STIMULATION OF LATE INTERPHASE *DICTYOSTELIUM DISCOIDEUM* AMOEBAE WITH AN EXTERNAL CYCLIC AMP SIGNAL

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ABSTRACT The microelectrode system described in the accompanying paper was used to investigate properties of fields of *Dictyostelium discoideum* amoebae in late interphase. Cells in the fields were competent to respond chemotactically to, and to relay, a c-AMP signal, but not to produce an aggregative signal autonomously. The experimental results are generally consistent with c-AMP being the sole compound required for chemotaxis and signal relaying. A periodic signal from the microelectrode can initiate and control aggregation and can compete with spontaneously arising aggregates. The electrode was used to measure the refractory period for relaying which decreases from 9 min or more to between 2 and 3 min with increasing developmental age, and to measure thresholds for chemotaxis and signal relaying. The results are discussed in relation to models for the control of aggregation in *D. discoideum*.

INTRODUCTION

In the accompanying paper (Cohen et al., 1975) we described the microelectrode system with which we have been measuring some parameters of the *D. discoideum* aggregative signal. In this paper we report some of our results with a general description of the experiments. In order to put our work into context so that the experiments may be understood properly, we first outline what is already known of aggregation and what we have been able to deduce from our own previous observations of films.

Amoebae remain in the vegetative state until their food supply is exhausted. The period between the vegetative and aggregative phases is called interphase (Bonner, 1963). Cells can be prevented from entering interphase by a dialysable substance or substances released by the food bacteria (Shaffer, 1966). They are therefore trophically influenced by a bacterial metabolite, although this has not been identified. To set a population of amoebae into interphase as synchronously as possible it is thus necessary to wash them by centrifuging them free of food and to plate them out on non-nutrient, plain buffered agar (Bonner, 1967).

A population of cells treated in this way develops during interphase a series of competences required for aggregation (Cohen and Robertson, 1972). We have called the proportion of amoebae in a population which has attained a competence $X_i(t)$, where $i = 1$ for chemotactic sensitivity, 2 for the capacity to relay an aggregative

signal, and 3 for the capacity to produce aggregative signals autonomously (Cohen and Robertson, 1974). Interphase lasts for 9 h at 21°C (Gerisch, 1968); after 9 h some cells begin to produce autonomous signals ($X_3 \neq 0$). There is good circumstantial evidence that the chemotactic attractant molecule released by amoebae is cyclic adenosine monophosphate (c-AMP) (Konijn et al., 1967). In addition the amoebae secrete a phosphodiesterase (PDE) which breaks down c-AMP (Chang, 1968).

The signal released by autonomous cells is periodic with a mode close to 5 min (Durston, 1974). Amoebae responding to an autonomous signal both move towards the signal source chemotactically and relay an apparently identical signal (Shaffer, 1957; Bonner, 1967; Cohen and Robertson, 1971 *a*). As there is a critical density of amoebae, N^*, below which an aggregative signal cannot be relayed, we can infer that, as the signal has a finite range, there is a threshold concentration of attractant which must be exceeded for relaying to be induced (Cohen and Robertson, 1971 *a*). The range will be a function of the ratio of signal amplitude to threshold concentration, the diffusion constant, D, of the signal molecule, PDE activity, and amoeba density. As a first approximation we can assume that the threshold, C^*_{Rel} is fixed once $X_2(t) = 1$, which occurs at about 9½ h (Gingle, 1975, unpublished results). In addition, there is a threshold for chemotaxis, C^*_{Chem}, such that $C^*_{\text{Chem}} \leq C^*_{\text{Rel}}$ as we have argued (Cohen and Robertson, 1971 *b*).

$X_3(t)$ never exceeds 1%, and in a dense population of amoebae it is much smaller (Konijn and Raper, 1961; Raman, Hashimoto, Cohen, and Robertson, unpublished experiments). Few cells, therefore, ever become capable of initiating aggregation; there is no evidence for or against the idea that these cells represent a special (predetermined) subset of amoebae that have passed through interphase.

