

Cell Behavior in *Dictyostelium discoideum*: Preaggregation Response to Localized Cyclic AMP Pulses

ROBERT P. FUTRELLE, J. TRAUT, and W. GEORGE McKEE

Department of Genetics and Development, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

ABSTRACT The motion of cells in the aggregation phase of *Dictyostelium discoideum* development is complex. To probe its mechanisms we applied precisely timed (± 1 s) and positioned (± 2 μm) pulses of cyclic AMP to fields of cells of moderate density using a micropipette. We recorded cell behavior by time-lapse microcinematography and extracted cell motion data from the film with our Galatea computer system. Analysis of these data reveals: (a) Chemotaxis lasts only about as long as the cyclic AMP signal; in particular, brief pulses (~ 5 s) do not induce chemotaxis. (b) Chemotactic competence increases gradually from within an hour after the initiation of development (starvation) to full competence at ~ 15 h when aggregation begins under our conditions. (c) Cell motion reverses rapidly (within 20 s) when the external gradient is reversed. There is no refractory period for motion. We present a new description of the process of aggregation consistent with our results and other recent findings. (d) The behavioral response to cyclic AMP includes a phenomenon we call "cringing." In a prototypical cringe the cell speed drops within 3 s after a brief cyclic AMP stimulus, and the cell stops and rounds and then resumes motion after 25 s. (e) The development of the speed response in cringing as the cells age closely parallels the development of the cyclic AMP-induced light-scattering response of cells in suspension. (f) Cringing occurs in natural populations during weak oriented movement. The computerized analysis of cell behavior proves to be a powerful technique which can reveal significant phenomena that are not apparent to the eye even after repeated examination of the film.

The cellular slime mold, and *Dictyostelium discoideum* in particular, has gained some notoriety as a system in which to study a range of phenomena including intercellular communication, chemotaxis, cellular differentiation, and the regulation of spatial patterns in development (6, 16, 32, 56, 66, 81). Detailed mathematical models have been developed for the aggregation process in this organism (12, 13, 66, 67). One purpose of this paper is to furnish the type of detailed data necessary to evaluate the models.

D. discoideum initiates development when starved. The cells (myxamebas) go through a period of interphase, aggregation to centers, formation and migration of a slug (grex or pseudoplasmodium), and fruiting body construction. During the interphase to aggregation period the cells move as individuals, colliding at times, so they can be filmed and the behavior of each cell analyzed. During this period the cells' behavior is coordinated on a field-wide basis—the cells behave as an integrated tissue.

Cyclic AMP appears to mediate aggregation in *D. discoi-*

deum. Cyclic AMP (cAMP) signals from single cells or groups diffuse to neighboring cells which relay the cAMP signal and move toward the source by chemotaxis. We applied cAMP to fields of cells using a micropipette so that the position and duration of the signal were under precise control. Movies of the behavior were made and analyzed by computer. We found that the chemotactic response lasts only as long as the signal. This implies that the duration of the natural signal is ~ 100 s. We found that cells rapidly reorient when the cAMP source is moved to a new position. In addition, we discovered a transient response to cAMP upshifts in which cells stop, round, and then respread and continue normal locomotion. This phenomenon, which we call "cringing," has a half-width of ~ 25 s. We followed the development of chemotaxis and cringing from the time of starvation to the time of aggregation. The speed during the cringe response changes during development in the same way that the rapid, initial part of the light-scattering response seen in cell suspensions changes. We conclude that the fast light-scattering response is the optical correlate of cringing. (A

brief description of these results has previously appeared [28].)

We discuss the previous models of *D. discoideum* aggregation and present a new one that embodies our results as well as those of Devreotes and co-workers (17–21, 87).

A number of the important results in the paper depended critically on having a large amount of very accurate cell motion data. This was achieved by using the Galatea computerized data gathering system.

MATERIALS AND METHODS

Cell Culture

Dictyostelium discoideum/B (*D. discoideum*, gift of E. R. Katz, State University of New York at Stony Brook) was grown on 35 ml of SM nutrient agar in 100-mm diameter Petri dishes with *E. coli* B/r (86). The plates were grown in the dark at $23 \pm 0.5^\circ\text{C}$ and harvested at 36 h when they had reached $\sim 7 \times 10^7$ cells/plate. (Terminal cell growth is 2.5 to 3×10^8 cells/plate at 43 h under the same conditions.) The cells were washed with cold Bonner's salt solution (BSS) (3), centrifuged three times at 250 g for 2 min, and resuspended in cold BSS.

