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Signal Propagation during Aggregation in the Slime Mould *Dictyostelium discoideum*

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SUMMARY

We have analysed the pattern of concentric waves visible at the onset of aggregation of amoebae of *Dictyostelium discoideum* and have shown that each wave consists of a light band of elongated, moving cells, and a darker interband zone of rounded cells. Our analysis supports earlier suggestions that aggregation occurs in response to chemotactic signals emanating from a centre and which are propagated outwards through the field by a relay mechanism. The width of each band of moving cells corresponds to the distance the signal is propagated during the time that the cells remain elongated after stimulation, and does not vary with signal frequency. The width of the interbands of non-moving cells depends upon the distance the signal is propagated *between* signalling events, and varies with the signal frequency which increases during aggregation. The velocity of signal propagation decreases slightly with increase in the density of the monolayer of aggregating cells. We have shown by time-lapse films taken at high magnification that the signal is relayed radially outwards in steps of approximately 57 μm (relay zones) and that the response of each successive zone occurs approximately 12 s after the previous one (relay time). We have attempted to demonstrate the existence of a refractory period for chemotactic responsiveness. Our results indicate that such a refractory period, if it exists, cannot be more than 12 s.

INTRODUCTION

In the life cycle of the cellular slime mould *Dictyostelium discoideum*, growth and differentiation occur separately. Unicellular amoebae feed and divide as long as food bacteria are present. When the bacteria are removed, the amoebae enter a period called interphase during which they develop the ability to aggregate at a central collecting point. The multicellular structure formed as a result of aggregation ultimately develops into a fruiting body from which spores may be dispersed to germinate as unicellular feeding amoebae (see reviews by Raper, 1940; Shaffer, 1962; Bonner, 1967; Gerisch, 1968; Newell, 1971).

Aggregation occurs by chemotaxis (Bonner, 1947). Signalling amoebae emit an attractant, called acrasin by Bonner (1947), which is probably cyclic AMP (Barkley, 1969; Konijn, Barkley, Chang & Bonner, 1968). Amoebae within range of a sufficient concentration of the diffusing signal respond by movement towards the signalling 'centre' cell(s). The responding cells show periodic movement steps suggesting that signal emission is itself periodic (Shaffer, 1962; Gerisch, 1965). The periodicity of response decreases during aggregation (Arndt's film, cited in Shaffer, 1962; Durston, 1974). Responding cells move towards each other and form streams moving towards the centre. The streams themselves also produce acrasin (Bonner, 1949; Shaffer, 1957*b*). These observations suggest that signalled amoebae respond not only by an inward movement step, but also by relaying the signal (Shaffer, 1957*b*),

perhaps again in the form of cyclic AMP. Thus there need not exist a steady gradient in concentration of acrasin from the centre outward; rather signalling 'secretory fronts' are propagated out by relay through the aggregation territory (Shaffer, 1957*b*; Gerisch, 1968; Cohen & Robertson, 1971*a*).

Unidirectional propagation of the signal, and centripetal cell movement, may require that cells become refractory for some time after each stimulation so as to prevent them from responding to acrasin released in turn by more distal cells (Gerisch, 1965; Cohen & Robertson, 1971*a*). They would have to remain refractory at least until the signal released distal to them is reduced to below threshold levels. This reduction is thought to be accomplished by inactivation of the attractant (Shaffer, 1957*b*). If this is cyclic AMP, inactivation would be brought about by the phosphodiesterase bound to the cell membrane and released into the medium (Chang, 1968; Malchow, Nägele, Schwarz & Gerisch, 1972; Pannbacker & Bravard, 1972; Malkinson & Ashworth, 1973). The phosphodiesterase, in destroying the cyclic AMP, would also permit detection of the next signal propagated from the centre.

Waves of inward movement spreading out from a signalling centre have been demonstrated by time-lapse films (Bonner, 1944, cited in Bonner, 1967). Moreover, early in aggregation, characteristic patterns of concentric bands may be visible (Gerisch, 1965). This paper reports an analysis of this band pattern and shows the individual relay events involved in signal propagation. We have also attempted to demonstrate the existence of a refractory period for chemotactic responsiveness.

METHODS

Strains. Dictyostelium discoideum strain NC4, the axenic strain AX2, and the bacterial associate, *Aerobacter aerogenes*, were kindly provided by Dr J. Ashworth.

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 (Sussman, 1951) contained (g/l KK_2 buffer): Bacto-peptone (Difco), 5; yeast extract (Difco), 0·5. For SM agar, 20 g Bacto-agar (Difco) were added to 1 l SM broth. After autoclaving (15 lb/in^2, 15 min), sterile glucose was added to SM broth and SM agar to 0·5% final concentration. Axenic broth, HL5 (Watts & Ashworth, 1970) contained (g/l water): bacteriological peptone (Oxoid), 14·3; yeast extract (Difco), 7·15; $Na_2HPO_4.12H_2O$, 1·28; KH_2PO_4, 0·49; D-glucose, 15·4; pH 6·7. Non-nutrient (NN) agar, contained: Bacto-agar (Difco), 10 g; KK_2 buffer, 1 l.