Once an amoeba has been stimulated by an attractant concentration above C^*_{Rel} it becomes refractory (Shaffer, 1957; Gerisch, 1965) to further stimulation for a time that may depend on age as measured from the time of centrifugation (Durston, 1974). The refractory period guarantees unisensal propagation of a signal from a center; if there were no refractory period, an amoeba could be stimulated by a reflection of its own signal from its peripheral neighbors (Cohen and Robertson, 1971 *a*).

These properties of the signal, and the amoebae's responses, lead to the periodic relaying of centrifugal waves of centripetally moving cells, which can clearly be seen in films of aggregating amoebae and have been described by many authors (reviewed by Bonner, 1967). The cells move stepwise, for about 2 min in response to each signal (Gerisch, 1968, 1971; Cohen and Robertson, 1971 *b*). The movement is probably ballistic in the sense that it represents, as does relaying, a relatively long all-or-nothing response to a brief signal.

Waves can be propagated from aggregation centers in forms topologically equivalent to spirals or expanding circles. If they are initially spiral, their observed period declines smoothly from longer than 7 min to about 2 min. If they are concentric, their period may first be approximately 10 min, switching abruptly to about 5 min, thereafter declining smoothly to 2 min (Durston, 1974). We have argued that the period of a spiral signal, for geometric reasons, represents the mean refractory period of an aggre-

gating field, while the switch of concentric periods from 10 to 5 min occurs when the mean refractory period of the field falls below 5 min (Robertson, 1974; Durston, 1974). We have interpreted these results as evidence for periodic autonomous signals which are at first gated by the refractory period of the field (Durston, 1974). That is, when the field's mean refractory period, T_r, is longer than the autonomous period, T, the field can only respond to every second autonomous signal; when $T_r < T$ then the field can respond to every autonomous signal. In addition we have suggested that the smooth decline in period from 5 min to $2\frac{1}{2}$ min occurs because the tip, which forms on top of the aggregate just after the switch to a 5 min period and which controls subsequent morphogenesis, releases attractant continuously such that C^*_{Rel} is always exceeded in the vicinity of the tip (Rubin and Robertson, 1975). The tip therefore causes signal relaying at T_r, which itself declines to about 2 min, as is implied by our observations of spiral periods.

These interpretations depend on results from many sources and constitute a model of the signaling system which is to be tested (Cohen and Robertson, 1971 *a*, *b*). In this paper we test the following features of the model, making quantitative measurements where appropriate and feasible: (*1*) Thresholds for chemotaxis and relaying, C^*_{Chem} and C^*_{Rel}. (*2*) Presence and length of refractory period, T_r. (*3*) All-or-nothing nature of signaling and relaying response. (*4*) Periodicity of centers. (*5*) What is the effect of a continuous, as opposed to a periodic, source? (*6*) Can an artificial c-AMP source replicate all the qualitative features of a natural center?

In most of the experiments reported in this paper, we used cells 8 h old, when $X_1(t) = 1, X_2(t)$ is increasing towards one, but $X_3(t)$ is still zero. Thus, the amoebae were competent to respond to signals, but did not release autonomous signals which would have interfered with the experiments. We used c-AMP exclusively not because we knew that it was the sole aggregative agent, but in order to determine whether or not all our results were consistent with this hypothesis, which is based on circumstantial evidence only. We shall also refer in the discussion to other features of aggregation which emerged during the course of our experiments, relating them where possible to observations in the literature, and to our own earlier results.

MATERIALS AND METHODS

D. discoideum, strain NC-4, was obtained from K. B. Raper and cultured in association with *Aerobacter aerogenes* on buffered nutrient agar, pH 6.5. Plates were incubated for 24 h, when all cells were still growing vegetatively. The amoebae were then washed by centrifugation, in buffer at 4°C, for three runs of 1 min at 600 *g*.