Filming Chambers

Most studies were done on agar in a culture dish chamber (Fig. 1). The design allowed the plate to be scanned over the cover-slipped area. Tissue culture dishes (but not bacterial dishes) allow a thin agar film to be poured on them easily. The dish was warmed to $\sim 65^\circ\text{C}$ and placed on a carefully leveled surface. 10 ml of agar at 90°C was pipetted directly down onto the cover slip from a glass pipette filled with 12 ml of agar. The thickness of the agar is ~ 0.8 mm over the cover slip. (When measuring the thickness of the agar by focusing through with the microscope, the actual thickness is given by $t = 1.33 \times d$, where 1.33 is the index of refraction (of water) and d is the apparent depth [46].) The dish was sealed by a vaselined lid "de-nibbed" by shaving off the three protrusions on the inside with a #10 scalpel blade. A glass slide chamber (Zigmond, [92]) was used for concentration upshift/downshift experiments.

Cell Preparations

The dish chambers used 2% Noble agar (Difco Laboratories, Detroit, MI) in BSS. The agar was overlaid with BSS for at least 12 h before use to facilitate cell spreading. The BSS was drained and replaced by 0.1 ml of cell suspension, final density of 800 to 1,000 cells/mm². Experiments were done at this "standard" density unless noted. During the experiments, control plates were kept on the bench in the same room.

Small population plates were prepared with 1% Noble agar in BSS, and chemotaxis assays were carried out as described by Konijn (50, 51). The assays were positive and served as an overall control.

Some observations were made of cells on small population ("small drop") plates made by spraying drops of a cell suspension onto the plate. Cells were suspended in BSS in a 12 × 75 mm plastic test tube fastened to the intake pipe of a DeVilbiss #15 atomizer (The DeVilbiss Co., Somerset, PA). Spraying was done one to three times horizontally ~ 20 cm above the open dish from 50 cm away. A few hundred to many thousand drops of various sizes can be deposited in a few seconds. Drops as small as 20 μm in diameter, containing one cell, can be made with no apparent damage to the cell. (In automated cell sorting, cells remain viable during drop formation and deposition [85]). For a given drop size, the number of cells is directly proportional to the concentration of cells in the suspension, not a simple function of drop size, however. A 5×10^7 cells/ml suspension gave ~ 50 cells in 120- μm Diam drops and ~ 150 cells in 240- μm drops. The number of cells in a given size drop depends on the condition of the agar surface (50). The smallest drops were circular; large ones were sometimes elliptical. It is also possible to spray two or more distinct cell suspensions or chemicals. The random positioning of the drops allows the plate to be scanned for desired combinations of drop size and interdrop distance.

A density of 700 cells/mm² was used in the bridge area for observations in the Zigmond chamber.

Stimulation

Cells in the dish chamber often deteriorated in appearance, assuming a circular shape with bright, indistinct edges, unless the agar was kept wet. For stimulus sets delivered every hour, wetting was done 20 min before the hour by injecting a small volume of BSS into the wetting tubes with a hypodermic syringe and blowing it through with an air-filled syringe.

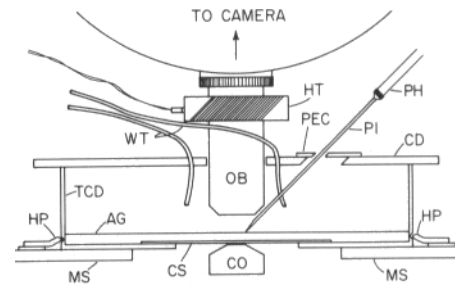


FIGURE 1 Schematic cross section of culture dish filming chamber. A 20 × 100 mm tissue culture dish (TCD) has a 35-mm diameter hole in the bottom (cut on a lathe). The hole is covered with a 40 × 50 mm #1 cover slip (CS) (A. Thomas, Philadelphia) held on with epoxy cement. The microscope condenser (CO) is raised close to this cover slip. The dish rests on the microscope stage (MS) and is positioned by holding pins (HP) fashioned from paper clips, which are epoxied to the arms of a movable slide stage. The holding pins rest in blind holes drilled slightly into the dish. The dish is covered by a $\frac{3}{16}$ " transparent plexiglass disk (CD) 20 cm in diameter (50), coated with vaseline in the area of contact. A hole is drilled in the center of the disk to allow the objective (OB) to enter without binding. The heater (HT), which prevents fogging of the objective lens, consists of a spring-metal broom-handle holder wrapped with teflon-covered chrome alloy wire embedded in Epoxy. The heater is driven with a low voltage (<12 VAC). The cells move on the agar (AG) and are stimulated by a micropipette (PI) inserted in a pipette holder (PH) mounted on a micromanipulator (E. Leitz). Three flexible wetting tubes (WT) (one behind objective not shown), which fit snugly through holes in the cover dish, are used for adding liquid to the preparation.