Growth conditions. Strain NC4 was grown on SM plates in association with *A. aerogenes*. Fifteen spore heads (approx. 3×10^6 spores) and 0·2 ml of an overnight bacterial culture were spread over the surface of each plate. Vegetative amoebae were harvested after 24 h incubation at 22 °C, and the bacteria removed by four centrifugations at 400 *g* for 1 min, resuspending the amoebae each time in ice-cold KK_2.

Strain AX2 was grown in liquid culture (in 10 ml HL5 in a 100 ml Erlenmeyer flask) at 22 °C on a rotary shaker (150 rev./min.). The amoebae were harvested in exponential phase (5×10^6/ml), washed by centrifugation, and resuspended in ice-cold KK_2.

Cell densities were determined in a Petroff–Hauser counting chamber and adjusted by dilution as required, before the cells were transferred to agar or coverslip for aggregation.

Aggregation. Amoeba suspension (0·5 ml) was spread over the surface of fresh NN agar plates (5 ml NN agar in $8 \times 5 \times 1{\cdot}8$ cm plastic boxes). The amoebae were allowed to settle and disperse for 30 min and the agar surface drained of excess liquid. Partial evaporation of the residual liquid film was allowed by leaving the boxes open for 20 min. Our technique regularly produced fields of amoebae of uniform density.

For AX2, aggregation could also be followed on a glass coverslip. The amoeba suspension (0·01 ml) was spotted on to an alcohol-washed coverslip, the amoebae were allowed to settle for 5 min, and then washed free of growth medium by immersion of the coverslip in KK_2 buffer. Excess liquid was drained and the coverslip then inverted over a humidity chamber. This chamber had been prepared by fixing a clean coverslip over a hole drilled in a plastic microscope slide and then partially filling the hole with 10% (w/v) gelatin. The upper coverslip, bearing the amoebae, was sealed to the slide with paraffin. The time required for aggregation depends on the density of amoebae in the monolayer and the volume of liquid remaining on the coverslip after draining. These conditions were carefully controlled.

The preparations developing aggregation competence were incubated at 22 °C. Aggregation commenced at times ranging from 6 h for 5×10^5 amoebae/cm^2 to 13 h for 5×10^4 amoebae/cm^2. High-density preparations were sometimes kept at 7 °C for 18 h and then incubated at 22 °C until aggregation commenced. This took place within 1 h. The wave patterns obtained by both methods were similar.

Cell densities were checked by counting cells in the field under study, using calibrated eyepiece graticules, and confirmed in the films and photographs used in the analyses presented in this paper. We were careful to ensure that the fields studied were uniform in density.

The intercellular distance was calculated as $2/\sqrt{(\pi N)}$, where N is the cell density (Cohen & Robertson, 1971*a*). In this formula we approximate to a uniform distribution of amoebae, in which each cell is equidistant from its neighbours, by regarding the field as a network of contiguous circles centred on the individual cells. The diameter of the circles then corresponds to the distance between the *centres* of neighbouring cells.

Optical methods. A Wild M20 microscope was used for phase-contrast observation. It was equipped with a humidity chamber fitting both the slide carrier and the boxes used for aggregation. Fogging in the objectives was avoided by a fitted warming sleeve which raised the temperature in the objective by not more than 0·5 °C. A Bolex H16 Reflex cine-camera and a Paillard/Wild Variotimer were used for time-lapse films, which were taken at a speed of 1 frame/4 s and analysed frame by frame, or in sets of frames, in a Litax film analyser. Photographs were taken with a Rada roll-film adaptor. Films and photographs were calibrated by filming or photographing a micrometer at the same magnification as used for the experimental material. For direct measurements under the microscope a calibrated eyepiece was used.

RESULTS

The concentric wave pattern

Territories exhibiting concentric wave patterns during the early stages of aggregation are shown in Fig. 1. Provided the amoebae are spread evenly and the surface moisture is controlled (see Methods) we consistently observed these patterns in populations of amoebae spread on the surface of buffered agar at densities ranging from 10^5 amoebae/cm^2 to 5×10^5 amoebae/cm^2 (an almost confluent monolayer). We used 1% rather than 2% (w/v) agar, for better optical definition. Gerisch (1965) published similar photographs of wave patterns that arose when amoebae were spread in dense multilayers. Microscopical examination of fields such as those in Fig. 1 showed that at any instant the light bands consisted of elongated moving cells, while the darker interband areas contained rounded cells exhibiting randomly-oriented pseudopodia. Contiguous areas of the two cell forms are seen in Fig. 2. The concentric wave pattern may easily be understood if each band of moving cells corresponds to the zone of influence, at a given time, of a wave of stimulation propagating outwards from the centre of the aggregation field. The darker interband areas would then contain cells

