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SIGNAL EMISSION AND SIGNAL PROPAGATION DURING EARLY AGGREGATION IN *DICTYOSTELIUM DISCOIDEUM*

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SUMMARY

Waves of chemotactic movement during the early phase of aggregation in *Dictyostelium discoideum* are of 2 kinds, concentric waves produced by cells that emit cyclic AMP signals spontaneously, and spirals generated by excitations relayed continuously around loops of excitable cells. The period of a spiral wave is the time taken for the excitation to make one complete circuit of the pacemaker loop. We have compared signal emission from the 2 types of source in time-lapse films made at a variety of temperatures. Our results show that spiral waves have a characteristic period length throughout most if not all of the early phase of aggregation, and that the period of concentric waves is generally longer and more variable. Temperature has a pronounced effect on period length and a lesser effect on propagation velocity. We find that each individual wave is propagated at constant velocity over distances of 1–2 cm but that the velocity of successive waves declines. This decline probably reflects some cumulative effect of the chemotactic excitations on the excitable properties of the aggregating cells.

INTRODUCTION

Amoebae of *Dictyostelium discoideum* multiply as isolated cells. Their developmental sequence is initiated when the food source is exhausted or removed, and involves extensive macromolecular turnover. A few hours after the onset of starvation, cells that have been spread on a solid surface begin to aggregate towards a number of centres and become partitioned into discrete territories (Shaffer, 1962). Aggregation occurs by chemotaxis (Bonner, 1947) and the attractant appears to be cyclic AMP (Konijn, 1972; Malchow & Gerisch, 1974). Signals emitted by the centres are relayed outward (Shaffer, 1962; Alcantara & Monk, 1974) giving rise to waves of inward movement visible in high-power time-lapse films. Cells at the advancing wave front remain elongated for about 100 s (Cohen & Robertson, 1971*b*; Alcantara & Monk, 1974). Propagating waves of alternating light and dark bands have been observed in dense cell multilayers (Gerisch, 1961, 1965) and in monolayers (Alcantara & Monk, 1974). The light bands consist of elongated cells, the dark bands of rounded cells (Alcantara & Monk, 1974).

The existence of a refractory phase during signal relay has been inferred from the fact that signal propagation is unidirectional as well as from the mutual annihilation of colliding waves (Gerisch, 1965; Cohen & Robertson, 1971*a*). During the early stages of aggregation, waves of chemotactic movement are of 2 types, concentric waves

which result from spontaneous release of cyclic AMP by individual cells or perhaps cell groups, and spiral waves generated by the continuous circulation of excitations around closed loops of cells (Fig. 1). These loops are thought to be set up when early waves from point sources interact with chance discontinuities in the distribution of excitable cells on the agar surface (Durston, 1973). Once formed, their pacemaker activity is independent of spontaneous signal release since each cell or zone of cells on the circuit is excited by the one adjacent to it, and the signalling period, i.e. the time for the excitation to make one complete circuit, depends simply on the velocity of signal propagation and the size of the circuit. It has been suggested (Durston, 1973) that all loops, whatever their initial size, should evolve to a 'minimum' configuration such that the excitation makes one complete circuit in the time required for an excited cell to recover normal responsiveness to a further signal.

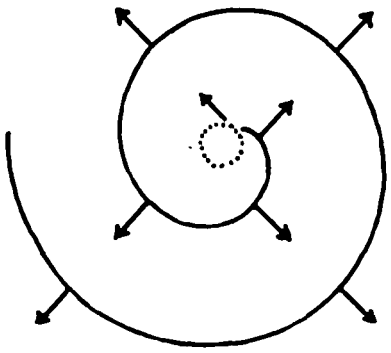

Fig. 1. A spiral wave generated by continuous relay of an excitation around a loop of cells. Each segment of the wave propagates outward tangentially from the pacemaker loop (arrows).