Micropipettes for cAMP were pulled from 1.2 mm O.D. glass tubing ("Omega-dot", Frederick Haer & Co., Brunswick, ME) to a tip O.D. of 1 to 2 μm . Filled with BSS, the pipettes gave DC resistances of 20 to 100 M Ω .

Micropipettes were filled with cAMP (Sigma Chemical Co., St. Louis, MO) in BSS. Concentrations ranged from 10^{-6} M to 0.1 M cAMP. For concentrations above 10^{-3} M cAMP, sodium salt was used. Experiments were done with 10^{-4} M cAMP, the "standard" concentration, unless noted. Representative pipettes were calibrated by filling them with dilute ³H-cAMP and allowing the cAMP to diffuse into BSS for up to 20 h (Appendix 1). The efflux varied from one pipette to the other but was never $>10^{-12}$ ml/s (equivalent flow rate.) This gives an efflux rate of 6×10^4 molecules/s for the standard concentration, 10^{-4} M. The concentration at a cell 100 μm from such a pipette was calculated to be 10^{-10} M.

A micropipette was normally used to stimulate the cells by touching it to the agar just inside the filmed area. Care was taken to avoid cell contact with the pipette. Lowering a cAMP-containing pipette onto (through) a cell lysed it immediately. When a cell reached a pipette which was already resting on the agar, it would sometimes enter the pipette and might even reemerge later. To achieve uniform timing, each stimulus was begun by touching down the pipette ~ 2 s before a film frame exposure and lifting it immediately after a later exposure. For 5-s pulses, we attempted to center the pulse around the time of exposure.

Injection and withdrawal of liquid from the Zigmond chamber was done by Dr. Zigmond's methods (92).

Optical Microscopy and Filming

A Leitz Ortholux II microscope was used with a 32 ×, 6.6 mm working distance Zernike phase contrast objective. The objective was warmed 0.2°C above the chamber temperature to prevent condensation on the primary element. The temperature difference was monitored by MT-3 thermocouple microprobes attached to a Bailey Model BAT-8 digital thermometer (Bailey Instruments, Inc., Saddle Brook, NJ). The image was directly projected onto the movie film at a magnification of 40 × by a 1.25 × trinocular stage (no relay optics). The camera was adjusted to be parfocal (using a small prism in the film gate) with an eyepiece reticle in one of the oculars (which remains parfocal at all interocular settings). Subsequent focusing was done through the oculars. The field viewed was 450 μm in diameter; the field filmed was 170 × 225 μm . Constant illumination was used, limited to the field of view. The quartz-halogen source was operated at a color temperature of 3,200K and then attenuated by 16 × of neutral density filtering followed by a heat filter.

A 16-mm Bolex H-16 motion picture camera was used, driven by an Emdeco TL 320 time-lapse drive set for a 1-s exposure. Most films were made at eight

frames per min (8 fr/min) on Kodak VNF 7240, ECO 7252 or Kodachrome 40 (KMA) stock on 100-foot reels (4,300 usable frames).

Data Extraction

THE GALATEA/ST COMPUTER SYSTEM: Films were projected onto a drawing-board-like electronic tablet. The user followed the apparent center of a cell by guiding a cursor as the film ran, usually at 1 fr/s. The tablet sensed the cursor's x,y position and sent the data to the computer (Fig. 2). The Galatea/ST system in the computer supported the data extraction process. It allowed the user to monitor the quality and completeness of the data and to re-enter data as needed. Galatea/ST at the University of Illinois in Urbana (9) is a variant of the more graphically oriented system at the University of Chicago designed by one of us (RPF) (25, 26) and further developed there (70). (A similar system was later built at MIT [2]).

