. No attempt was made to return eyes to normal culture medium after they had been cultured in D$_2$O medium.

Effect of temperature on the CAP frequency rhythm

Cooling the isolated eye resulted in a decrease in frequency of CAPs as shown for two eyes in Fig. 10. These are continuous records of CAP output, each lasting 1·7 h, during which time the culture medium was cooled from 15·0 to 5·5 °C. This decrease in CAP frequency is similar in form to changes in CAP frequency under the influence of the circadian clock (as illustrated in Fig. 1 for frequency increase). The frequency of bursts and the number of CAPs per burst decreased simultaneously.

Sensitivity of the CAP generating mechanism to temperature differed slightly from one eye to another. In most experiments, all activity ceased at about 8 °C, but in some preparations normal CAPs at very low frequency were observed at 6 °C, especially after the eye had been at this temperature for 24 h or more. Cold pulses of 2 h duration at 6 °C suppressed CAP production but left the phase and period of the rhythm virtually unaffected.

The influence of constant temperature level on the period of the CAP frequency rhythm is shown in Fig. 11. The four records, with centroid points marked, show th
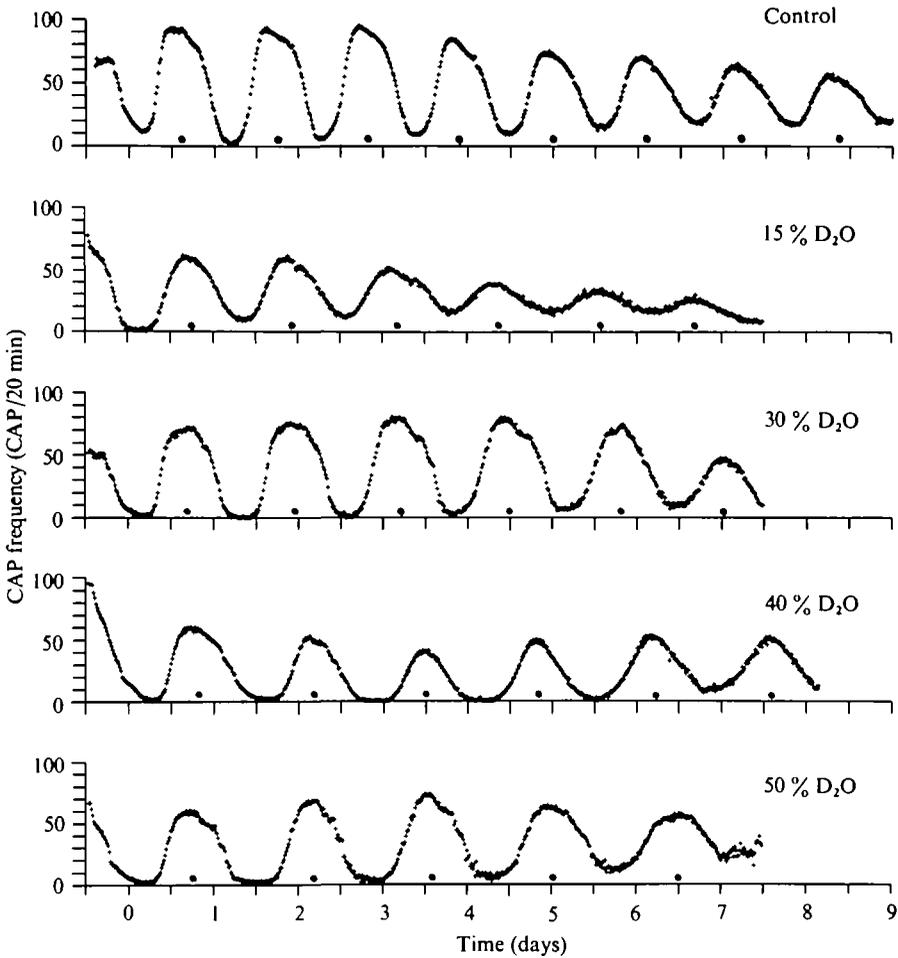the amplitude of the rhythm decreased, and the period increased, as temperature wa

Fig. 7. Records of the CAP frequency rhythm from eyes in culture medium containing various concentrations of D₂O. The eyes were placed directly into the D₂O-containing medium following dissection. Free-running period increases with increasing concentration of D₂O. Control as in Fig. 2.

lowered. At the lowest temperature shown, 9·5 °C, the frequency of CAPs was low, but four cycles of the rhythm are sufficiently pronounced for period measurements to be made. Rhythms of CAP output from eyes kept at 9 °C damped out after one or two cycles. At high temperatures the amplitude of the rhythm was increased, although the minimum frequency was still at or near zero.

