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

IV. A MODEL OF THE CLOCK: DIFFERENTIAL SENSITIVITY TO LIGHT AND LOW TEMPERATURE PULSES

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SUMMARY

1. A relaxation oscillator, feed-back model for the circadian clock in the eye of *Aplysia* is proposed to account for the experimental findings described earlier. Further data on the effects of light pulses and temperature pulses are reported here to test the hypothesis that light and temperature perturb the clock oscillation at different points in the feed-back loop.

2. The rising phase of the CAP frequency rhythm is postulated to be due to an energy-requiring, synthesis process, and the falling phase to a passive, diffusional process. Synthesis produces a substance, *C*, which controls CAP frequency, and the level of which oscillates about a reference level, *R*.

3. The synthesis phase of the oscillation is suggested to be temperature compensated from about 9 °C to at least 22·5 °C. Cooling the eye to 6 °C for long periods therefore inhibits synthesis so that the clock eventually stops at its lowest phase point.

4. 12 h cold pulses of 4 °C applied during the rising phase of the rhythm (i.e. during synthesis) cause large phase delays (9 h), while similar cold pulses applied during the falling phase (i.e. during diffusion) cause only small phase delays (2 h).

5. The action of light is to lower the value of the reference level, *R*, so that the constant illumination damps the oscillation until the clock is stopped at its lowest phase point.

6. The model predicts that light pulses applied during the rising phase will effectively accelerate the increase in level of *C*, thus causing phase advances, while phase delays will result from light pulses applied during the falling phase. A phase response curve for 2 h, 1100 lux light pulses confirms this. A rhythm splitting effect due to an appropriately timed light pulse is predicted and tested.

7. Possibilities of clock control of the CAP generating mechanism are discussed with reference to recent findings on the regulation of membrane potential oscillations in molluscan bursting pacemaker neurones.

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INTRODUCTION

The circadian clock in the eye of *Aplysia californica* appears to consist of a population of coupled oscillators. This functions as a coherent time keeping mechanism, modulating the frequency and amplitude of the compound action potentials (CAPs) transmitted via the optic nerve to the cerebral ganglion (Jacklet & Geronimo, 1971). These CAPs are the product of synchronous discharge by the 1000 secondary neurones in the eye, which may also be the sites of the individual oscillators comprising the circadian clock (Benson & Jacklet, 1977*b*). During a steady-state free-run, the population of oscillators behaves as a single self-sustaining oscillator. Only an appropriately timed perturbation reveals its composite character, by transiently dividing the single rhythm into two parts of slightly different periods (Benson & Jacklet, 1977*b*). In this paper a feedback model will be presented, which describes the behaviour of the synchronized population. By assuming that the endogenous activity and responses of the population reflect qualitatively the behaviour of the individual oscillators, it is possible to attribute physiological connotations to the elements of the black box diagram. This assumption will be discussed below. Following an outline of the various elements of the model, the properties of the *Aplysia* clock will be compared with those of the model, and results of two sets of experiments investigating properties of the clock will be given.

A number of different approaches have been made to modelling circadian rhythms. Bünning's 'kinetic approach', essentially considering types of relaxation oscillators (Bünning, 1973), and Pittendrigh's early model, involving master and slave oscillators (Pittendrigh, 1960), were expressed in qualitative terms, and accounted well for many properties of circadian rhythms. Pittendrigh has recently provided a detailed account of a new model based on the circadian locomotor activity of nocturnal rodents. It involves two oscillators or coherent groups of oscillators which show opposite phase shifting responses to light. Both phase response curves and period changes in response to intensity of constant illumination are explained in terms of this single feature of the model (Pittendrigh & Daan, 1976).

Various mathematical descriptions of non-linear oscillators have been applied to circadian rhythms. Wever (1965, 1972) utilized an extended van der Pol equation which allowed him to draw important conclusions regarding changes in shape and amplitude of clock oscillations, and hence of their phase response curves, in the entrained state and following single perturbations. Pavlidis (1967, 1968, 1973) and Winfree (1970, 1973, 1974) have made extensive analyses of clock behaviour in terms of limit cycles. The topological approach led to the discovery of a singularity at which clock oscillators are stationary with varying degrees of stability. The eye clock of *Aplysia* is a self-sustaining, non-linear oscillator which shows transient amplitudes. As such it could be described in terms of a limit cycle (Minorsky, 1962), although for practical reasons it is not possible to make the large number of measurements required to define numerically all of the state variables. Although a generalized biochemical model based on limit cycle behaviour has been developed (Pavlidis, 1971), mathematical models tell us very little about the possible biophysical/biochemical nature of the clock.

Physiological or biochemical models of varying degrees of specificity have been

put forward. The chronon theory of Ehret & Trucco (1967) requires sequential transcription of RNA from a chronon, a hypothetical linear sequence of DNA of fixed length. Barnett, Ehret & Wille (1971) attempted to test some aspects of this model, but convincing evidence for the existence of chronons remains elusive. In fact, persistence of circadian rhythms in ennucleated cells argues against the direct participation of transcription in the clock mechanism. Other physiological models, bearing strong formal similarities to one another, have been proposed by Njus, Sulzman & Hastings (1974), Sweeney (1974), and Gander & Lewis (in prep.). In each case, a form of relaxation oscillator is involved, with an energy requiring phase and a passive relaxation phase. The oscillation is visualized as a periodically varying concentration of some unspecified compound or ion, the synthesis or active transport of which is switched on and off when the level of the substance takes on a particular relation with a reference level. Destruction or dissipation of the substance is considered to be a slow, passive process. The feedback model of Johnsson & Karlsson (1972; Karlsson & Johnsson, 1972) for the rhythm of petal movement in *Kalanchoë* belongs in this category. Although their model is given in control systems language, reference is made to possible physiological counterparts for the information flow within the system. The Karlsson and Johnsson model accounts remarkably well for the known properties of the *Kalanchoë* rhythm, including the singularity of limit cycles, and reversing transients (Aschoff, 1965) which are not compatible with most other models. The feedback model to be proposed for the eye clock of *Aplysia* derives most of its features from this class of physiological model.