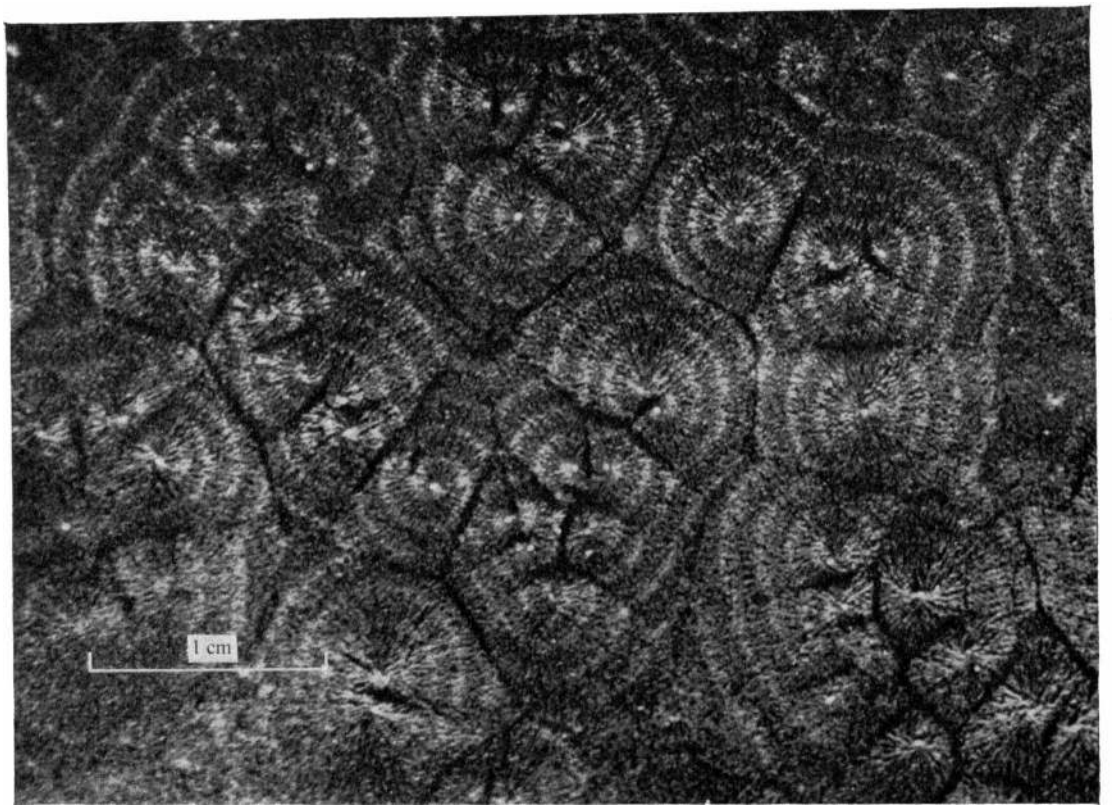

Fig. 1. The concentric wave pattern in fields of *D. discoideum* NC4 aggregating on the surface of NN agar (cell density 10^5 amoebae/cm^2). The light bands are bands of moving cells. Some spiral wave patterns are also present (see Gerisch, 1971). The photograph (by J. Kinross) was obtained with a camera fitted with a 4 × 5 in sheet film back and Ilford FP4 film (exposure 1/8 s at $f = 4{\cdot}7$). A Kodak cold light illuminator and a 180 mm condenser were set to provide a form of dark-field illumination.

which have ceased to move in response to a signal that has just passed them and which have not yet been stimulated by the next signal. This explanation was confirmed by examining the same group of cells for some time, either directly or in time-lapse films. The cells alternated between the elongated form during active inward movement, and a more rounded shape during their less active, 'non-moving' phase. Similar regular changes of form have been observed previously by Gerisch (1964), studying aggregating cells of *Dictyostelium purpureum*. We detected no systematic difference in cell density between light areas of moving cells and darker areas of non-moving cells. The visible pattern presumably depends on differences in the light-scattering properties of the two types of area.

The width of bands of moving cells represents the distance the signal travels in the time that cells remain elongated after stimulation; it should therefore not vary with signal frequency. The interband distance, on the other hand, *should* depend on signal frequency since it reflects the distance that waves are propagated in the time elapsing between successive signals. This prediction can be tested since we observed that during the course of aggregation the period of signalling within a given territory decreases from 10 min to 3 min or less, by which time the cells are beginning to form streams and it becomes hard to discern individual movement steps (see also Durston, 1974). Table 1 shows results obtained by

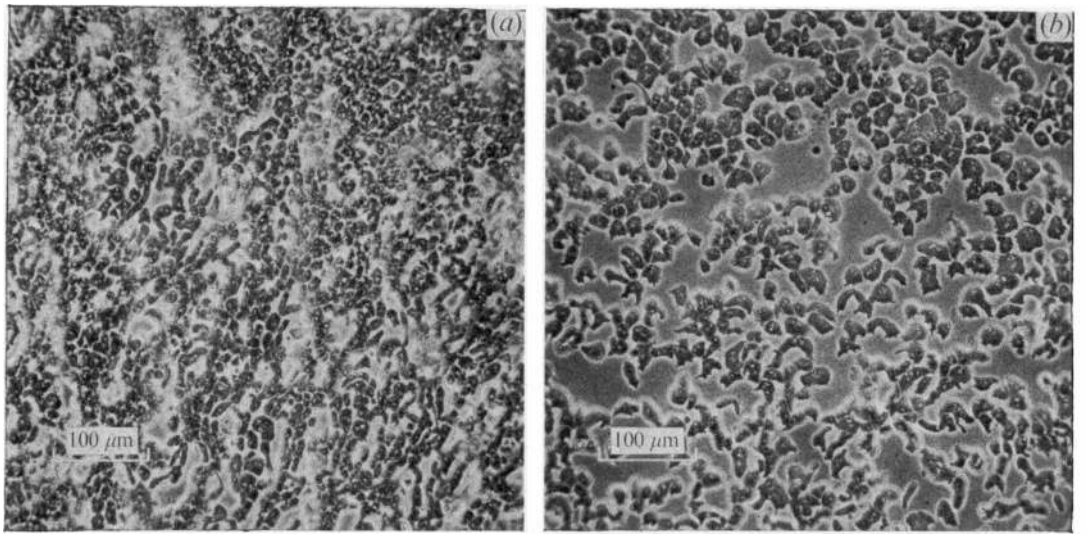

Fig. 2. The junction between a movement band and an interband in the concentric wave pattern of *D. discoideum* NC4. Cell densities: (*a*) 5×10^5 amoebae/cm^2; (*b*) $1{\cdot}25 \times 10^5$ amoebae/cm^2.