Period measurements for various temperatures ranging from 9·5 to 22 °C are shown in Fig. 12. The relationship between temperature and period is non-linear, with a Q_{10} of 1·07 between 15 and 22·5 °C, but with greater temperature dependence below 15 °C, the Q_{10} between 9·5 and 15 °C being 1·35. The average Q_{10} for the range observed is 1·20 ($Q_{10} = [X_{T+\Delta T}/X_T]^{10/\Delta T}$), where X_T is rate at temperature T, and $X_{T+\Delta T}$ is rate at temperature $T + \Delta T$).
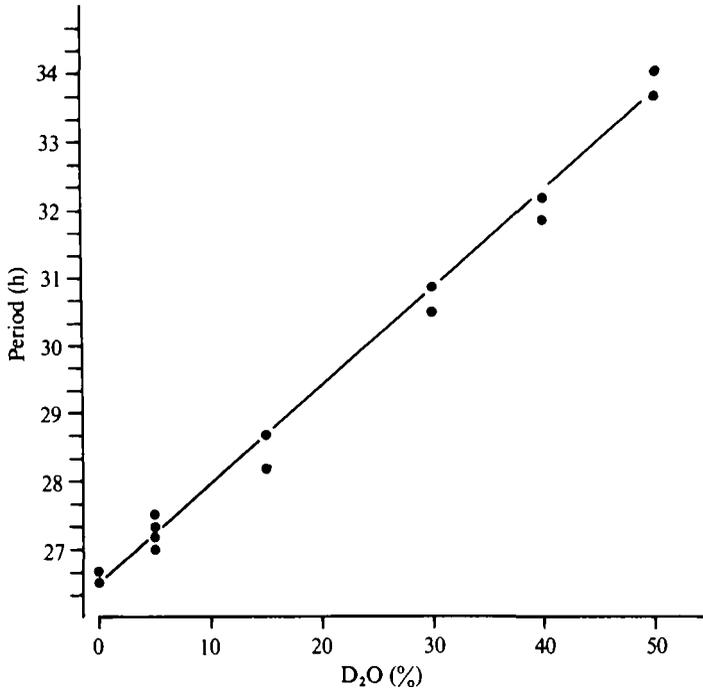
Fig. 8. Change in circadian period with concentration of D_2O in the culture medium. Periods obtained by periodogram analysis of complete experimental records such as those illustrated in Fig. 7 are plotted against the concentration of D_2O in the culture medium. Each point on the graph represents the average period for one experimental record.
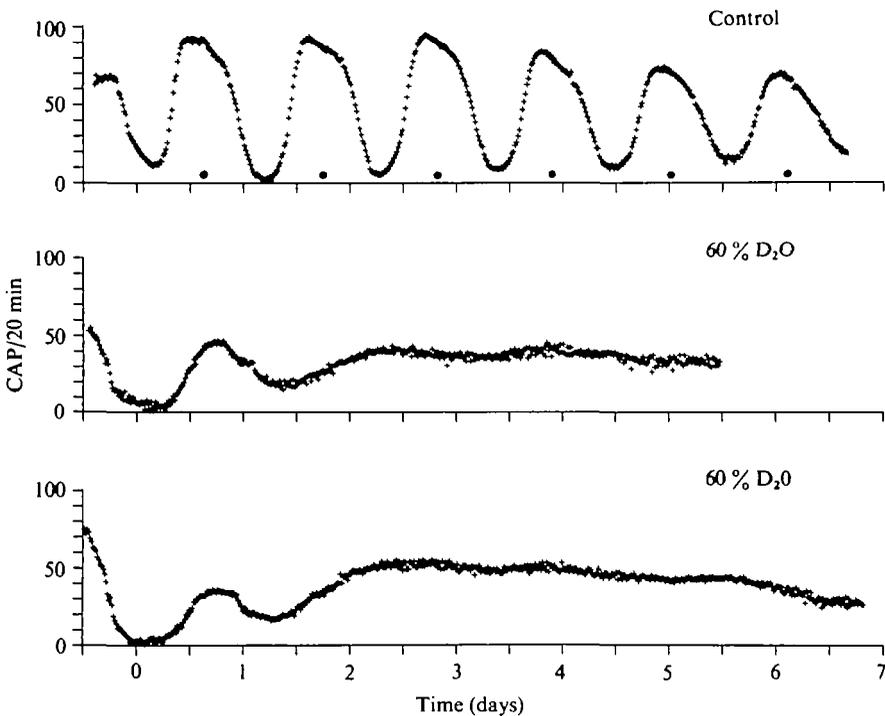