CIRCADIAN CLOCK MODEL

Fig. 1A is the block diagram of a simple, time-delayed feedback loop which was designed to incorporate, in a single oscillating system, most of the known properties of the circadian rhythm of CAP output frequency from the eye of *Aplysia*. For the purposes of the present paper, the elements of the loop have not been characterized mathematically, and attention will be confined to qualitative specification of the transformations carried out by each of them.

Synthesis of a compound, C, takes place by means of a temperature compensated mechanism designated 'synthesizer' (a rate function describing change in concentration or level of C with time). Level of C rises in an 'accumulator' (an integrating function giving level of C as output), from which it diffuses or is transported so that directly or through a series of steps it influences the 'effector', which is the CAP generating mechanism at the cell membrane. In physiological terms, C is depleted by loss to the effectors. This represented in the model by a 'loss' function which reduces the level of C in the accumulator, probably in direct proportion to that level. The level of C at the effector membrane is directly proportional to the level of C in the accumulator and the time delay between the supply of C and the cell membrane is small. In other words, the rhythm is postulated to be in phase with the oscillating level of the clock (i.e. substance C). The level of C is constantly compared with a 'reference' level R, and the difference is transformed by a non-linear function designated 'switch' (Fig. 1C) and delayed by the 'time delay'. When the concentration of C falls below R, a signal passes from the switch through the time delay

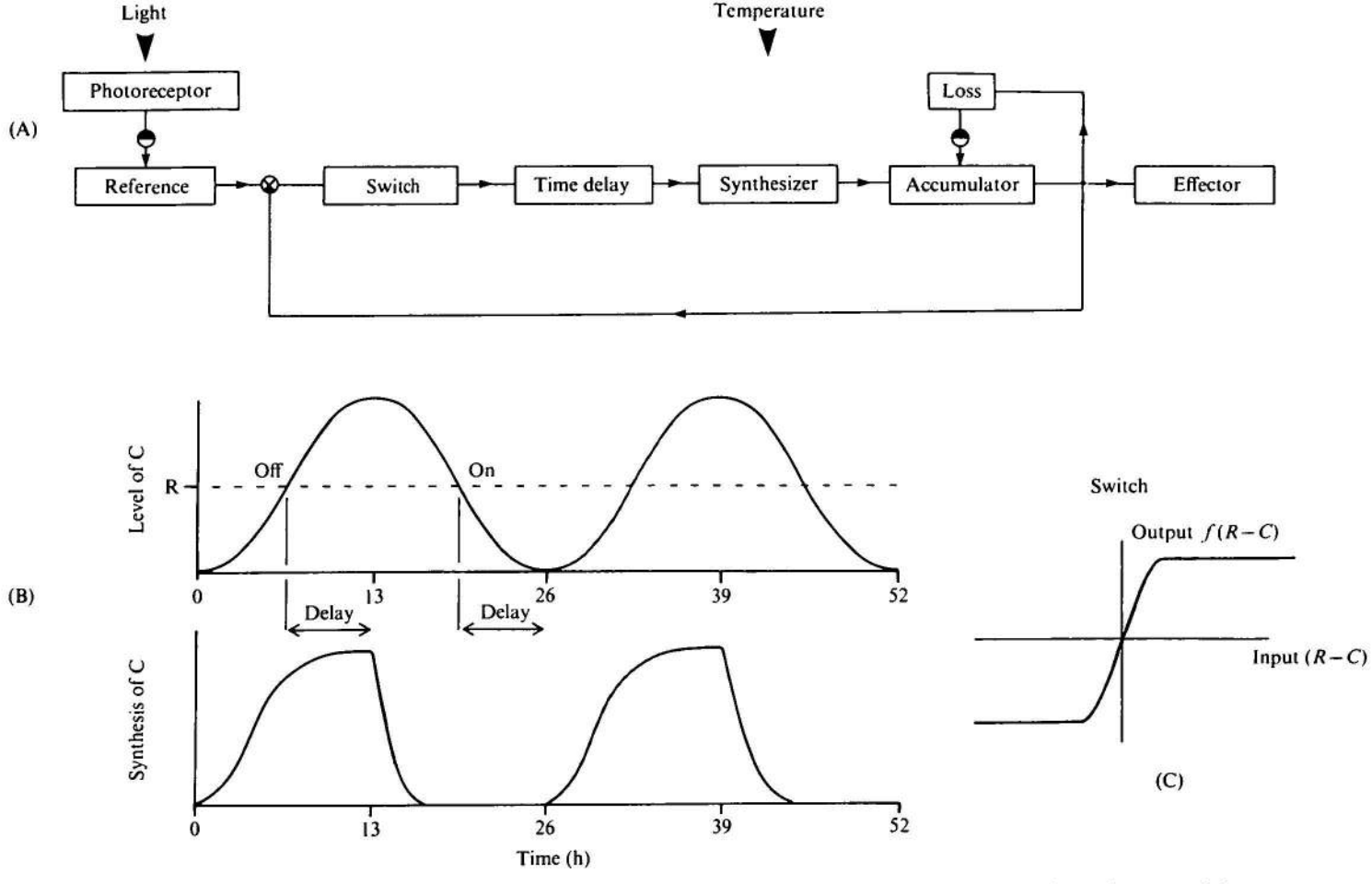

Fig. 1. Feed-back model for the circadian clock. (A) Black box flow-chart for the model. (B) Output of two elements of the model. (C) 'Switch' function. See text for explanation.