The development by Alcantara & Monk (1974) of a procedure for obtaining large aggregation territories with waves that are visible under low magnification has permitted us to study the evolution of many separate territories in the same field of aggregating cells. Our results are consistent with Durston's suggestion, and also throw some light on the nature of autonomous pacemaker activity. In addition they reveal a remarkable decline in the velocity of propagation of successive waves.

MATERIALS AND METHODS

Media

KK_2 buffer contained (g/l. water); KH_2PO_4, 2·25; K_2HPO_4, 0·67; $MgSO_4.7H_2O$, 0·5; pH 6·1. Standard medium, SM, contained (g/l. KK_2 buffer): Bactopeptone (Difco), 5; yeast extract (Difco), 0·5. For SM agar, 20 g Bacto-agar (Difco) were added to 1 l. SM broth. After autoclaving (103 kN m^{-2} (15 lb $in.^{-2}$), 15 min), sterile glucose was added to SM broth and SM agar to 0·5% final concentration. Non-nutrient (KK_2) agar contained: Bacto-agar (Difco), 10 g; KK_2 buffer, 1 l.

Strains

Dictyostelium discoideum strain NC4 obtained from Drs Alcantara and Monk was kept as a spore stock on silica gel. Sublines 1 and 2 were derived at different times by transferring a few crystals from this stock to an SM agar plate previously spread with 0·2 ml of a dense culture of *Aerobacter aerogenes*. Subsequent subcultures were made at weekly intervals by picking 10 spore heads (approx. 2×10^6 spores) with a loop and streaking them on an SM agar plate inoculated with *A. aerogenes*.

Growth conditions

Amoebae were grown with *A. aerogenes* on Petri dishes containing 40 ml of SM agar. Approximately 3×10^6 spores were spread with the bacteria over the surface of each plate and the amoebae harvested after 30 h at 22 °C. Bacteria were removed by 4 centrifugations at 200 **g** for 3 min, resuspending the amoebae each time in KK_2 buffer at room temperature. Cell density was measured with a Petroff Hauser counting chamber.

Aggregation

Half a millilitre of a suspension of washed amoebae at 2×10^7 per ml was spread over the surface of freshly prepared non-nutrient agar (5 ml) in 8 × 5 cm plastic boxes with hinged lids (Kabi Ltd, Potters Bar, Herts). Uniform spreading was achieved with a gentle to-and-fro motion of the 'heel' of a glass spreader rather than its flat surface. The amoebae were allowed to settle for 20 min and the agar surface carefully drained of liquid. The boxes were kept open until only a very thin uniform film of liquid was left, and placed at 7 °C overnight. Visible waves developed upon further incubation at 7 °C or after transferring the cells to higher temperatures (see Results). We had little success in obtaining waves when the cells were placed at 22 °C without prior incubation at 7 °C.

Optical methods

Wave patterns are easily seen if the boxes are held up to a light at a suitable angle. However, the actual difference in refractivity between 'light' and 'dark' bands is so slight that a dark-field illuminating system capable of detecting quite small deflexions of the illuminating beam is required to photograph them. In the system used (Fig. 2) a double plano-convex lantern condenser lens of 14 cm diameter and about 12·5 cm focal length is supported on a shelf in a rectangular tower made of 'Speedframe'. A shelf just above it holds the box with amoebae and another, below, supports a narrow annulus of 3 cm diameter covered with a dark green filter and lit by an opal lamp. An image of the annulus, of equal size, is produced at the lens of the H16 Bolex Reflex camera. The camera is fitted with a wide angle Yvar lens, and a Wild Vario-Timer. Films were made at 1 frame per 10 s on Kodak High Contrast (type 7457) film, and processed by reversal to a direct positive. Exposures were determined using a previously calibrated photometer (Salford Electrical Instruments) on the reflex screen image.