The washed amoebae were plated on non-nutrient buffered agar at densities between 1 and 2×10^6 cells/cm^2. At the end of 8 h a microelectrode was introduced as described in the preceding paper (Cohen et al., 1975). The electrolyte contained the same buffer as the agar and 10^{-3}, 10^{-4}, 10^{-5}, or 10^{-6} M c-AMP. The tip of the microelectrode was biased positive with respect to the grounded agar to retain the negatively charged c-AMP ions. The bias was reversed for 1.5 s every 5 min to drive out fixed amounts of c-AMP. Total current flow through the pipet tip was 10μA. All other experimental details were as described in the preceding paper.

The preparations were either filmed by time-lapse cinemicrography with a Bolex 16 mm

camera (Bolex-Paillard S.A., Zurich, Switzerland) at 8 frames/min and a total magnification of 2.5 or were recorded on video tape with a time compression of 80. Magnifications were measured by filming a grid of known spacing through both the optical systems used. All microscopes and lenses were manufactured by Nikon (Nikon, Inc., Garden City, N.Y.). Films were played back on a Traid stop-motion projector (Traid Corp., Glendale, Calif.) for analysis. Measurements from video tape records were made directly on the face of a 12-in monitor.

Further details of technique may be found in the descriptions of experimental results.

RESULTS

General Description of Experiments

In all the experiments reported in the first three parts of the results we kept amoeba density and age, pulse duration, and current constant. We varied the concentration of c-AMP in the microelectrode to vary signal amplitude, that is the number (η_p) of c-AMP ions released in each pulse. The concentrations used were 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M. In most experiments, including all those described in the first two parts of this section, we used a fixed pulse period of 5 min. We therefore describe first the typical responses to a periodic c-AMP source supplying a signal that causes both chemotaxis and signal relaying in the responding field of amoebae. As we discovered, these conditions were always met by experiments in which 10^{-3} M and 10^{-4} M c-AMP was used, but not always in experiments in which the lower c-AMP concentrations were used.

With 10^{-3} M and 10^{-4} M electrodes 8 h postcentrifugation amoebae showed immediate chemotactic responses by moving towards the electrode after a pulse. Responses were always observed by the time the third pulse had been produced, and usually after the first. However, signal relaying, which is marked by the propagation away from the electrode of a constant velocity signal, seen as a wave of inward cellular movement, never occurred in response to the first pulse, but took from 3 to 11 pulses, with a mean of 8. The relayed signal, once initiated, caused immediate, field-wide wave propagation. These results are summarized in Table I.

Once a relayed signal was propagated, stream formation occurred. Amoebae moved towards their nearest central neighbors rather than directly towards the microelec-

TABLE I

RESPONSES OF AMOEBAE TO PERIODIC c-AMP SOURCE

Concentration	Time to chemotaxis	Time to relaying	Territory size	Appearance of autonomous cells
M	*min*	*min*	*mm*	
10^{-3}	5	40	>1.5	1 h
10^{-4}	8	38	1.2	1 h 8 min
10^{-5}	11	54	1.0	1 h 30 min
10^{-6}	10	—	—	1 h 20 min

All times are measured from the time of electrode introduction, 8 h after centrifugation. Results are means from 10, 10, 10, and 4 experiments for 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M c-AMP, respectively.

trode, as each amoeba acted as a point source of signal. The amoebae condensed into randomly bifurcating streams leading towards the microelectrode. This is also the normal pattern of aggregation towards a spontaneously arising natural center.

Spontaneously arising autonomously signaling cells begin to appear in fields from 9 h. If they are close to the microelectrode their own signals may compete with those from the electrode. Then one sees competition for amoebae and entrainment of one center's field by another center, again exactly as occurs during spontaneous aggregation. This occurred in many of our experiments and will be described in the Frequency Dependence section of the Results.

Once the electrode had attracted cells to form an aggregate of normal appearance, the aggregate proceeded to produce a tip (Raper, 1940; Farnsworth, 1973; Rubin and Robertson, 1975). Once the tip had formed the observed signal period decreased steadily to about 2 min. The signal from the tip dominated that from the microelectrode, entraining all cells attracted by the microelectrode. The aggregate proceeded through the normal morphogenetic cycle independently of the microelectrode. This happened in all our experiments with a periodically signaling electrode.