Fig. 9. Effect of 60 % D_2O on the CAP rhythm. After a single cycle, the rhythm was eliminated and the CAP frequency remained at a high level. Control as in Fig. 2.
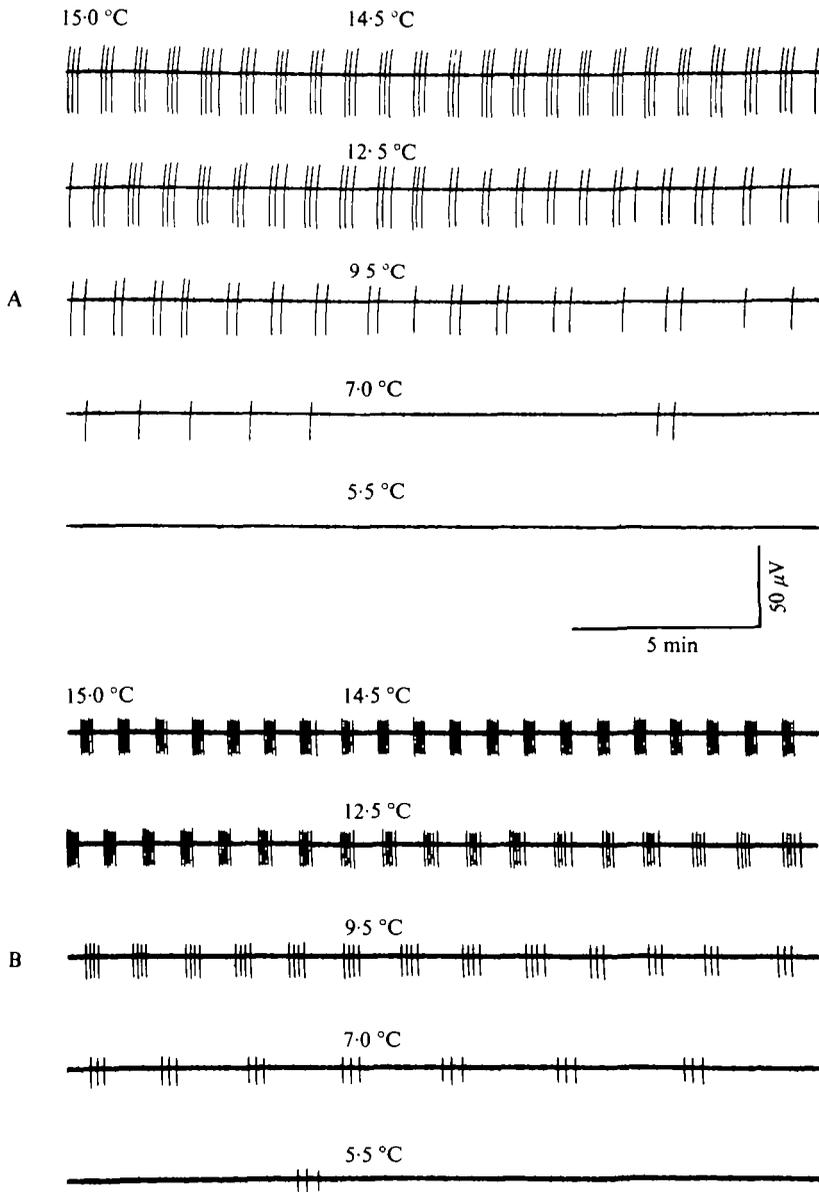
Fig. 10. Effect of lowering temperature on the CAP frequency. Two continuous records of CAP output from different eyes subjected to gradual, uninterrupted temperature decrease indicate that cooling decreases the burst frequency and the number of CAPs per burst.