To film below room temperature the assembly was transferred to a cold room at 4 °C, and the upper storey of the Speedframe tower enclosed in a Perspex box with the funnel of a domestic hair dryer inserted into it. The heater of the hair drier is governed by a proportional controller with its sensing thermistor inside the box. A separate electric thermistor (Light Instruments, Brighton) monitors the temperature of the cells by means of a small surface contact probe attached to the culture box with 'Blutack'. Films were analysed by projecting them (× 4 magnification) with a Specto Mk 3 projector at 16 frames per s on to a sheet of paper. Lines were drawn from the centres of the territories to be analysed, and marked off at appropriate intervals. The films were then run, and the passage of the fronts of successive 'light' bands past the markers recorded manually by means of a cable release operating a pen on a kymograph trace moving at a known speed.

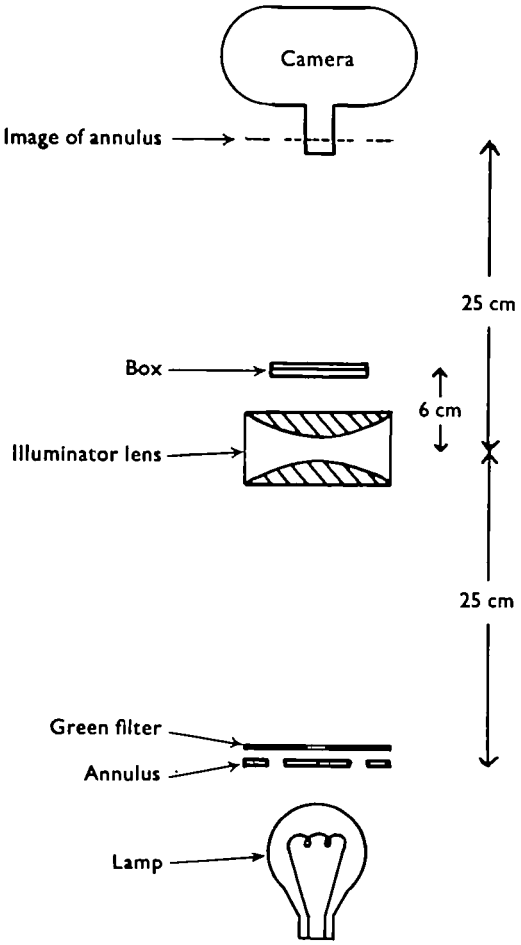

Fig. 2. Schematic diagram of the apparatus used for dark-ground time-lapse photography. See text for details.

RESULTS

In all experiments washed amoebae were incubated on non-nutrient agar at 7 °C for 16 h (see Materials and methods). To film wave propagation at temperatures of 15 °C or higher, an agar box was transferred to 21 °C until waves appeared, and placed in the filming cabinet equilibrated at the appropriate temperature. The entire field of cells was filmed until waves could no longer be discerned. Wave propagation at 7 and 10 °C was filmed by transferring boxes directly from the 7 °C incubator to the constant-temperature cabinet. Visible waves appeared sooner when cells were transferred to 21 °C than when they were kept at 7 or 10 °C. Most waves were spiral. At the start of wave propagation the cells appeared to be uniformly distributed, but as wave propagation proceeded they gathered together, eventually forming tight streams in which waves could not be discerned under the form of low-power microscopy employed.

Velocity of propagation and period of spiral waves

A spiral wave is a continuous structure, each segment of which propagates outward tangentially from the central pacemaker loop (Fig. 1). However, when one examines the wave from fixed vantage points on an arbitrarily chosen line, as we have done in making our measurements (see Materials and methods) it appears as a succession of discrete waves, and it is in this sense that we shall employ the term 'waves' as applied to spirals. In Fig. 3 we reproduce the successive positions of the waves of a spiral in a film of cells aggregating at 22 °C. It is evident that each wave travels with constant