Table 1. *Variation of wave pattern with signal period in D. discoideum* NC4

Cell density* (amoebae/cm^2)	Signal period (s)	Movement band† (μm)	Interband (μm)	Wavelength‡ (μm)	Velocity§ (μm/s)
$1{\cdot}25 \times 10^5$	224	536	780	1316	5·9
(32)	184	489	451	940	5·1
$1{\cdot}5 \times 10^5$	480	470	1645	2115	4·4
(29)	330	470	1410	1880	5·7
	290	470	705	1175	4·1
	239	470	564	1034	4·3
$1{\cdot}7 \times 10^5$	300	705	752	1457	4·9
(27)	290	658	658	1316	4·5
	285	658	611	1269	4·5
	275	658	658	1316	4·8
	270	658	611	1269	4·7
	260	611	752	1363	5·2
$2{\cdot}5 \times 10^5$	600	470	1645	2115	3·5
(22·5)	530	470	1880	2350	4·4
	530	470	1457	1927	3·6
	330	470	940	1410	4·3
	310	470	658	1128	3·6
	285	470	752	1222	4·3
5×10^5	600	470	2021	2491	4·2
(16)	580	470	2021	2491	4·3
	290	470	564	1034	3·6
	275	470	564	1034	3·8

* The intercellular distances, in μm, are given in parentheses.

† The velocity of signal propagation can be calculated by dividing the movement band widths in this column by 100 s, the movement duration (see text).

‡ Sum of the widths of a movement band and the corresponding interband.

§ Derived from wavelength/signal period.

Table 2. *Velocity of signal propagation determined by measuring the time interval between movement responses of individual cells*

Strain	Cell density* (amoebae/cm²)	Distance between chosen cells (μm)	Interval between responses† (s)	Velocity‡ (μm/s)
NC4	5×10^4	143	16(2)	8·9
	(51)	128	16(3)	8·0
		114	16(3)	7·1
		70	16	4·4
		50	12(7)	4·2
NC4	$1{\cdot}25 \times 10^5$	230	32	7·2
	(32)	215	40	5·4
		138	24	5·8
		136	32	4·3
		130	28	4·6
		100	12	8·3
NC4	$1{\cdot}5 \times 10^5$	180	32(4)	5·6
	(29)	115	24(3)	4·8
		115	20(3)	5·8
		108	24(3)	4·5
		108	20(2)	5·4
		108	16(2)	6·8
		186	34(2)	5·5
		100	17	5·9
		93	17(2)	5·5
		86	17	5·1
AX2	2×10^5	180	47(5)	3·8
	(25)	160	46(2)	3·5
NC4	4×10^5	255	64(8)	4·0
	(18)	210	68(8)	3·1
		158	32(8)	4·9
		115	32(8)	3·6
		65	20(8)	3·3
		74	12(8)	6·2
NC4	5×10^5	104	32	3·3
	(16)	104	28	3·7

* The intercellular distances, in μm, are given in parentheses.

† Where more than one determination has been made the number of determinations is indicated in parentheses.

‡ Since we show below that the signal is actually propagated in steps of about 57 μm this method of determining the velocity is subject to error, especially for pairs of cells that are close together.

microscopic observation (see Fig. 5) of fields exhibiting wave patterns. The signal period was measured by timing the interval between movements of a selected cell in response to two successive signal fronts. The width of the corresponding movement band was immediately recorded and the width of the corresponding interband measured by scanning the preparation outwards from the movement band. Variation in signal frequency affects the width of the interbands of non-moving cells, whereas the width of the movement bands is almost constant.

It is possible to derive several independent estimates of the velocity of signal propagation. First, since the wavelength corresponds to the distance over which a signal is propagated before the next signal leaves the centre, the velocity is given by dividing the wavelength by the signal period, as in Table 1. Second, if the width of the movement band corresponds to the distance the signal is propagated in the time cells remain elongated, the velocity of wave