(6·5 h) initiating synthesis of C, and when C rises above R the output from the time delay stops synthesis of C about 6·5 h later. One action of light is to generate a signal via the 'photoreceptor' which decreases R. In darkness R returns to its steady-state value.

These time relations are illustrated in Fig. 1 B. The level of C fluctuates about R. As the level of C rises through R due to ongoing synthesis, the transformed signal via the time delay terminates synthesis 6·5 h later. The level of C immediately begins to fall due to loss of C to the effector system. As the level of C passes below R, the signal to begin synthesis is released, and when synthesis is reactivated after a 6·5 h delay, level of C begins to rise once more. The shape of the complete oscillation depends on the synthesis and loss functions. The oscillation as given in Fig. 1 B is an approximation since, in the CAP frequency rhythm in *DD* at 15 °C, the rising phase is usually somewhat steeper than the falling phase, suggesting that the diffusional process is slower than synthesis. This assymmetry is typical of relaxation oscillators. However, providing that the falling phase (decrease in level of C) is more or less similar to the rising phase, as is the case in experimental records, the feedback loop will generate an oscillation with a period of approximately 26 h.

Non-linearities should probably be included in all three units of the loop. The switch is the major non-linear function transforming the difference signal from the comparator to an abrupt step. The slope of the switching function is important in determining the stability of the feedback loop. Synthesis probably takes some time to reach peak rate, and again some time to fall to zero. Furthermore, it is influenced by the shape of the off/on signal from the comparator and switch. The change of level of C in the accumulator depends both on synthesis rate and on the properties of the loss pathway. Simple diffusion would be concentration dependent, and active transport would introduce other non-linearities at that point. There are several possibilities for the physiological nature of the comparison between level of C and R. For example, a change in charge ratio across a membrane separating C and R could initiate slow structural changes in the membrane that would ultimately trigger the synthesis mechanism. At present, speculation about the comparator is not easily tested, although the absence of circadian rhythms in prokaryotes implies that intracellular membranes may be an essential feature of biological clocks. The synthesis mechanism is amenable to experimental investigation, some approaches to which are discussed below.

EXPERIMENTAL EVIDENCE AND DISCUSSION

(*a*) *Effects of temperature*

Despite the similarity between ambient and body temperatures in eurythermal poikilotherms, many of them are able to survive and function at widely different habitat temperatures. This is largely related to the fact that when a poikilotherm is transferred from one thermal environment to another, many aspects of its physiology and biochemistry are altered in a manner that often compensates for the temperature change (Hazel & Prosser, 1974). In intertidal invertebrates especially, this compensation appears to be instantaneous. The molluscs *Cardium*, *Littorina*, and *Mytilus* ll show a Q_{10} of close to 1·0 for standard oxygen consumption over a range of temperatures similar to those of their natural habitat (Newell, 1969; Newell & North-

croft, 1967). In view of the widespread occurrence of temperature compensation in metabolic pathways of poikilotherms, the relative independence of the circadian period from ambient temperature does not seem surprising from a biochemical standpoint, just as it is to be expected in view of the selection pressures for an accurate time keeper.

According to the *Aplysia* clock model, the synthesis mechanism is the major energy requiring element in the loop and as such is the principal site of temperature compensation. There are several mechanisms by which biological reactions may be rendered temperature independent. Those arising from the properties of enzymatic proteins are reviewed by Hazel & Prosser (1974). Temperature compensation holds the Q_{10} of the eye clock near 1·0 for temperatures upwards from about 10 °C to at least as high as 22 °C (Benson & Jacklet, 1977*a*), which probably includes the environmental temperature range encountered by *Aplysia*. However, as the temperature approaches 8–9 °C, compensation begins to break down. Such thermotropic transitions are characteristic of systems involving biomembranes (Melchior & Stein, 1976), and have been postulated to contribute to the mechanism of circadian clocks (Wisnieski & Fox, 1976). Furthermore temperature has a direct, uncompensated action on the CAP production mechanism, decreasing CAP frequency with decrease in temperature to a minimum at 8–9 °C, with complete cessation of CAP production below 7 °C. It is interesting to note that there is an abrupt prolongation of post-tetanic potentiation of chemical synaptic potentials measured in cell R15 of *Aplysia* at about 9 °C when temperature is lowered in steps from 15 °C (Schlapfer *et al.* 1976).

For the synthesis mechanism of the eye clock, a slight, more or less linear dependence on temperature is suggested for normal temperatures, with a transition at between 8 and 10 °C to decrease in the synthesis rate with decrease in temperature at low temperature. It is suggested that when the temperature is reduced to 6 °C, for example, synthesis decreases to a very low level, and the level of *C* in the accumulator of the feedback loop is depleted by loss to the effector, so that after a few hours the clock oscillation drops to near its lowest phase point. This is postulated to be the mechanism underlying clock stopping. While *C* is at its lowest level, the signal from the switch calls for maximum synthesis. Hence when the temperature is returned to normal and synthesis can take place once more, the increase in level of *C* should be more rapid than during a normal rising phase. A rapid rise in CAP frequency following the end of a prolonged cold pulse was observed experimentally, and the reinitiated rhythm was consequently always 2–4 h in advance of the phase predicted from a normal rise rate (Benson & Jacklet, 1977*b*).