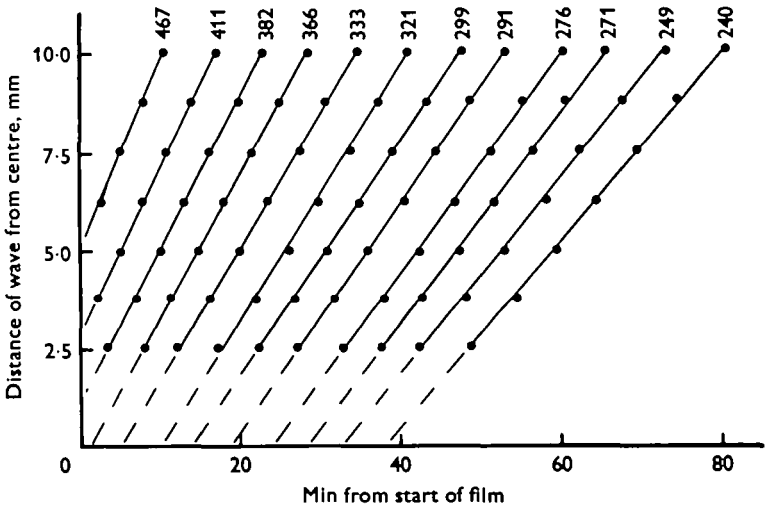

Fig. 3. Propagation of successive waves of a spiral through an aggregation territory in a film of cells of subline 2 at 22 °C. The data plotted are means of 2 measurements and the lines were fitted by regression analysis. Numbers above each line represent the velocities of the corresponding waves in μm/min, obtained from the slopes of the lines. The first 2 waves shown had already emerged at the start of filming. For further details see Materials and methods.

velocity, but that the velocity of successive waves declines progressively. The actual velocities, calculated by regression analysis, are shown above each wave, and plotted in Fig. 4A. We have analysed the propagation of 10 waves of each of 5 other spirals in the same field of aggregating cells. For convenience each wave was measured as it passed 4 rather than 7 reference positions in the field, and the data were plotted as in Fig. 3, regression lines obtained, and the mean velocities of each wave calculated. The mean velocities for all 6 spirals, with their standard errors, are plotted in Fig. 4C from which it is apparent that a similar decline in velocity occurs in all the spirals, and that the mean velocity declines in an approximately linear manner.

The period of a spiral pacemaker loop may be defined as the time for the excitation to make one complete circuit of the loop, or equally, the time elapsing between the emergence of successive excitation fronts from any fixed point on the loop (see Fig. 1). If successive waves travelled with the same velocity the period could be determined by measuring the interval between the arrival of successive waves at any point in the

field. However, because of the decline in velocity of successive waves the period measured in this way, as well as the distance between waves, depends on the distance of the point of measurement from the centre (see Fig. 3). It is therefore necessary to measure period close to the centre, or better still to extrapolate the path of each wave, plotted as in Fig. 3, back to the centre. The validity of the latter procedure depends on the assumption that wave velocity is the same near the centre as it is in the rest of the field (see Discussion).

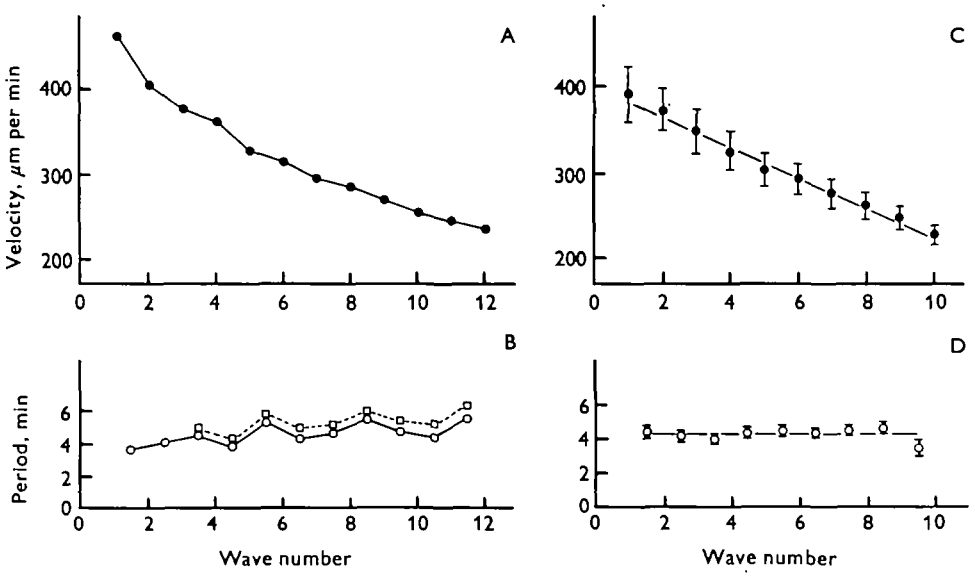

Fig. 4A. Velocities of the successive waves of Fig. 3.