The microelectrode can therefore replicate the normal initiation of aggregation, but does not control morphogenesis after tip formation.

We next explored the concentration dependence of the qualitative effects observed, in particular the thresholds for chemotaxis and signal relaying.

Concentration Dependence

Uniform fields of amoebae at densities between 1 and 1.5 × 10^6 cells · cm^{-2} were stimulated with 1½ pulses at 5-min periods. Stimulation was started 8 h after centrifugation, when the cells were competent to perform chemotaxis towards a c-AMP source and becoming competent to relay a pulsatile signal, but were not yet competent to signal autonomously. Table II shows the qualitative results observed. Relaying was seen in 2 out of 10 experiments with 10^{-5} M c-AMP.

For each concentration of c-AMP in the electrode a well-defined circular area round the pipet tip was cleared of amoebae during the first few pulses as cells responded purely by chemotaxis. The chemotactic radii are shown in Table III.

From our calibration curve in the preceding paper we know that for the 10^{-4} M and 10^{-5} M electrodes the mean pulse sizes η_p were 8 × 10^{10} and 8 × 10^9 molecules of

TABLE II

RESPONSES TO ELECTRODES CONTAINING 10^{-3}, 10^{-4}, 10^{-5}, AND 10^{-6} M c-AMP

c-AMP concentration	Chemotaxis	Relaying
M		
10^{-3}	+	+
10^{-4}	+	+
10^{-5}	+	+
10^{-6}	+	0

TABLE III

CHEMOTACTIC RADII FOR EACH CONCENTRATION OF c-AMP

[c-AMP]	Chemotactic radius	
M		
10^{-3}	Larger than frame size	
10^{-4}	0.485 mm	Av. of 10
10^{-5}	0.24 mm	Av. of 10
10^{-6}	0.1 mm	Av. of 4

c-AMP, respectively. If we neglect the extracellular phosphodiesterase activity which removes c-AMP from the medium, we can calculate threshold concentrations for chemotaxis as follows:

Time to reach maximal c-AMP concentration is

$$t = r^2/6D,$$

where r is chemotactic radius and D the diffusion constant of c-AMP in agar, for diffusion from a point source into a semi-infinite medium; and the threshold concentration of c-AMP for chemotaxis,

$$C^*_{\text{Chem}} = (2\eta_p/[4\pi Dt]^{3/2}) \exp(-r^2/4Dt).$$

The results for the 10^{-4} and 10^{-5} M electrodes, which gave the most reliable data, are shown in Table IV. The values listed for C^*_{Chem} are upper limits because of the neglect of PDE activity.

Frequency Dependence

Refractory Period. Fig. 1 shows a record of the responses obtained from a field of amoebae to pulses from a microelectrode every 3 min. The electrode contained 10^{-3} M c-AMP and was placed in the field 8 hr after centrifugation. Superficially the aggregate produced resembled a natural center. It can be seen that responses were initially to every third, then to every second, and finally to each pulse from the electrode. The refractory period of the field was therefore initially more than 6 min but less than 9 min, then between 6 min and 3 min, and finally less than 3 min.

TABLE IV

CHEMOTACTIC THRESHOLDS CALCULATED FOR 10^{-4} AND 10^{-5} M c-AMP ELECTRODES

[c-AMP]	η_p	r	t	C^*_{Chem}
M		*mm*	*s*	*M*
10^{-4}	8×10^{10}	0.485	39.2	$<3.4 \times 10^{-7}$
10^{-5}	8×10^{9}	0.24	9.6	$<2.8 \times 10^{-7}$

η_p = pulse size, r = chemotactic radius, t = time to reach maximal concentration, C^*_{Chem} = chemotactic threshold.