The slight increase in period with temperature decrease from 22–10 °C is considered to be due partly to the slowing of synthesis, but also to temperature effects on other parts of the loop, since such non-energy requiring processes as diffusion have a Q_{10} of about 1·3. At lower temperatures, the increase in period is accompanied by a decrease in amplitude of the clock oscillation which would be expected to accompany a decrease in synthesis rate. This is apart from the decrease in rhythm amplitude caused by the direct action of decrease in temperature on the CAP generating mechanism.

It is postulated that the rising phase of the oscillation in level of *C* is due to active synthesis, while the falling phase is a product of a passive process such as diffusion.

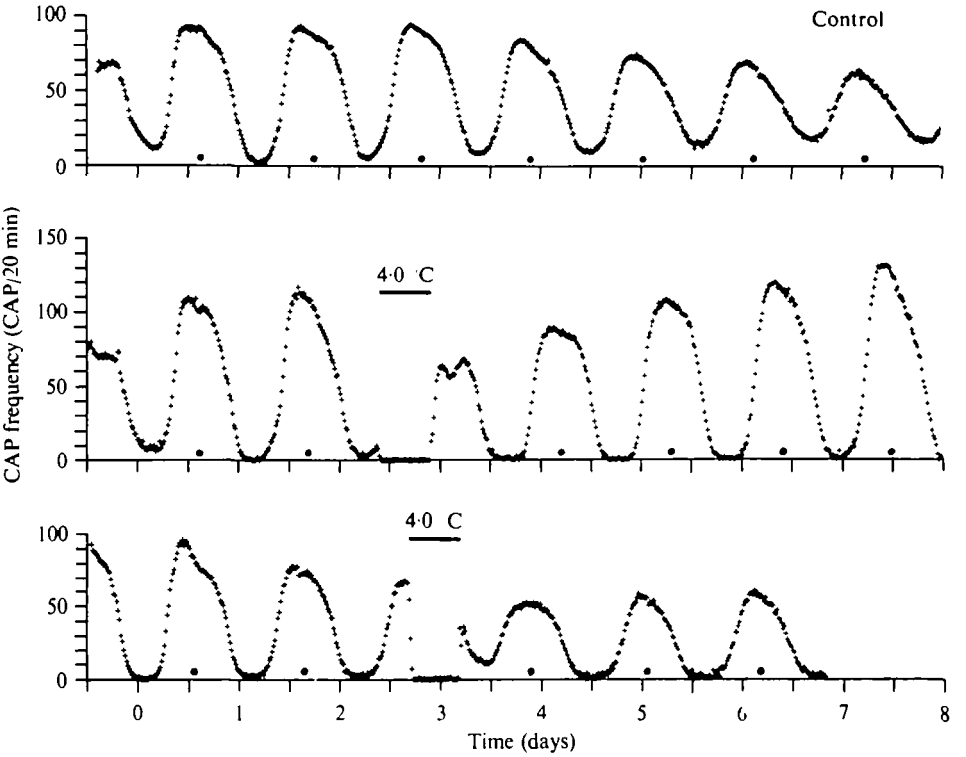

Fig. 2. Differential effect of 12 h cold pulses of 4 °C on the rising and falling phases of the rhythm. The second record shows a 12 h cold pulse of 4 °C applied during the rising phase of the rhythm, causing a 9·0 h phase delay. A 12 h cold pulse of 4 °C applied during the falling phase caused a delay of only 1·7 h as shown in the third record. Numerical data for these experiments and others are given in Table 1.

Above 9–10 °C, both rising and falling phases were slowed more or less equally by decrease in temperature, since the wave form of the rhythm at 9 °C and above remained symmetrical. This means that the temperature compensated rising phase has a Q_{10} close to that of the passive falling phase over this temperature range.

To test the hypothesis of an active rising and a passive falling phase, cold pulses of 4 °C and 12 h duration were applied, beginning at the base of the rising phase and at the peak of activity. Typical records for these experiments are illustrated in Fig. 2. In the second record, the cold pulse began at the base of the rising phase. At the end of the pulse, CAP frequency increased due to the removal of the inhibitory effect of low temperature on the CAP generating mechanism. The short duration peak immediately following the cessation of the cold pulse was a transient effect at the CAP generating site, and not a reflexion of the shape of the clock oscillation (Benson & Jacklet, 1977*b*). The phase delay of subsequent cycles of the rhythm was approximately 9 h in comparison with controls, for experiments of this kind. The influence of a 4 °C cold pulse beginning at the uppermost phase point of the rhythm is depicted in the third record of Fig. 2 and the data for other records are given in Table 1. The direct effect of temperature inhibited the activity of the CAP producing mechanism for the duration of the pulse, as before. However, the phase delay caused by this type

Table 1. *Differential effect of cold pulses on the rising and falling phases of the circadian rhythm*