B. Intervals between successive pairs of waves of Fig. 3. These were obtained either by extrapolating each regression line back to the centre (open circles) or by direct measurement 2·5 mm from the centre (open squares). Since the first 2 waves had passed beyond the 2·5-mm point when filming began they are not included in the measurements made by the second method.

C. Mean velocities of successive waves in 6 spirals. The spirals were in the same field as the spiral of A, and include that spiral. Data were recorded and analysed as in Fig. 3, and the waves that had emerged at the start of filming were not included. Vertical bars represent standard errors.

D. Mean intervals between equivalent pairs of waves in the 6 spirals of C, with their standard errors. The intervals were measured by extrapolation.

The intervals between successive pairs of waves in Fig. 3, measured in these 2 ways, are shown in Fig. 4B. Intervals measured 2·5 mm from the centre are 9% longer on average than the values obtained by extrapolation, and neither sequence of intervals shows a pronounced trend. The intervals between successive pairs of waves were measured in the same fashion for each of the 6 spirals analysed in Fig. 4C, and gave similar results, again without any obvious trends. Fig. 4D gives the means of the interval measurements for corresponding pairs of waves in the 6 spirals. The mean intervals for individual spirals varied from $4{\cdot}0 \pm 0{\cdot}3$ to $5{\cdot}0 \pm 0{\cdot}1$, and an analysis of variance indicates that this variation was significant ($0{\cdot}05 > P > 0{\cdot}01$).

Effect of temperature on wave velocity and period

We have analysed films of wave propagation at a variety of temperatures. The velocity of individual waves through the large territories examined was found to be remarkably constant. We have timed 54 waves past a minimum of five points and all gave co-efficients of correlation of distance travelled with elapsed time only fractionally below 1·0. The velocity of successive waves declined in every sequence of waves that was analysed except one, and in general both the average velocity and the rate of

Table 1. *Effect of temperature on period and propagation velocity of spiral waves*

°C	NC4 subline	No. of spirals analysed	No. of waves per spiral	Mean period,* min	Mean velocity* of first wave, μm/min	Mean velocity* of last wave, μm/min
24	2	2	9	3·9 ± 0·2	297 ± 9	217†
22	1	4	11–13	6·4 ± 0·3	400 ± 21	174 ± 10
22	2	6	9	4·3 ± 0·2	393 ± 33	228 ± 15
19	1	3	7–10	8·2 ± 0·3	317 ± 9	187 ± 17
19	2	3	4–7	4·5 ± 0·4	286 ± 9	215 ± 8
15	1	2	11–12	9·3 ± 0·4	292 ± 3	235 ± 17
15	1	6	8–12	10·9 ± 0·4	330 ± 19	207 ± 16
10	2	3	14–15	16·0 ± 0·4	169 ± 14	123 ± 16
7	1	3	6–12	28·0 ± 0·7	176 ± 4	138 ± 12
7	2	3	9–10	21·1 ± 1·9	149 ± 1	157 ± 15
7	2	3	12–14	20·3 ± 1·4	158 ± 14	93 ± 15

* Values are given ± standard error of the mean.
† No variation was observed.

decline in velocity increased with increasing temperature (Table 1). However, these parameters varied considerably under ostensibly similar conditions. Although the decline in velocity was generally fairly regular this was not always so. A good example of an irregular decline is shown in Fig. 5 where abrupt drops in velocity occur between waves 7 and 8, and again between waves 10 and 11. Closely comparable behaviour was shown by each of 4 other spirals analysed in the same field. We have also analysed waves from 2 concentric sources in a field of cells at 22 °C. Their velocities declined in the same fashion as those of spirals in the same field.