DISCUSSION

The normal pattern of bursting of CAPs suggests that the cells in the eye of *Aplysia* which produce these synchronized action potentials are similar in mechanism of endogenous activity to other bursting neurones in the CNS of *Aplysia*, *Tritonia* and *Otala*. Intracellular recordings from secondary cells in the retina show a strong
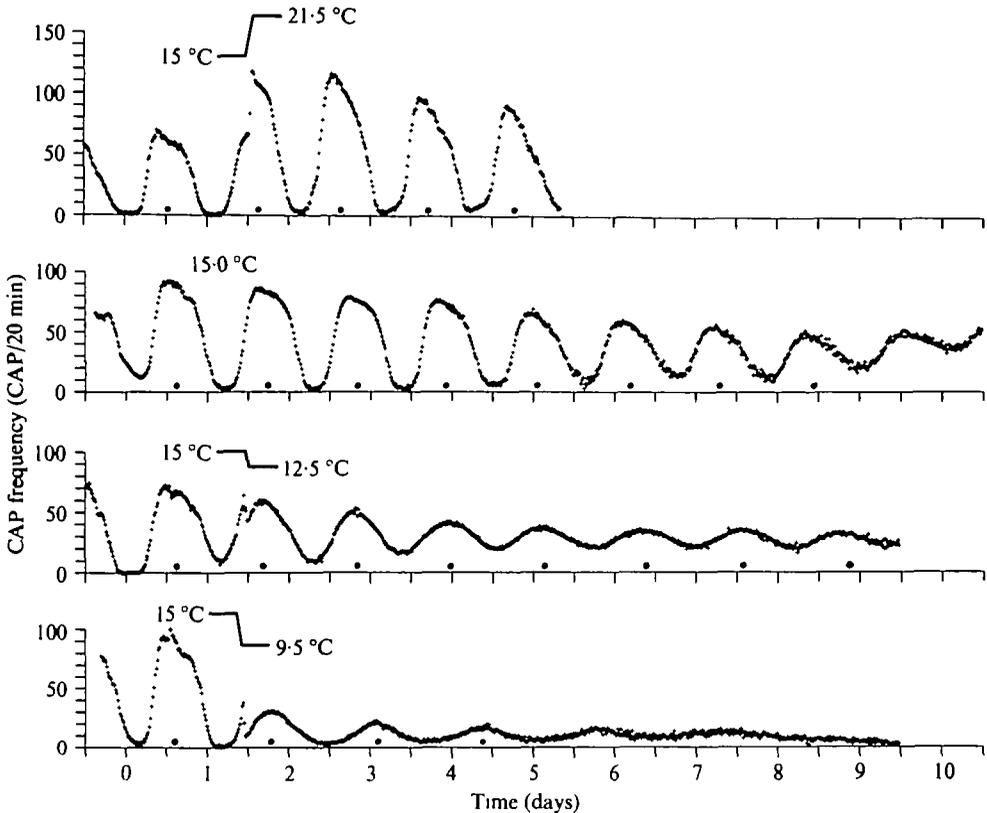
Fig. 11. Effects of temperature on the period and amplitude of the CAP frequency rhythm. After one cycle of the rhythm, the temperature was changed from 15 °C to a new constant value. The amplitude of the rhythm was altered both by the direct effect of temperature on the CAP-generating mechanism and by the damping effects of low temperature on the clock. At 9·5 °C, the rhythm eventually damped out entirely.

correlation between their action potentials and CAPs measured simultaneously in the optic nerve, and a regular membrane potential oscillation has been recorded intra-cellularly from secondary cells of the eye (Jacklet, 1973, 1976a, 1976b). It is therefore likely that an electrotonically connected population of secondary cells produces the compound action potentials (Jacklet, 1971).