The movie projector was a 224A Mark IV (LW International, Woodland Hills, CA). It was interfaced so that each frame change was signaled to the computer along with the film direction. The data tablet had a 22 inch square (56×56 cm) working area and 0.001 in. (~ 0.025 mm) resolution (Talos Inc., Scottsdale, AZ). The computer was a PDP-11/60 (Digital Equipment Corp., Maynard, MA) with 32,768 16 bit words of memory, 2.5 M words of permanent disk storage, and 1.25 M words of removable disk cartridge storage. Galatea/ST was written in the structured assembly language BIOMAC (by Scott Herman-Giddens, available from the Digital Equipment Company Users Society, DECUS program 11-208, DECUS, Marlboro, MA) and ran under the RT-11 operating system (Digital Equipment Corp.).

To avoid accidental destruction of valuable data, original films were archived and work prints used for data entry. The image on the data tablet was 36×47.5 cm, a total magnification of 2110 times. A sheet of paper was taped to the tablet for recording cell outlines and other alignment information. In each entry session, Galatea was first used in "comment mode" and a description of how the particular data were to be taken was typed in. Then Galatea was used in "moving point mode" to track selected cells. The x,y data corresponding to a given frame were automatically read from the tablet at the moment that frame changed to the next to allow the user maximum time to position the cursor accurately. Data for a single experiment were contained in a single Galatea file. Each file was further divided into "tracks". Typically, the stream of moving point data corresponding to a single cell was entered into its own track. Data entry could be done at various rates up to 24 fr/s, but the cell motion we studied could not be tracked with sufficient accuracy at the higher speeds.

Galatea gives summaries of the data, listing inadvertent gaps, and reporting the maximum difference between cell positions in successive frames to avoid accidental jumps in the data due to misalignment or misidentification of cells. Tracking by eye and hand was at best accurate to 1 mm, which corresponded to $0.5 \mu\text{m}$ in the original experiments (Appendix 3).

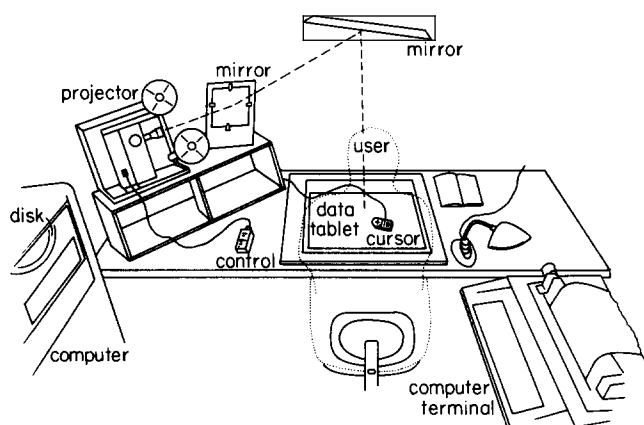


FIGURE 2 The Galatea/ST computer system for interactive data extraction from movie films. The user can easily monitor the projector film path and the mechanical frame counter. The image is reflected twice to give a normal, upright, and undistorted image. The projector is normally run at 1 frame/s, and a cell tracked by sliding the cursor on the data tablet so that the cell image is centered on the black cross drawn on the white top of the cursor. The x,y cell position data are taken in by the computer and stored on the magnetic disk.

Data Records

Besides the laboratory notebook, a six page film form (plus continuation sheets as needed) was used with ~ 50 specific items to be filled in for each filming session, covering the experiment from cell culture conditions to final data analysis. Each separate analysis of any subsection of a film was given a unique experiment number (shown on the data plots along with the date and time of data processing).

Data and Error Analysis

The raw position values (x,y pairs) from the data tablet were stored as 12 bit numbers, a resolution of ~ 0.1 mm on the data table—well beyond the film resolution. The data were sent to the University's Cyber 175 computer in ASCII print format by telephone at 300 baud ($\sim 7,000$ position values/h). On the Cyber 175 the data were managed, processed, and plotted by the programming system, Sigma (CERN, Geneva, Switzerland) (43). The speed (a scalar) was calculated as the distance between successive x,y positions divided by the time interval, typically 7.5 s. The speeds from a number of cells or from replicate data on the same cell were averaged together for most of the plots. The chemotaxis index was calculated from the velocity vector and the chosen source position as shown in Fig. 3. The index has a maximum of +1.0 when a cell moves directly toward the source and -1.0 when it moves directly away from the source (cf. Appendices 2 and 3).