In only one circumstance did we observe any marked trend in spiral period. This was with cells of subline 2 aggregating at 7 °C, where the period of the spirals in each of 2 independent films started at about 16 min and rose gradually to about twice this value. In all other cases the intervals between successive waves, whether measured close to the centre or by extrapolation to the centre, fluctuated irregularly about a characteristic value. Occasional very long intervals were observed. These invariably followed sharp drops in propagation velocity. This effect can be seen after waves 8 and 11 in Fig. 5. Table 1 summarizes data for the periods of spirals, obtained by extrapolation of wave trajectories back to the centre. The period values given were computed by excluding the abnormally long periods following sharp velocity drops. In no

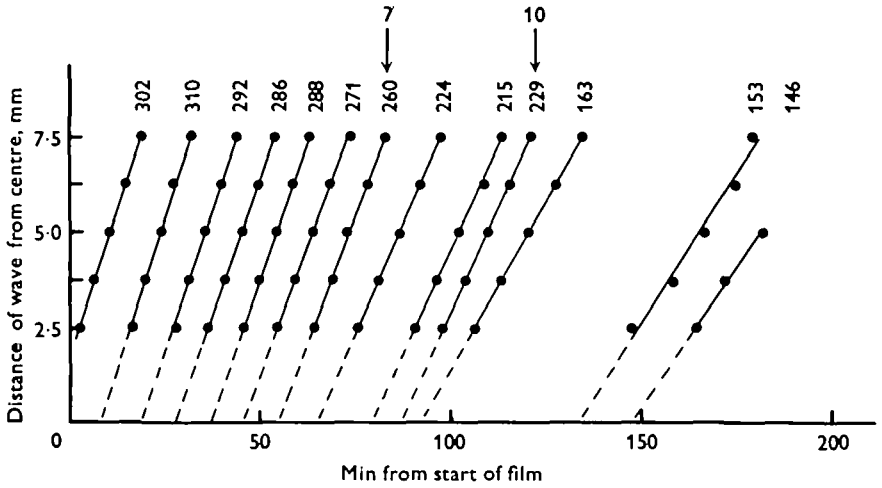

Fig. 5. Propagation of successive waves of a spiral of cells of subline 1 at 15 °C. For ease of reference waves 7 and 10 are individually labelled. Details as for Fig. 3.

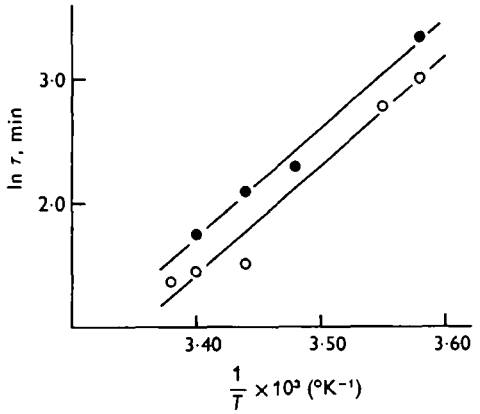

Fig. 6. Dependence of spiral period on temperature. The Arrhenius equation was applied to the data of Table 1 in the form

$$\ln \tau = \frac{E}{RT} + \text{constant},$$

where τ is the mean spiral period, R the gas constant and T the absolute temperature. Solid circles are data for cells of subline 1, open circles for cells of subline 2: regression lines are drawn separately for each.

case would inclusion of these long periods increase the estimated mean values by more than 10%. It is evident from the data in the Table that period length shows a more pronounced temperature dependence than does wave propagation velocity.

The data for spiral periods are presented as an Arrhenius plot in Fig. 6. The plots of the natural logarithm of period against the reciprocal of absolute temperature are approximately linear for cells of both sublimes used, and yield similar estimates of 16·9 and 17·4 kcal/mol for the activation energy.