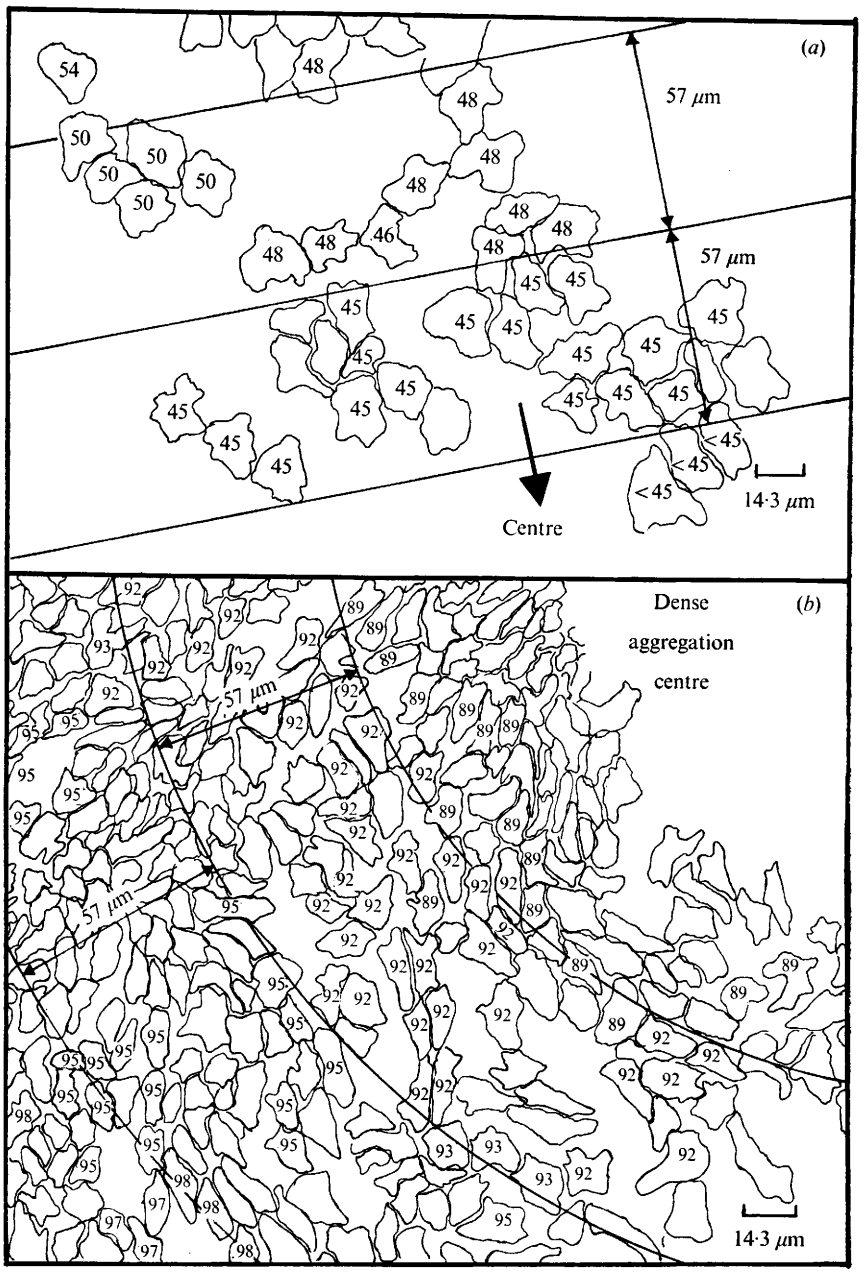

Fig. 3. The range of the relayed signal and the relay time. A field of amoebae was traced from a time-lapse film of aggregating *D. discoideum* NC4. The film was run repeatedly through the same sequence and the frame (frames at 4 s intervals) in which each amoeba started to move in response to a particular signal front was recorded; (*a*) and (*b*) correspond to independent experiments. In (*a*) unnumbered amoebae showed no marked movement response. In (*b*), not all of the amoebae were analysed and in many cases discrete movement steps could not be distinguished owing to the close proximity of the cells. Cell densities: (*a*) $2{\cdot}3 \times 10^5$ amoebae/cm^2; (*b*) $7{\cdot}3 \times 10^5$ amoebae/cm^2. The lines have been drawn knowing the position of the centre, and in such a way as to include as many 'simultaneously' responding amoebae as possible.

Table 3. *Variation of velocity of signal propagation with intercellular distance*

Cell density (amoebae/cm²)	Intercellular distance (μm)	Velocity* (μm/s)
5×10^4†	51	6·89 ± 0·67(3) 6·30 ± 0·42(7) 5·64 ± 0·41(5) 5·47 ± 0·39(16)
$1{\cdot}25 \times 10^5$	32	5·77 ± 0·18(43) 5·23 ± 0·20(7)
$1{\cdot}5 \times 10^5$	29	6·44 ± 0·40(7) 4·75 ± 0·10(10) 4·70‡(4)
$1{\cdot}9 \times 10^5$	26	5·28 ± 0·27(11)
$2{\cdot}5 \times 10^5$	22·5	6·48 ± 0·47(8) 5·63 ± 0·16(68) 4·76 ± 0·14(8)
5×10^5	16	5·51 ± 0·28(8) 4·70‡(5) 4·70‡(5)

* Measured movement band widths divided by 100 s, the movement duration (see above). The results are expressed as mean velocities with standard errors for 95 % confidence limits. Separate experiments are recorded on different lines. Number of determinations are in parentheses.

† The wave pattern in fields of this density is not visible to the naked eye but can be seen clearly under the microscope. Aggregation is delayed, compared with fields of higher densities, and can take up to 13 h.