Clock regulation of the frequency of CAP production by the secondary neurones may be at the level of a cell membrane potential oscillation, with bursts of action potentials occurring during maximum depolarization, as in endogenous bursting neurones. Under clock control, the frequency of the membrane potential oscillation might change with a circadian periodicity, with the more rapid depolarization at higher frequencies producing a larger number of action potentials at peak depolariza-tion. This would account for the circadian rhythms of both burst and CAP frequencies.

The rhythm of CAP amplitude modulation probably reflects a change in the number of secondary cells contributing to the compound action potential (Jacklet, 1974a). The separate peaks in the amplitude rhythm may reflect a bimodality in the clock oscillation, but since the CAP frequency rhythm is unimodal it seems more likely that they represent subpopulations that are out of phase during the first few cycles.
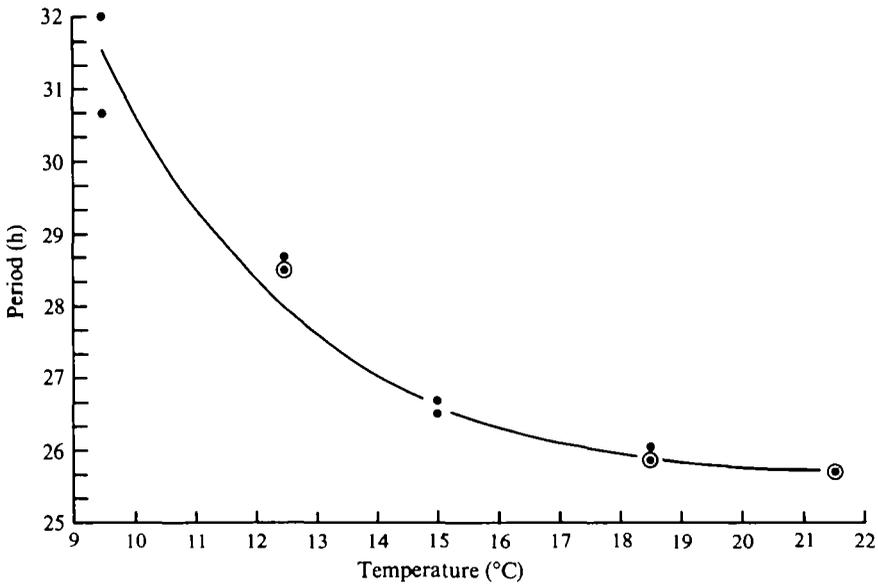
Fig. 12. Periods obtained by periodogram analysis of complete experimental records such as those illustrated in Fig. 11 are plotted against the temperature of the culture medium. Each point on the graph represents the average period of one experimental record. Where points coincide, concentric circles are used.

Possibly the smaller peak disappears as the members of the smaller subpopulation are drawn into the larger and therefore more stable subpopulation.

A reduction in temperature decreased and finally abolished the output of CAPs from the isolated eye. It is suggested that this decrease in CAP frequency in response to lowered temperature is a result of the direct influence of temperature on the CAP generating mechanism, and thus should not be confused with the effect of temperature change on the circadian clock mechanism. The response of the CAP generating mechanism to temperature change was immediate, whereas longer periods of cooling were required to perturb the clock mechanism. Decrease in temperature from 15 °C caused a reduction in number of CAPs per burst as well as an increase in the interburst interval. This is similar to the change in activity of single endogenous bursting neurones measured by intracellular recording during decrease in temperature. However, Anderson (1976) observed, in *Tritonia*, a decrease in interburst interval as the number of action potentials per burst decreased with decrease in temperature. The extra-cellular recordings from the optic nerve thus probably reflect a decrease in frequency of slow membrane potential oscillations in the secondary cell population, in response to decrease in temperature.

Barker & Gainer (1975) have suggested that the effect of temperature on the slow membrane potential oscillation in endogenous bursting neurones may be mediated in part by the temperature dependence of the regulatory role of Ca^{2+}. Since the general form of the change in CAP frequency with temperature is very similar to the change in CAP frequency under the control of the circadian clock, it is possible that the circadian clocks exert control over the CAP frequency via the same regulator.