Period of concentric waves

As already mentioned, the majority of the pacemakers in our film sequences are spirals. In some of the sequences a few concentric sources emit a number of waves and are then swamped by neighbouring spirals or replaced by a new concentric source some distance away. However, a few films include a number of stable concentrics whose periods have been compared with spirals in the same sequences. Fig. 7 presents the results of an analysis of one of the most favourable sequences. The cells in this case had been incubated at 7 °C until wave propagation was under way, with 6 large

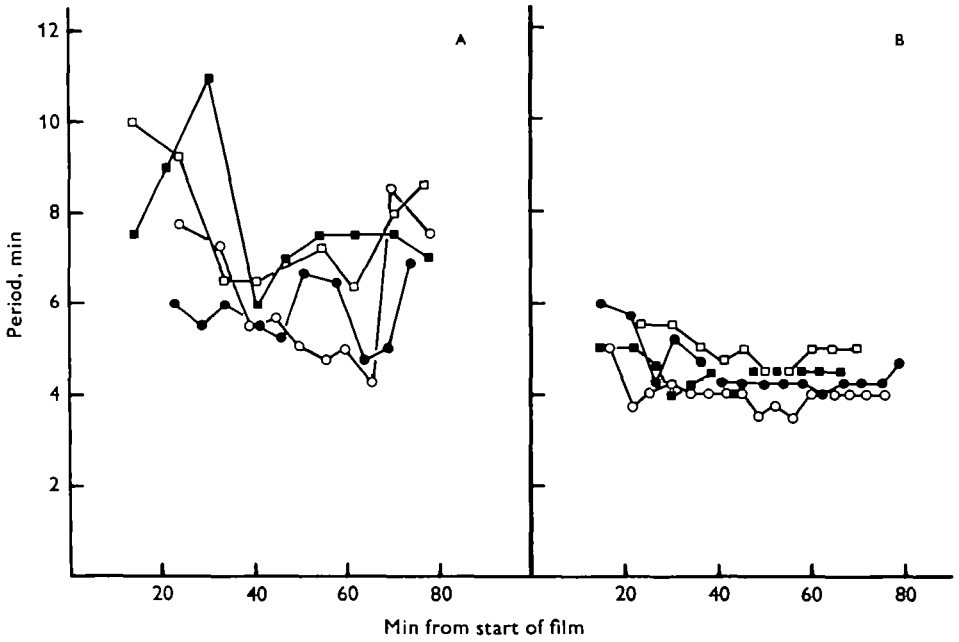

Fig. 7. Intervals between successive pairs of waves in cells of subline 1 after transfer from 7 to 22 °C. A, concentric waves; B, spiral waves. Filming was begun 7 min after transferring the box with aggregating cells to the incubator at 22 °C. At this time the temperature at the surface of the agar was about 17·5 °C and it reached 22 °C a few minutes later. Intervals were measured 1·25 mm from each centre. The different symbols refer to different centres.

spirals extending over the entire plate, and then transferred to the filming cabinet at 22 °C. The original pattern of waves established at 7 °C broke up within 15 min into numerous sets of waves which stabilized some 10 min later into a new pattern consisting of several dozen centres, approximately half of which were concentrics. Three of the original 6 spiral centres survived. Because of the small size of the territories and the large number examined we have not attempted to extrapolate the period measurements back to the sources but instead present measurements made 1·25 mm from each centre. All 4 spirals shown in Fig. 7B were formed after transfer to 22 °C. Note that their periods were more or less constant from the outset. The mean period was 4·4 min, but this result is not entered in Table 1 because of the unusual protocol followed.

The periods of the concentrics in Fig. 7A are substantially longer than those of the spirals, and more variable. Six other concentrics were analysed with similar results. In 3 other films the concentric periods were again longer and more variable than the spiral periods. However, 2 further sequences included several concentrics with the same short and relatively constant period as the spirals in the same fields of cells. We have never observed any concentric with shorter signal periods than spirals formed under the same conditions.