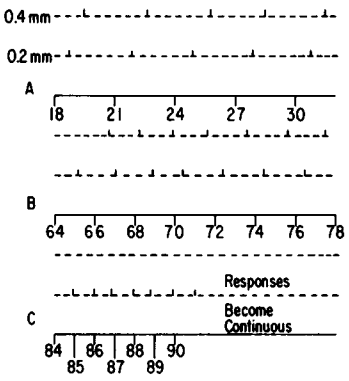

FIGURE 1

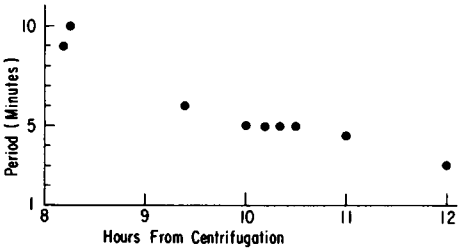

FIGURE 2

FIGURE 1 Three sequences showing responses to a microelectrode signal with 3 min period. The signals started 8 h after centrifugation. Time is indicated by the pulse numbers on the lower scale for each run. Mean wave velocities were calculated for each run and are 95 μm/min, 55 μm/min, and 38 μm/min for *A*, *B*, and *C*, respectively, showing a decrease with increasing cell density. Responses are shown at positions 0.2 mm and 0.4 mm from the microelectrode tip in each case.

FIGURE 2 Graph of refractory period as a function of time after centrifugation. Data are presented as the first observed responses to a signal of the given period. They are taken from six films where there was no interference from signals arising from autonomous centers.

While we are measuring T_r more accurately (to be reported separately), we did note a similar effect in many of our experiments, in that, to an electrode with a 5 min period, responses were first to every second pulse and then to each pulse. The switch always occurred between 10.0 and 11.5 h after centrifugation. T_r is plotted in Fig. 2.

In addition, in all experiments with a 5 min electrode the period of waves propagating from the induced aggregates after the latter had formed their own tips declined to about 2 min, as occurs in normal aggregation. The pseudoplasmodia formed remained independent of the microelectrode and migrated.

Entrainment. Fig. 3 shows tracings of cell movements in response to a microelectrode containing 10^{-4} M c-AMP and signaling every 2 min. The cells were initially responding to a spontaneous aggregation center. The electrode was placed 20 μm from a stream. Amoebae entering the stream were entrained by the microelectrode, propagating waves to every second pulse, that is, with a period of 4 min (mean of 10 periods = 4.17 min). Waves in streams leading to the aggregate had been propagating spontaneously with a mean period of 6½ min. The refractory period of the cells was therefore between 2 and 4 min which is consistent with our measurements of T_r (Fig. 2). Similar results were seen in two other experiments where a "faster" electrode was placed in the field of a spontaneous aggregate.

In all cases a boundary appeared between the two signal sources, marked by normal movement of cells towards the sources. The boundary moved towards the source with the lower frequency, as would be expected during entrainment by a higher frequency

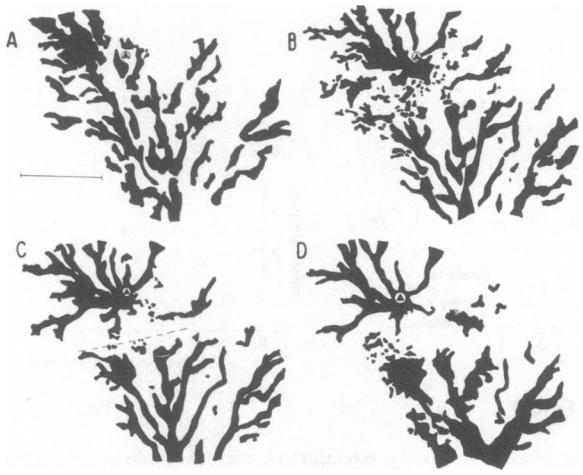

FIGURE 3 Entrainment of aggregating amoebae by an electrode signaling with a 2 min period. Details are in the text. *A* is 540 min after centrifugation, *B* 24 min later, *C* 44 min later, and *D* 64 min later. Each represents a tracing of a single frame from the TV monitor by Marc Edelsten. The length bar is 300 μm. The ▲ indicates the position of the microelectrode tip.