‡ No variation was observed.

propagation would also be obtained by dividing this time into the movement-band width. We have measured the movement duration (maintenance of elongated shape) by analysis of time-lapse films and found a mean value of 99·5 (S.D. 14·1) s, in agreement with the value of 100 s reported by Cohen & Robertson (1971*b*). The velocities of signal propagation which would be obtained by dividing the movement-band widths in Table 1 by this value agree well with those listed in the last column. Finally, we determined the velocity by measuring the time interval between the movement responses of individual cells a known distance apart in the region of the advancing wave front (Table 2).

Range of relayed signal and relay time

If the signal emanating from a centre is propagated outwards by a succession of discrete relay steps (Shaffer, 1962; Gerisch, 1965; Cohen & Robertson, 1971*a*) its velocity should depend on the number of relay events per unit time and on the range of influence of each event. We have attempted to obtain independent measurements of these two parameters Figure 3 shows analyses of time-lapse films of aggregating *D. discoideum* NC4 where we have determined the film frame in which individual amoebae start to move in response to a signal propagating across the field. Two independent experiments were analysed. Blocks of amoebae measuring about 57 μm across generally respond in the same film frame (i.e. within 4 s of each other). This demonstrates signal relay directly, and shows that the range of the relayed signal is about 57 μm. The responses of successive blocks of amoebae are separated by two, or more frequently three, film frames. The relay time is therefore less than, but close to, 12 s. The range of influence of the relayed signal divided by the relay time yields a minimum estimate of the velocity of signal propagation (57 μm/12 s = 4·75 μm/s). This is in satisfactory agreement with the previous measurements of velocity.

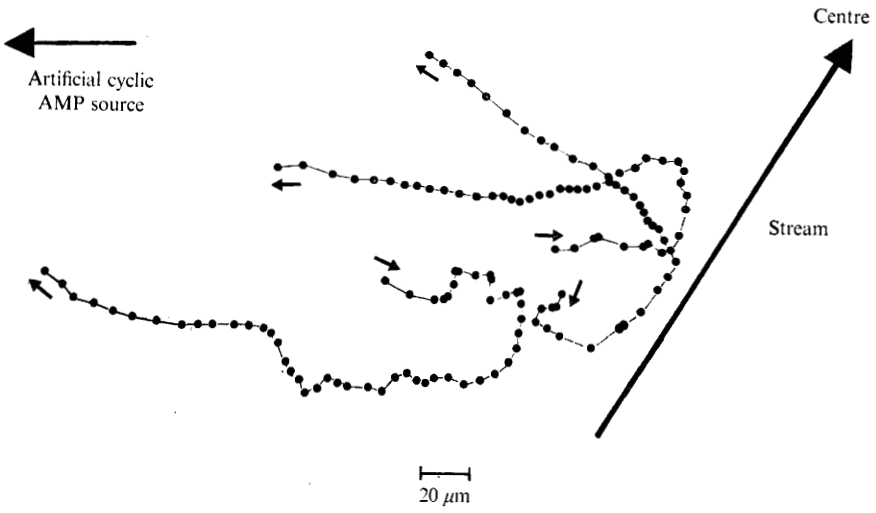

Fig. 4. Analysis of a time-lapse film showing the path tracks of three AX2 amoebae initially carrying out discrete movement steps towards a natural centre with a period of about 3 min. A small filter-paper square saturated with a cyclic AMP solution (2 μl of a 10^{-3} M solution) was placed in the field at a distance of 1 mm in the direction indicated. Within 25 min the amoebae were diverted from the natural centre by the artificial cyclic AMP source and moved continuously and with increasing speed towards it. Later, outside the field shown, their speed reached a maximum of 0·5 μm/s. The points represent the position of the centre of each cell at 60 s intervals.

Effect of cell density on velocity of signal propagation

Since the range of influence of the relayed signal as well as the relay time are approximately the same in the two populations analysed in Fig. 3, we have derived the same estimate of velocity for them. It is possible to evaluate more reliably the dependence of velocity of signal propagation on cell density from the data given in Tables 1 and 2 as these were obtained in populations with cell densities varying from 5×10^4 to 5×10^5 amoebae/cm^2. The corresponding intercellular distances (see Methods) range from 51 to 16 μm respectively. There is no marked dependence of velocity on density, but the velocity declines somewhat with increase in density. More extensive data point to the same conclusion (Table 3). Student's 't' test on the correlation coefficient (eqn 39, Bailey, 1972) between velocity and intercellular distance in each of the three tables indicated that the correlation for these two variables was significantly different from zero ($P \leqslant 0{\cdot}001$). We conclude that there is a dependence of velocity on density.

Refractory period for movement response

To ensure centripetal movement of responding cells and outward propagation of the signal, it would seem that cells must become refractory to stimulation for some time after being signalled (Gerisch, 1965). The refractory periods for the two components of the response, namely oriented movement and signal relay, need not be the same. Robertson *et al.* (1972) and Durston (1974) presented evidence for a refractory period for relay and showed that it decreases during aggregation from about 7 to 2 min.

Table 4. *Consecutive directional path times in cells stimulated from different directions*

Strain	Cell	Directional path time* (s)	Minimum directional path time (s)
NC4	1	24, 12, 20, 12, 16, 16, 20, 16	12
	2	16, 16, 12	12
	3	16, 12, 36, 16, 24, 32, 24, 20	12
AX2	4	12, 20, 32	12
	5	16, 20, 16, 12, 32, 44, 44, 28, 40, 28	12
	6	68, 16, 36, 28, 24, 60, 20, 32, 20, 28, 24, 20, 28, 32, 44, 40, 24	16
	7	12, 36, 36, 24, 16, 12, 56, 44, 56, 24, 32, 16, 120, 16, 20, 16	12
	8	16, 48, 48, 48	16

* The short stationary periods between directional displacements which occur in some directional changes were added to the path time in the new direction.