DISCUSSION

Nature of spiral and autonomous pacemakers

We have found that all the spiral waves formed in a given field of aggregating cells have similar periods. This finding is consistent with the idea that the pacemaker loop of a spiral has a configuration such that the excitation makes one complete circuit in the time required for an excited cell to recover normal responsiveness to further stimulation. This recovery time includes both the 'response' time of an excited cell, as represented for example by the phase of cyclic AMP release (Roos, Nanjundiah, Malchow & Gerisch, 1975; Shaffer, 1975), and the ensuing refractory period (Gerisch & Hess, 1974). The fact that we observed no appreciable change in period in sequences comprising 10–15 successive periods suggests that the length of this combined interval is constant during the early stage of aggregation that we have examined. We cannot exclude the possibility that it is greater at earlier times (Durston, 1974; Robertson & Drage, 1975).

Our estimate of the activation energy of spiral periods is very similar to that recently reported for autonomous oscillations in cell suspensions (Wurster, 1976) and for autonomous pacemakers on agar (Nanjundiah, Hara & Konijn, 1976). This result suggests common elements in the underlying mechanisms. In fact slight changes in the parameters of an excitable system can permit it to oscillate spontaneously (Wever, 1965; Winfree, 1974). For example, in the model of Goldbeter (1975) stable oscillations occur only within a well defined range of values of ATP input and adenyl cyclase activity. All such parameters vary to some extent from cell to cell, and autonomous pacemakers may simply represent those at the extreme of a distribution of some such critical parameter.

Evolution of wave propagation velocity

We have observed that each individual 'wave' of a spiral or concentric territory propagates with constant velocity over distances of 1 or 2 cm but that the velocity of successive waves declines, the decline being gradual in some instances, and rather abrupt in others. This behaviour has interesting implications, which can best be described by reference to Fig. 3. In this figure, wave 6, for example, as it propagates outwards passes at all points during its passage through cells that propagate it at about 320 μm/min despite the fact that these same cells have previously propagated wave 4 at about 370 μm/min and will shortly propagate wave 8 at about 290 μm/min. The field of cells constituting the aggregation territory evolves, so to speak, from

the centre outward, as though the cells are synchronized not with respect to 'clock time' but rather with respect to 'wave time'. The truly remarkable synchrony achieved is most evident in cases such as the one illustrated in Fig. 5 where the entire field changes its propagation velocity abruptly between waves 7 and 8, and again between waves 10 and 11. Evidently the change is brought about by the excitations themselves.

As cells respond to successive excitations their distribution changes in 2 ways. Their density increases towards the centre as the cells move inwards by chemotaxis (Cohen & Robertson, 1971*b*; Nanjundiah, 1976), and in addition, local signal relay causes the cells to gather together in streams (Nanjundiah, 1973). We do not believe that either density effect can be responsible for the decline in wave propagation velocities seen in our films since each, if it were responsible, would result in variation of individual wave velocity with distance from the centre (Cohen & Robertson, 1971*b*; Nanjundiah, 1976) and also, presumably, in a gradual evolution of wave velocity with time, never abrupt changes. Instead we believe that the excitations probably act by causing a progressive change in some aspect of the intracellular excitation cycle of the cells, for example, in the length of the delay before onset of the phase of cAMP release (Roos *et al.* 1975; Shaffer, 1975) or in the initial rate of release. Alternatively the excitations may activate release of some component, such as cyclic AMP phosphodiesterase, that could affect signal range. Whatever its precise nature, this process appears to be an example of the effect of cAMP signals on the evolution of aggregation competence (Darmon, Brachet & Pereira da Silva, 1975; Gerisch, Fromm, Huesgen & Wick, 1975). Any explanation of it must encompass the fact that the decline in propagation velocity can be either gradual or abrupt, as well as that the abrupt changes, when they occur, are invariably associated with a pronounced delay in the emergence of the next wave. This latter observation, incidentally, provides evidence that the changes in propagation velocity detected by us take place in the cells of the pacemaker loop as well as elsewhere in the field.

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