(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 delay (h)
	(rising)			
2	16·3	12·0	4	−9·0
	17·0	12·0	4	−8·7
	17·0	12·0	4	−9·0
	16·7	12·0	4	−9·3
	20·0	6·0	7	−4·3
	21·0	6·0	7	−3·7
	(falling)			
3	25·7	12·0	4	−1·7
	25·7	12·0	4	−2·0
	24·3	12·0	4	−5·7
	25·3	12·0	4	−4·7
	3·3	6·0	5·5	−0·7
	3·3	6·0	5·5	−1·3

of pulse was 2 h or less. The differential sensitivity of the two phases to low temperature strongly supports the concept proposed here for the synthesizing and loss elements. An active process during the rising phase was greatly reduced in rate for the duration of a cold pulse below the range of temperature compensation, causing a phase delay close to the duration of the pulse, while the passive relaxation phase, most likely with a Q_{10} of about 1·3, was slowed slightly. Karakashian & Schweiger (1976) have suggested that the cycloheximide-sensitive phase of the circadian rhythm in *Acetabularia* may be increased in length and delayed by low temperature. The biochemical nature of the synthesizing process is amenable to further experimental investigation. Rothman & Strumwasser (1976) found that 12 h pulses of the protein synthesis inhibitors puromycin and cyclohexamide could cause phase delays when applied during the rising phase of the rhythm. However, puromycin applied at other phases showed some phase advances. The phase response curve for puromycin pulses coincides approximately with that for high K^+ pulses (Eskin, 1972), and puromycin at high concentrations had a considerable effect on the CAP generating mechanism. This suggests that puromycin may have effects other than inhibiting protein synthesis, such as changing potassium permeability in the photoreceptors or secondary cells (Dahl, 1969). Protein synthesis inhibitors such as anisomycin do not irreversibly affect the activity of bursting pacemaker neurones in the CNS of *Aplysia* (Schwartz, Castellucci & Kandel, 1971) or in the eye (Jacklet, 1977). Six h pulses of 10^{-6} M-anisomycin produced large phase delays (maximum 15 h) during the rising phase, with a sharp change near peak CAP frequency to small advances (maximum 5 h) and then small delays during the falling phase (Jacklet, 1977). It is thus possible that the synthesis process of the model is ribosomal dependent protein synthesis, or a chain of active events arising from such protein synthesis.

The stable phase point at which the CAP rhythm stopped during prolonged cooling can now be identified according to the model as the lowest level to which concentration of C falls when synthesis is greatly reduced or ceases. This point

should not be confused with the singularity of a limit cycle. In the former case, the rhythm reinitiates as soon as normal temperature is regained, while in the latter case, the rhythm has been driven to a unique point by a particular duration and intensity of perturbation applied at a specific phase point, and it remains arrhythmic until driven away from the singularity.

(*b*) *Effects of deuterium oxide*

The period of the free-running rhythm was lengthened in linear proportion to the concentration of D_2O in the culture medium, as far as concentrations of 50%. At 60 and 70% the rhythm appeared to stop at its uppermost phase point after a single cycle (Benson & Jacklet, 1977*a*). As at temperatures above 9 °C, the rhythm retained its symmetrical form at all concentrations of D_2O up to 50%. However, the maximum period increase due to D_2O was much greater than that due to low temperature; clearly there is no universal homeostatic mechanism for the conservation of period (cf. Pittendrigh & Caldarola, 1973). Since most, but not all, biological reactions are slowed by D_2O, and purely physical processes such as diffusion are also slowed, the general period lengthening action of D_2O cannot be localized to any particular point in the model. Probably all elements of the loop were affected to some extent.

The apparent clock stopping effect of high concentration of D_2O may be due to one of a number of causes. For example, evidently it takes approximately 36 h for D_2O to take full effect on the cells of the eye. If the time delay were increased so as to effectively cut the feedback loop after this time, the synthesis process would be left in an activated state, though considerably slowed by the action of D_2O, so that the clock would be stopped at its uppermost phase point. The time taken for the D_2O to take full effect is crucial since, if the increase in time delay occurred at some time when C fell below R, the clock would stop at its low phase. Another possibility is that the D_2O effect takes place at the effector stage, holding the cell membrane in a depolarized state producing constant CAP output. However, the lack of change in the range of the rhythm at 50% D_2O suggests that the CAP producing mechanism is essentially unaffected by D_2O, and that the action of high concentration of D_2O is on the clock mechanism itself.

(*c*) *Effects of light*

The action of light on the clock is mediated by a receptor system which may be either the receptor cells of the eye or the secondary neurones themselves. The receptor system which mediates the photoresponse measured in the optic nerve as an increase in CAP frequency, light adapts within 20 min, lowering the light induced CAP frequency by 15 to 25% (Benson & Jacklet, 1977*c*). If the photoreceptor system for the clock is the same as that for the CAP frequency response, then the action of many short pulses should be different from that of continuous illumination (*LL*) even though the total duration of illumination and the light intensities are the same.

Constant light

As was the case with the action of temperature change, the response to light of the CAP producing mechanism, a phasic response followed by a tonic increase in CAP frequency (Jacklet, 1971), is distinct from the response to light of the eye clock in terms of changes in phase, period, amplitude and shape of oscillation. In general

terms, the reference level R can be considered to be the bias of the oscillation, and may be expressible, for example, as a function of reaction rates. In the simplified terms of the model as proposed here, R is expressed by the concentration of some specific substance, but it may be a complex system of reaction limiting enzymes (e.g. light sensitive phosphodiesterases). For present purposes, it is hypothesized that the action of light on the clock is to lower the level of R, by increasing normal loss rates or by active destruction. LL therefore lowers R over several days, so that the oscillating level of C passes R sooner in its upward trajectory than it would in constant darkness (DD). R is lowered further by the time the level of C passes it on its downward path for any given cycle. The result of LL is thus a slow damping of the rhythm, but with a greater shortening of the rising phase of the rhythm than of the falling phase, which has been observed experimentally (Benson & Jacklet, 1977*c*). The nett change in period due to this process is a slight decrease, because although the 'on' signal is released sooner, the 'off' signal is delayed. Whether the advance is larger than the delay depends on the rate of decrease of R.