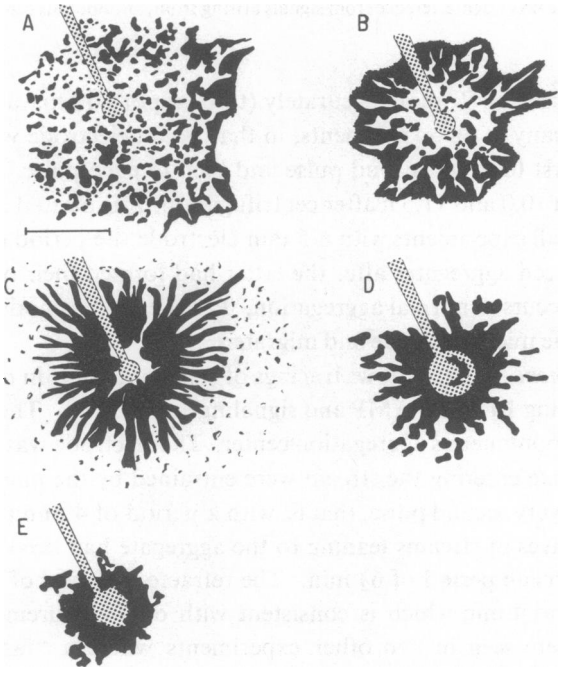

FIGURE 4 Responses by amoebae to c-AMP "leaked" continuously from a microelectrode. Details are in the text; *A* is 576 min after centrifugation, *B* 27 min later, *C* 54 min later, *D* 101 min later, and *E* 127 min later. Each represents a tracing of a single frame from the TV monitor by Marc Edelsten. The length bar is 300 μm.

signal. Waves did not propagate through the boundary, presumably because cells were refractory to further stimulation.

Responses to a Continuous Signal

In four experiments we introduced a microelectrode containing 10^{-4} M c-AMP into 8 h fields of cells and drove out the c-AMP with a continuous current of 1 μA. The responses in all cases were similar and are illustrated in Fig. 4. The amoebae were initially attracted, showing continuous chemotactic movement towards the microelectrode tip. Approximately 90 min after the beginning of stimulation a relayed signal spread from a roughly annular region of cells with a mean diameter of 300 μm, centered on the electrode tip. The relayed signal induced the formation of streams of cells directed towards the relaying cells and attracted some amoebae from the area between them and the microelectrode tip. As cells moved into the annulus through the streams its width increased and the cell mass moved inwards towards the microelectrode tip. The final configuration resembled a normal aggregate whose center was at the electrode tip. The continuous signal thus caused continuous chemotaxis, but periodic signal relaying. The observed periods corresponded with the expected values of T_τ shown in Fig. 2. Therefore a continuous, suprathreshold c-AMP signal can cause periodic signal relaying, presumably stimulating amoebae as soon as they emerge from the refractory state. A continuous c-AMP source can therefore initiate and control aggregation, but in an abnormal way, with a clear separation of the chemotactic and relaying responses of the amoebae.

This experiment provides a lower value for the relaying threshold, C^*_{Rel}. Using Eq. 8 from the previous paper, and a relaxation time τ_F of 0.37 s as measured for this cell density (5×10^5 cells/cm^2) (Gingle, unpublished measurements), we obtain a threshold of approximately 10^{-7} M c-AMP. This is a more realistic value than that obtained in the Concentration Dependence section above, implying a pulse size of approximately $\eta_p = 10^8$ molecules for the signal from a single amoeba for a value of 1.5×10^4 for C^*_{Rel} (Cohen and Robertson, 1973). We shall take up this point in the discussion.

DISCUSSION

These experiments provide evidence for the following components of the aggregation control system in *D. discoideum*. (*1*) A brief, periodic signal which stimulates chemotaxis and signal relaying. (*2*) Distinct thresholds for chemotaxis and signal relaying with the relaying threshold concentration possibly two orders of magnitude greater than the chemotactic threshold. (*3*) c-AMP can cause both signal relaying and chemotaxis. (*4*) A refractory period for signal relaying which declines with developmental age. (*5*) A clear distinction between responses to periodic and continuous signals. A periodic, brief, signal can cause both periodic chemotaxis and periodic signal relaying. The period of relaying is a function of both signal period and of refractory period. A continuous signal causes continuous chemotaxis, but periodic signal relaying.