We have attempted to detect a refractory period for movement response. A cell responding periodically to a natural centre with a movement duration of 100 s can be made to move continuously towards a constant artificial cyclic AMP source (Fig. 4). The refractory period for movement response for the cells in Fig. 4, initially moving with a period of 3 min, was $\leqslant$ 100 s. We also attempted to measure the minimum unidirectional path time of cells whose movement response demonstrated that they were being signalled successively from different directions. By analysing successive frames of time-lapse films we have determined the directional path times of isolated cells in the neighbourhood of streams in an aggregation field. Such cells (cells 1 to 5, Table 4) first move obliquely to the stream, in the direction of the centre. After that they make further movement steps more nearly perpendicular to the stream and even biased away from the centre, as they are stimulated by the relayed signal passing down the stream. The directional path times for cells 6, 7 and 8 in Table 4 correspond to directional changes observed in the 'zig-zag' path tracks of cells which were approximately equidistant from two streams moving continuously towards an artificial cyclic AMP source.

The minimum unidirectional path time for individual cell tracks was most commonly 12 s (Table 4). We conclude therefore that the refractory period for movement response is probably not more than 12 s. However, we have also observed (unpublished) that 12 s is the minimum time required for cell displacement, that is, for contraction of the back of the cell following directional pseudopod formation. This approach therefore establishes only an upper limit for the refractory period for movement response.

DISCUSSION

Our interpretation of the concentric wave pattern analysed in this paper is given in Fig. 5. Near the bottom of the field is an aggregation centre, depicted formally as a single cell, to which other cells in the field are responding. The field is divided into concentric zones representing the discrete 57 μm steps by which signals are relayed out from the centre. The visible pattern depends on the alternation of bands of elongated moving cells (MB) and of relatively non-motile, rounded cells (IB). Each movement band comprises the cells responding at a given moment to a signal relayed out from the centre. The band itself moves outwards as