Deuterium oxide in the culture medium left the CAPs essentially unaffected, but had a marked effect on the period of the circadian rhythm of CAP frequency. Periods were increased linearly with increase in D_2O concentration, with a maximum increase of 7 h in 50% D_2O. The increase in period in 30% D_2O culture medium was 15%, which is more than twice the increase measured in other organisms. After administration of 30% D_2O, Suter & Rawson (1968) found that the free-running period increased by 6·45% in *Peromyscus*; Palmer & Dowse (1969) reported a 6·0% increase for the waxbill, *Estrilda*; Enright (1971) found a 6·33% increase in the tidal rhythm of the isopod, *Excirolana*; Bünning & Baltes (1963) measured a 6·6% increase in the bean leaf movement rhythm; and Dowse & Palmer (1972) found that 30% D_2O gave a 7·4% increase in free-running period in the mouse. It is possible that diffusion of D_2O into the isolated eye from the surrounding culture medium is more complete than the penetration of D_2O into the clock mechanism when whole organisms are tested.

When eyes were cultured in 60 or 70% D_2O, the rhythm of CAP frequency was abolished after a single cycle. There are at least two possible explanations for this. First, the clock may have stopped after one cycle at its uppermost phase point. In this case the CAP output would have reflected exactly the state of the underlying circadian clock. Secondly, a high concentration of D_2O may have uncoupled the CAP production mechanism from the clock, leaving the secondary cell population to fire freely at a high frequency. Chen *et al.* (1973) showed that the effect of D_2O on endogenous bursting neurones elsewhere in the CNS of *Aplysia* was to depress spontaneous activity. Furthermore, at lower concentrations of D_2O, there was no evidence of partial uncoupling of the CAP production mechanism from the clock; the amplitude of the rhythm, from near zero to almost a hundred CAPs per 20 min interval, in concentrations of 50% and below, is normal. Although it thus seems likely that the 60 and 70% concentrations of D_2O did in fact stop the circadian clock at its uppermost phase point, the possibility of D_2O-induced uncoupling cannot be dismissed. A series of experiments in which the D_2O was washed out at various projected phases might show whether uncoupling or clock-stopping was involved.

Temperature reduction resulted in a decrease in amplitude of the rhythm, and a lengthening of period, with apparent damping out of the rhythm at temperatures of about 9 °C. Part of the decrease in amplitude was clearly due to the direct effect of cooling on the CAP production mechanism, as can be seen in Fig. 11 where CAP frequency drops sharply during the downward temperature step. However, during the time that the temperature was held constant at 9·5 °C, the peak values of CAP frequency diminished with each cycle of the rhythm. This is evidence that the clock oscillation did actually damp out during the course of the experiment. After several days at 9·5 °C, the rhythm appeared to be completely abolished. It is suggested that the circadian clock stops at low temperatures. Results of experiments testing this hypothesis are given in the second paper in this series.

The free-running period of the CAP frequency rhythm was increased with decrease in temperature. The Q_{10} between 9·5 and 22 °C was 1·20, which is similar to the values obtained for other circadian systems (Sweeney & Hastings, 1960). It appears that temperature compensation in the *Aplysia* eye clock is more complete at temperatures of 15 °C and above where the Q_{10} was 1·07. Temperature compensation is an essential feature of any reliable biological clock and must have been strongly selected for in

most systems, so that very little can be concluded about the nature of the clock mechanism from Q_{10} values, particularly within naturally occurring temperature ranges (Bünning, 1974). More important conclusions regarding the nature of the clock mechanism will be drawn from the results of the application of stimuli such as are not encountered by the organism in its natural environment. Results of experiments will be given in a subsequent paper (Benson & Jacklet, 1977*b*), in which temperature perturbations were applied during selected phases of the CAP frequency rhythm. The next paper in this series (Benson & Jacklet, 1977*a*) will describe the clock-stopping and rhythm-splitting actions of cold pulses of temperatures below the natural range for *Aplysia*.

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