A detailed error analysis was made (Appendix 3). The most notable result was that the errors produce a bias in the reported speeds, especially at low speeds. Speed minima shown as $2 \mu\text{m}/\text{min}$ are in fact nearer to $0.5 \mu\text{m}/\text{min}$ if corrected for all error sources. Our plots have not been so corrected because an inordinate amount of additional data collection would have been necessary.

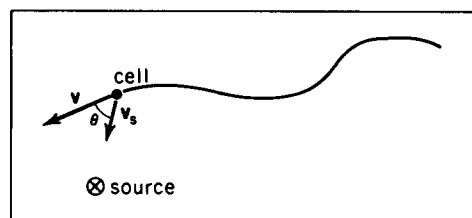


FIGURE 3 Directed cell motion and the chemotaxis index (CI). At the moment shown in this schematic, the cell is partly directed towards the chemoattractant source. The chemotaxis index is defined as $CI = \cos \theta$, which is the ratio of the cell's speed $\|v_s\|$ towards the source to its total speed $\|v\|$. At this moment, $CI \approx +0.75$.

RESULTS

The Chemotactic Response Lasts Approximately as Long as the Stimulus

The duration of chemotactic movement is approximately as long as the applied cAMP stimulus. Fig. 4 illustrates this for the application of a 1- and 2-min cAMP signal to 9-h cells. We have seen brief responses to 20-s signals and extended responses with constant motion towards the pipette source for many hours. The long responses agree with observations that *D. discoideum* cells move steadily towards continuous sources (51). In Fig. 4 the second response of ~ 120 s requires a stimulus of that duration to produce it. We conclude that in natural aggregation, where the movement "step" is ~ 100 s long (1), the cAMP signal seen and relayed by the cells is ~ 100 s long. This is in marked contrast to the original brief pulse theory (13). Our results are in accord with measurements of autonomous (37) and induced (relayed) cAMP production seen in stirred cell suspensions (77) and with measurements on induced cAMP release by cells on plates (82). These events, as well as the light-scattering changes that accompany them (55), are 1–3 min long. In an ingenious experiment, Tomchik and Devreotes were recently able to measure the distribution of cAMP in waves on aggregation plates and found that they were indeed 1–3 min long (87).

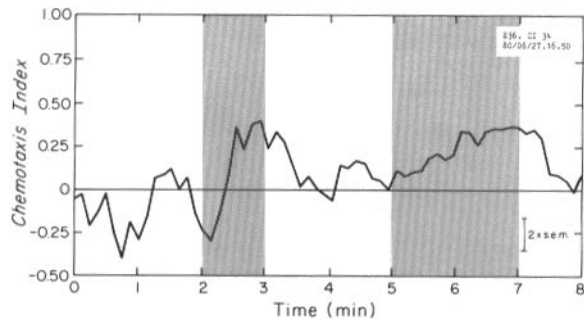


FIGURE 4 Chemotaxis in response to 1-min and 2-min cAMP pulses (gray bars). The average chemotaxis index (CI) of 34 cells at 9-h of development. The maximum value reached, $CI \approx 0.35$, implies that the average angle of a cell's path deviates 70° from the direction toward the pipette, or 20° away from a random, unoriented motion. Standard conditions of cell density, pipette size, cAMP concentration, and standard magnification for filming are used in this and all later figures unless otherwise noted.

The data in Fig. 4 are noisy due to the modest number of cells included in the average but it is clear that the half-time to rise to the maximum and the half-time to decay are both in the neighborhood of 20 s for the response to the first (1-min) signal. The response to the 2 min signal has a half-time for the initial rise of closer to 50 s, but a half-time to decay nearer the first, ~ 30 s. We have often seen such systematic differences in the responses to sequential signals, and these vary with developmental age, pulse strength, and timing. It might be guessed from the figure, and it can be seen more clearly in data presented later, that chemotaxis begins within seconds after the stimulus onset.