If LL is sufficiently prolonged, according to the model the level of R eventually falls to close to zero, so that the clock oscillation is effectively stopped at its lowermost phase point. As soon as LL ceases, the level of R rises in 1 or 2 h to its normal constant level. Since the level of C is then below that of R, the comparator signal is transformed and delayed to initiate synthesis 6·5 h later. A latency of 6–8 h after the cessation of prolonged LL was observed before the rhythm began again. This is in contrast to the observations of rhythm re-initiation after prolonged low temperature, when the rhythm always began again immediately, despite the fact that the clock was stopped at a similar phase point in both cases.

Twenty min light pulses (1100 lux), beginning at 2 h intervals appeared to damp out the rhythm more rapidly than constant light of the same intensity (Benson & Jacklet, 1977*c*). There was also a considerable nett phase advance after 2 days of light pulses. This suggests that the photoreceptor system involved in damping the rhythm dark adapts, and that the input to the clock from the photoreceptor is greater at the onset of light than during the course of a long light pulse. Similar actions of light have been described by Daan & Pittendrigh (1976) as 'nonparametric' and 'parametric' respectively and were investigated by Chandrashekeran, Johnsson & Engelmann (1973) as 'dawn' and 'dusk' effects. It is not clear whether such effects are due entirely to the state of adaptation of the photoreceptor, or whether the clock itself is involved.

Phase response to light pulses

An essential property of all circadian rhythms is a periodic sensitivity to external perturbations such as single short light pulses. A phase response curve illustrating such periodic sensitivity of the clock to a particular stimulus is obtained when the positive and negative phase shifts resulting from the application of a standardized stimulus are plotted against the circadian time of application of the stimulus. The phase response curve is thus an assay of the clock oscillation itself which may be out of phase with the externally manifested rhythm that it controls. Jacklet (1974) measured phase shifts of the CAP rhythm caused by 1 h pulses of 600 lux light and found delays during the falling phase and advances during the rising phase.

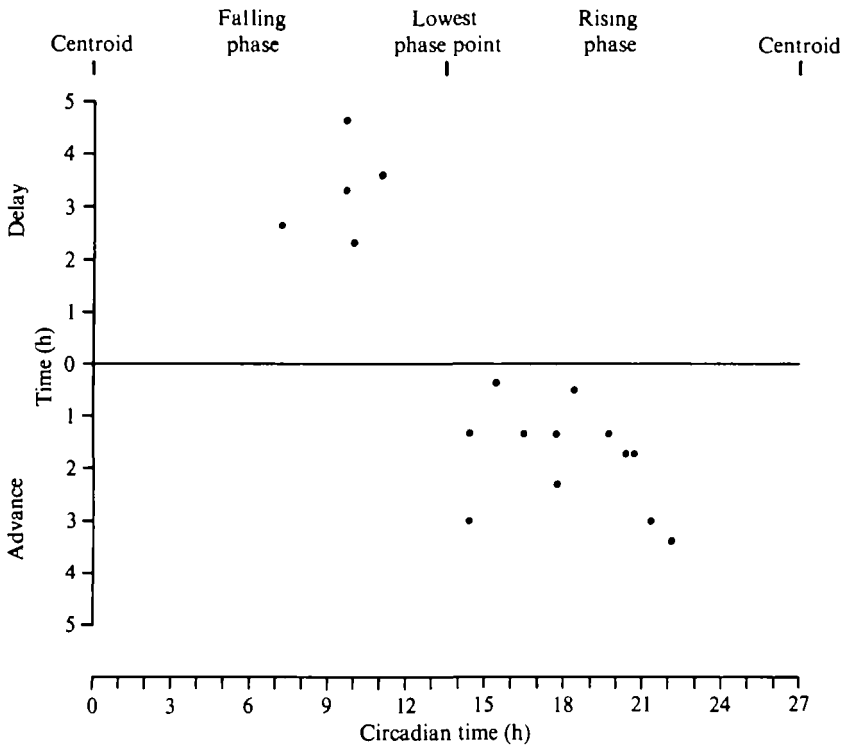

Fig. 3. Phase response curve for light pulses of 2 h at 1100 lux. Light pulses of 2 h duration at 1100 lux were applied during the late falling phase and early rising phase of the rhythm to determine the 'phase jump' point, between maximum phase delays and maximum phase advances. The phase shift caused by a light pulse is plotted against the circadian time (centroid at 0·0 h) of light onset for that pulse. The time of 'phase jump' was close to the lower-most phase point of the rhythm.