(6) Thus, a periodic applied c-AMP signal can initiate and control aggregation in a way quite similar to that shown by a normal aggregation center.

Our measurements for C^*_{Chem} and C^*_{Rel} show the importance of knowing PDE activity and distribution (Table IV).

If we repeat the calculation, taking PDE activity into account, we are faced with two uncertainties. First, we need a value for the activity at the given developmental age and cell density. This has been obtained in our laboratory by Gingle whose results will be published separately. Second, we need to know the distribution of PDE activity in the agar beneath the amoebae, that is we need the diffusion constant of PDE, D_{PDE}. While we can estimate this for diffusion in water, it is difficult to know how much hindrance will be offered by the gel structures of the agar, although the diffusion constant will be substantially reduced. If the chemotactic range, R, of the c-AMP signal from the microelectrode is such that $R \ll (D_{\text{PDE}}t)^{1/2}$ then the measured PDE relaxation time, τ_f, applies. If $R \gg (D_{\text{PDE}}t)^{1/2}$, then it can be shown that the new relaxation time, $\tau_s = 4D\tau_F 2/\pi D_{\text{PDE}}t$. The chemotactic thresholds can then be recalculated, using the expression

$$C^*_{\text{Chem}} = \eta_p 10 D\tau_s/[(2\pi/5)e^{R^2}]^{3/2}e^{R^2}.$$

The new thresholds are 1.1×10^{-9} M and 3.7×10^{-9} M for the 10^{-4} M and 10^{-5} M electrodes, respectively. These are in closer agreement with Konijn's estimate of less than 10^{-8} M. The discrepancy is probably due to differences in experimental technique. On the other hand, if we had supposed $R \ll (D_{\text{PDE}}t)^{1/2}$ we would have obtained ridiculously low values for C^*_{Chem}. This implies that the distribution of PDE is indeed limited by diffusion and that $R > (D_{\text{PDE}}t)^{1/2}$. We can conclude that the diffusion constant for PDE is such that $(D_{\text{PDE}}t)^{1/2} \simeq 200\,\mu$m, because the value of C^*_{Chem} for the 10^{-5} M c-AMP microelectrode is higher than that for the 10^{-4} M electrode. This implies that we have underestimated PDE activity in the 10^{-5} M case because diffusion of PDE is sufficiently slow that all the PDE is still within a distance from the agar surface that is comparable to the chemotactic range. However, the effect also suggests that $(D_{\text{PDE}}t)^{1/2} > 75\ \mu$m, which is the signal range at critical density, as would be expected. This emphasizes the important role of PDE in removing c-AMP between signals, preventing saturation of the receptor system.

Estimates of the relaying threshold from both brief and continuous signals agree quite well. On a very short time scale the kinetics of the interactions between c-AMP and its receptor will of course determine a more complex relationship between signal strength and duration, just as in the case of the excitation of a neural membrane. The determination of a "strength-duration" curve will depend on observing responses to carefully controlled stimulation with very short pulses. It is clear that although during normal aggregation the effect of the PDE is to remove c-AMP between pulses, giving an all-or-nothing signal, a continuous suprathreshold signal will also induce relaying. This behavior is perfectly consistent with that seen in other excitable systems and does not invalidate the concept of a threshold.

In addition the results are consistent with c-AMP being both the chemotactic and relayed signal. The value of 10^{-7} M for the relaying threshold is probably an upper limit, but it is physiologically possible because it implies a pulse size of about 10^8 molecules. Thus, if an amoeba released 100 pulses during aggregation 10^{10} molecules of c-AMP would be expended. This is less than 10% of the dry weight of a single amoeba.

A. Robertson is an Alfred P. Sloan Fellow, 1973-75.
This work was supported by National Science Foundation grant GB-30784 to the University of Chicago.

Received for publication 2 January 1975 and in revised form 21 April 1975.

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