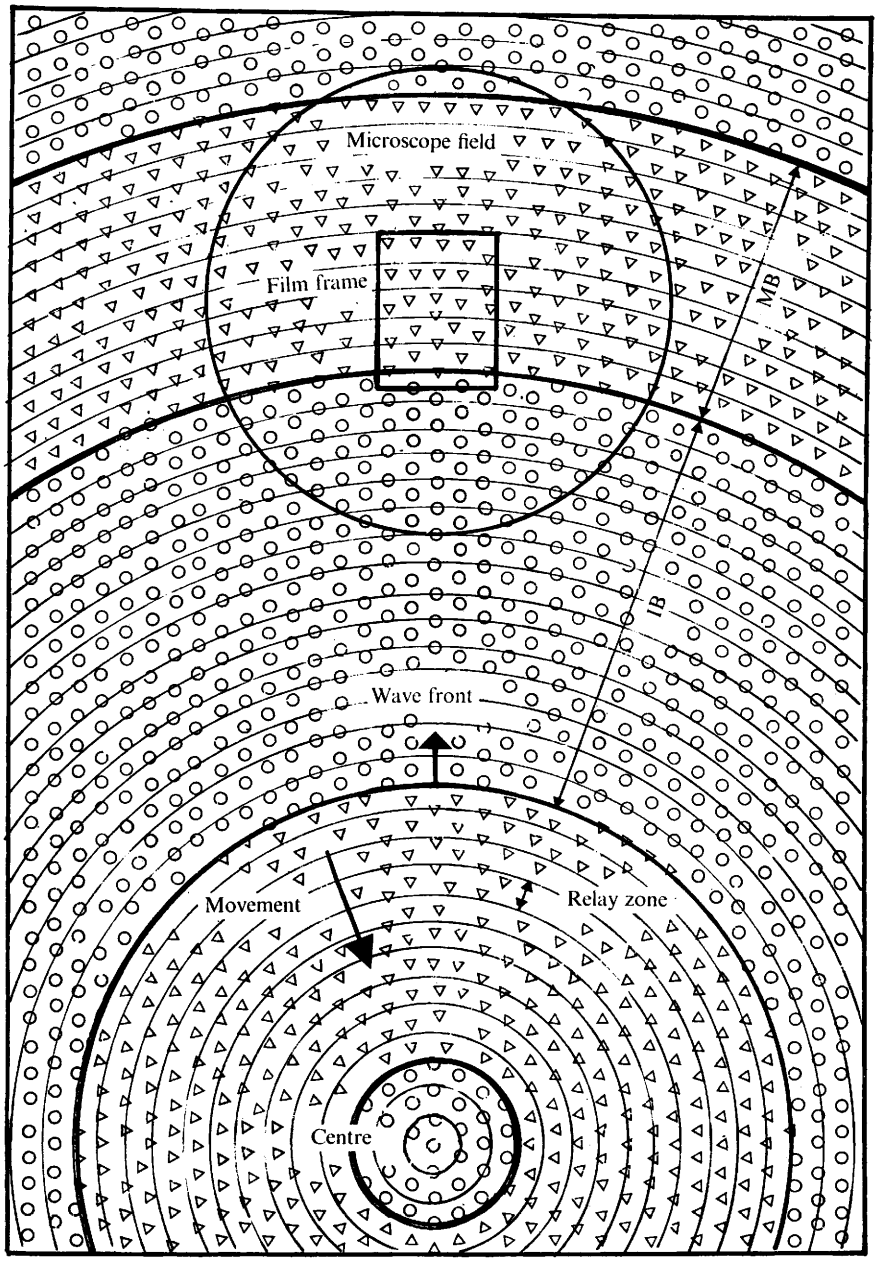

Fig. 5. Model of signal propagation. The field of amoebae is divided into concentric relay zones. Each relay zone is depicted as encompassing only one or two amoebae. This would be true for sparse aggregation territories. For denser territories see text and Fig. 3. Two wave fronts corresponding to two consecutive signals from the centre are shown propagating outwards. The direction of cell movement is inward as shown. The sizes of the microscope field and film frame used to collect the data presented in this work are indicated, in relation to the size of the aggregation territory as a whole. MB, movement band; IB, interband; △, moving cell; ○, non-moving cell.

cells in its most proximal zone complete their inward movement step and cells in its most distal zone relay the signal ahead of them. We have drawn each movement band as comprising 10 relay zones; this is the approximate number obtained by dividing the movement duration of 100 s by the relay time of slightly less than 12 s, as well as by dividing the movement band width (470 to 705 μm) by the range of influence of the relayed signal (57 μm). The value of 57 μm for signal range agrees well with our measurement of 51 μm for the maximum intercellular distance permitting aggregation to occur (unpublished observations; see also Konijn & Raper, 1961, Gerisch, 1961). With a signal propagation velocity of about 350 μm/min and an initial territory size of up to 10000 μm in radius, one signal could take 30 to 40 min to reach the edge of the aggregate; if the signalling period were 5 min, 6 to 8 signals would be propagating outwards at the same time (see Gerisch, 1968).

Cohen & Robertson (1971*a*) have calculated the delay between a cell s being signalled and its relaying the signal (relay delay time) to be 15 s. This is to be compared with our upper estimate of 12 s for the relay time, which includes this delay as well as the presumably very short time required for the signal to diffuse from one zone to the next. The calculation of Cohen & Robertson is based on the assumption that the signal is relayed from cell to cell (10 μm) in a dense population, and on the velocity of signal propagation of 43 μm/min obtained by Gerisch (1965). Our results show that in almost confluent populations the range of the relayed signal encompasses 4 to 6 cells, and Cohen & Robertson (1971*a*) have themselves reported 'near simultaneous pulsatile movement of entire areas of amoebae occurring during aggregation'. Gerisch (1965) was using multilayered populations, where signal range may well be decreased. Certainly our velocity of signal propagation of about 350 μm/min is considerably greater than the 43 μm/min observed by Gerisch (1965) for *D. discoideum* strain V12 and the 28 to 50 μm/min observed by Samuel (1961) for *D. purpureum*. On the other hand it is somewhat slower than the 500 μm/min observed in films by Bonner (cited in Shaffer, 1962). These differences may be due to the strain employed or to experimental conditions. The velocity of signal propagation decreases somewhat with increase in cell density (Tables 1, 2 and 3). We cannot account for this dependence but it is clear that the velocity of signal propagation could be affected by changes in the balance between cyclic AMP output (assuming that cyclic AMP is the signal) and phosphodiesterase activity (Cohen & Robertson, 1971*a*). It is also possible that a feedback control mechanism operates, e.g. cyclic AMP output per cell may be inversely related to cell density due to control exerted by external cyclic AMP (Shaffer, 1962).

The minimum refractory period required to ensure centripetal cell movement in uniform fields of amoebae can be derived by the following argument, using our upper limit of 12 s for the relay time. A cell stimulated at time $t = 0$ s must become refractory at some time before emitting its own pulse of cyclic AMP i.e. by $t = 12$ s; it must remain refractory until cells in the relay zone distal to it have themselves signalled (at $t = 24$ s), and until that signal has been degraded by phosphodiesterase to below threshold level. Since Cohen & Robertson (1971*b*) have calculated the duration of the chemotactic signal to be a fraction of a second, the minimum refractory period would be about 12 s. We have analysed the movement behaviour of single cells in the neighbourhood of streams. They showed several changes of direction, presumably due to successive stimulations by a signal propagating down the stream. From the minimum directional path time of these cells we have concluded that the upper limit to the cell refractory period for movement response is 12 s. It is therefore close to the minimum tolerable duration of the refractory period. Given a short refractory period for movement response, the net effect of individual cells sensing a signal passing down a stream would be to draw them into the stream. This would explain the

previously somewhat puzzling observations of Shaffer (1957*a*), that responding cells appear to approach a stream perpendicularly rather than biased towards the centre.

There remains the question of why cells in early aggregation remain elongated for 100 s when the signal is of very short duration. We have observed that the movement step of cells travelling in one direction frequently shows a fast component lasting for around 20 to 40 s, followed by progressively slower movement until the cell comes to rest (unpublished observations). The latter phase could represent residual directional movement and elongation that is maintained unless the cell is otherwise stimulated.

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