The phase response curves was a 'Type 1' or weak stimulus curve (Winfree, 1970), with the 'phase jump' from delays to advances occurring near the lowermost phase point of the rhythm. These results are confirmed by Fig. 3 which shows the phase shifts due to 2 h light pulses of 1100 lux. The phase shifts were measured as the difference between the expected period and the period calculated from the observed intercentroid time lapse between the final pre-pulse cycle and the first steady-state cycle after the pulse. The phase point of application of the pulse was measured from the preceding centroid point. In free-running rhythms there was always some variation in period so that the calculated phase shifts show a considerable range, even for light pulses applied at the same circadian time. The intention of these phase shifting experiments was to locate the point of abrupt change from delays to advances, and to discover which phases of the rhythm showed, in effect, angular acceleration and deceleration of the clock oscillation in response to light perturbations. The clock is effectively speeded up (phase advanced) during the rising phase and slowed down (phase delayed) during the falling phase. The sharp change from maximum delays to maximum advances occurs at the lowermost phase point.

Although it takes 3 or 4 days of continuous 1100 lux light to lower R to close to zero, it is possible that 2 h light pulses of a similar intensity would cause transient

decreases in the level of R, producing instantaneous period changes in the clock oscillation. This is likely particularly in view of the relatively greater effect of the initial part of illumination on R, and of the small range of the phase response curves for 600 and 1100 lux in comparison with those of other organisms. A light pulse during the rising phase would lower R, which is equivalent to accelerating the increase in level of C. The closer the time of application to the base of the rising phase, the greater the possible phase advance. Within limits, larger phase advances would be produced by higher intensity light, and there would be a reciprocity between intensity and length of the perturbation pulses, as has been found in other systems (Karlsson & Johnsson, 1972; Englemann, Karlsson & Johnsson, 1973). During the falling phase, lowering R would effectively decelerate the oscillator, causing a delay in the phase of the rhythm. Again, the maximum phase shift would be closer to the base of the falling phase, with the cross-over between maximum delays and advances occurring at the lowermost phase point. This hypothesized cross-over on the clock oscillation corresponds with the observed cross-over point on the rhythm (Fig. 3), so that if the model is correct in terms of the postulated phase shifting mechanism, the clock oscillation and rhythm must be in phase. This conclusion is supported by the correspondence between theory and data for the differential effects of cold pulses during the rising and falling phases.

The effects of light pulses on the signal from the comparator are transformed and delayed by the switch and time delay, so that the response of the oscillator in terms of phase and amplitude changes are long term. Experimental data show that any transients that may follow the perturbed cycle do not vary in period outside the normal range of period variation. This is the case for weak responses, but possibly for very large phase shifts transients would be more pronounced.

Rhythm splitting effect of a light pulse

If the circadian clock consists of a population of coupled oscillators, as has been suggested (Jacklet & Geronimo, 1971; Benson & Jacklet, 1977*b*), there would be some variation in the phase relations of the individual oscillators, so that the abrupt cross-over between phase delaying behaviour and phase advancing behaviour of the individual oscillators would be distributed on either side of the lowermost phase point of the rhythm. A light pulse applied at precisely this point would be expected to advance some oscillators and delay others, thereby causing a 'split' in the rhythm (i.e. desynchronization). Fig. 4 depicts the record of a single case in which the light pulse fell on the projected cross-over point. It is tentatively suggested that a slight split was induced by the light pulse so that the population was split into two subpopulations that oscillated out of phase for two or three cycles, before drawing one another back into phase, as indicated in the hypothetical curves in the third record. The split appears to reach its maximum on the second cycle after the pulse, which may be due to transients in the response of the subpopulations to the light pulse. In all other light pulse experiments the perturbation fell on one side or the other of the critical phase point, since variation in period of the rhythm generated by the population of oscillators as a whole makes it difficult to predict precisely the real time of the critical phase point.

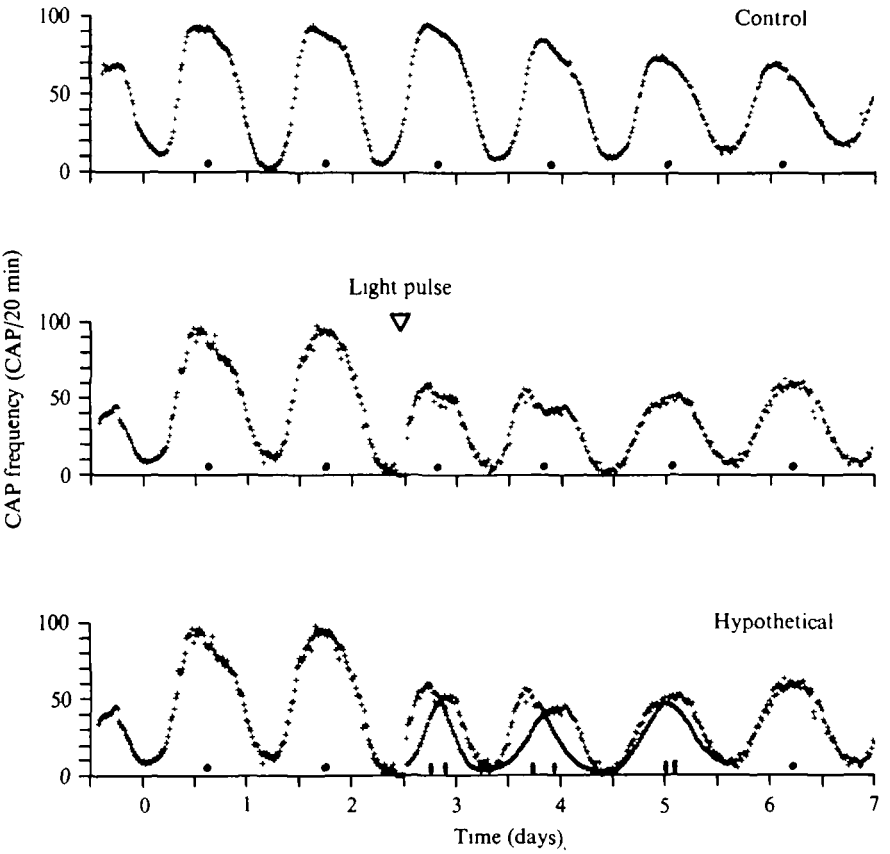

Fig. 4. Rhythm splitting effect of a single light pulse. The second record illustrates the rhythm-splitting effect of a 2 h, 1100 lux light pulse applied at the circadian time of 'phase jump' from maximum phase delays to maximum phase advances in response to light pulses. The third record was constructed by fitting two periodic curves to the second record. See text for explanation.

(d) Coupling of clock to effector

Finally, it is possible to speculate about the coupling between the clock oscillation and the CAP producing mechanism, based on experiments into the regulatory mechanisms of bursting pacemaker activity in neurones of *Aplysia*, *Otala*, and *Helix*, since the secondary neurones of the eye probably have many features in common with these endogenous bursters.

The important feature of pacemaker activity in molluscan neurones is the membrane potential oscillation which gives rise to bursts of action potentials during maximum depolarization. Blocking action potential production with TTX can leave the membrane potential oscillation unaffected (Strumwasser, 1974; Barker & Gainer, 1975*a*).

Divalent cations, particularly Ca^{2+}, can regulate the appearance of bursting pacemaker activity in neurones of *Aplysia* and *Otala* (Meech, 1972). These ions are necessary for burst generation and will inhibit its appearance at high concentrations (Barker & Gainer, 1975*b*). Changes in Ca^{2+} level have a pronounced effect on the rate of CAP production in the eye of *Aplysia* (Jacklet, 1973). It now appears likely

that the membrane potential oscillation underlying burst formation in pacemaker neurones depends on a cyclic variation in potassium permeability together with an inward current of Ca^{2+} (Eckert & Lux, 1976; Johnston, 1976) or Na^{+} ions (Smith, Barker & Gainer, 1975).

It has also been shown that peptide hormones such as vasopressin and oxytocin increase the amplitude of the membrane potential oscillation in bursting neurones in *Aplysia* and *Otala*, and initiate such activity in aestivated or semiaestivated neurones (Barker & Gainer, 1974; Barker, Ifshin and Gainer, 1975). Their effects long outlast the period of application. Peptide factors in extracts from molluscan tissue have similar effects on a specific neurosecretory cell in *Otala* (Ifshin, Gainer & Barker, 1975). Untreated extracts from the parieto-visceral ganglion of *Aplysia* caused an increase in CAP production lasting several hours in isolated eyes of *Aplysia* (Benson, unpublished experiments).

Finally, Treistman & Levitan (1976) have found that the addition of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) caused a marked increase in duration and amplitude of the interburst hyperpolarization of R15 in *Aplysia*, and that there was an accumulation of cAMP after treatment with IBMX. Derivatives of cAMP that are resistant to breakdown by phosphodiesterases caused similar modifications of the bursting activity when injected intracellularly.

The processes underlying the coupling of the reception of a peptide at the membrane to the change in membrane potential oscillation remain to be elucidated. However, it is possible that peptides alter permeability to Ca^{2+}, or Ca^{2+}-binding, either directly or via cAMP, or that they act on a cAMP-calcium feedback loop underlying an endogenous oscillation in the intracellular level of Ca^{2+} (Rapp & Berridge, 1977). Conceivably the output from the clocks in the secondary neurones of the eye of *Aplysia* could be peptides which control the frequency and amplitude of the membrane potential oscillation in the individual neurons, and hence the CAP production of the population. The direct effect of temperature change on CAP frequency could be mediated in part by a temperature dependence of the regulatory role of Ca^{2+}, as has been postulated by Barker & Gainer (1975*b*) for other bursting neurones on the CNS of *Aplysia*.

CONCLUSION

A simple feedback model has been proposed to account for the behaviour of the CAP frequency rhythm in the isolated eye of *Aplysia*, in different constant conditions and when subjected to a variety of external stimuli. It is suggested that although this rhythmic behaviour results from the interaction of a population of coupled oscillators the period and range of which decrease slightly when the population size is decreased (Jacklet & Geronimo, 1971), it may reflect at least qualitatively the behaviour of the individual oscillators. Boon & Strackee (1976) have described the interaction of populations of relaxation oscillators, in which the population behaviour was also of the relaxation type. The *Aplysia* eye rhythm shares some properties with such oscillators.

In the present series of papers, the primary concerns have been as follows: (1) to characterize the cells involved in CAP production as typical bursting pacemaker neurones; (2) to provide further evidence that a population of cells, most likely the

condary neurones which are the source of the CAPs, act in synchrony to produce the observed rhythm; (3) to investigate the properties of the basic oscillation, particularly with respect to differences in its various phases; and (4) to examine the possible connexions between the clock and the receptors and effectors of the circadian system in the eye. Although practical considerations preclude the large-scale experimentation required to construct a precise mathematical model of the eye rhythm, the isolated eye preparation is remarkably suitable for investigation of physiological mechanisms. It now appears likely that active synthesis or energy-consuming ion transport occurs during the rising phase of the rhythm but that the falling phase is the result of a non-energy requiring process. Very little is known about these aspects of circadian clocks in any system, but the success of the experiments reported here and of preliminary investigations into protein synthesis in the eye suggest that the eye system may provide more clues as to the nature of the circadian clock.

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