To understand the response, we have to understand the stimulus. Close to the pipette ($30 \mu\text{m}$), the cAMP concentration and gradient build up within ~ 1 s. The signal near the edge of the field ($180 \mu\text{m}$) is 6-fold weaker in concentration and 36-fold weaker in its gradient. The signal build-up time near the edge is ~ 36 times slower. Thus the cells in various parts of the field are exposed to different primary signals. The data from cells at various distances have been averaged together in Fig. 4, which may lead to a broadening and smoothing of the actual response. Between the two pulses the concentration of cAMP in the agar at a typical cell ($90 \mu\text{m}$ from the pipette) decreases to 5% of its earlier maximum, so that the 2-min pulse represents a major upshift in cAMP concentration and gradient strength. The decrease is predicted from diffusion of the primary signal alone and does not take into account possible cAMP relay (secondary signal) or degradation by phosphodiesterases. One might think that the response to the second (2-min) signal was weaker because the cAMP in the pipette was expended by the first signal. This is not the case; the pipette recovers within a few tenths of a second after the first pulse (Appendix 1). In viewing the film we did see characteristic differences in cell responses between cells near the pipette and cells near the edge of the field. Different cells at the same distance from the pipette also appeared to respond differently to the same stimulus. This may be a manifestation of innate, nongenetic heterogeneity, or range variation, which has been discussed for the cellular slime molds (5, 6) and for the bacterial chemotactic response (83). Both sources of variability contribute to the response shown in the figure (and certainly, to some extent, to the noise in the data).

Even after the averaging is done, there remains a systematic difference between the responses to the two signals, even in the

first minute of each, when the stimuli are the same. The simplest explanation of the difference is to say that the cells are in a partially refractory state after the first stimulus, less responsive to the second stimulus—a view that originated with Shaffer (79). Refractoriness (adaptation) has been studied for the cAMP relaying response and found to be a graded phenomenon, with no absolute refractory state (20). The chemotactic response seems to be of this nature also.

Brief Pulses Do Not Induce Chemotaxis

Shaffer (79) reasoned that acrasin (now known to be cAMP in *D. discoideum*) was produced and relayed in "pulses". Cohen and Robertson (12, 13) assumed that the pulses were brief, typically a few seconds long. The response to the brief signal was assumed to be a preprogrammed step 100 s long. They reported confirmation of the theory in "pulser" experiments in which 1.5-s pulses of cAMP were released by iontophoresis (74, 75). Coordinated chemotactic motion toward the pipette was reported. Those experiments have not been replicated by any other group. In the "pulser" experiments, pulse sizes (in number of cAMP molecules) ranged from 2×10^9 /pulse to 1.5×10^{12} /pulse and the "leak" from the back-biased electrode between the pulses ranged from 2×10^6 /s to 6×10^9 /s. Maximal cAMP release during aggregation is probably $\sim 10^4$ /s per cell (17, 37) and a duration of the relayed cAMP release of 1–3 min (17, 77, 82).

We stimulated cells with 5-s pulses using a wide range of cAMP concentrations and saw no evidence of chemotaxis by cells at any distance from the pipette. Fig. 5 shows the results of one such experiment using standard conditions. Short pulses do induce a different response, cringing, discussed in later sections of this paper.

The major differences between our approach and the "pulser" experiments are that we reliably shut off the stimulus by lifting the pipette and we analyzed the motion of individual cells rather than visually observing wave patterns in films running at 24 fr/s (39).

The result that brief pulses do not induce chemotaxis is in harmony with studies on cAMP-induced cAMP relaying (17). There, brief pulses of cAMP induced only small and equally brief relay responses rather than a full quantal release independent of the stimulus. Other micropipette experiments, only briefly described, and done on mutant strain *ga 93* (35, 36), also suggest that the short pulse response involves a brief movement response at best and no preprogrammed step. The brief response reported by Gerisch gave a net cell movement

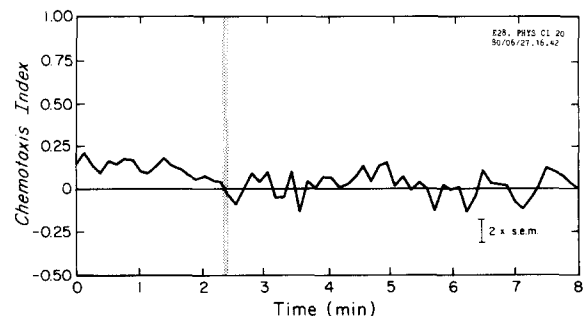


FIGURE 5 The average chemotaxis index of 20 cells, at 13-h development, in a $170 \mu\text{m} \times 225 \mu\text{m}$ viewing field stimulated for 5 s (vertical bar) with a standard pipette. The net motion of this group of cells toward the pipette in the 100 s after the stimulus is $< 1 \mu\text{m}$.

of a micrometer or less, which would be lost in the “noise” in Fig. 5.

Development of the Chemotactic Response

There have been few studies of the development of the chemotactic response in the cellular slime molds. The first used the Konijn assay (51) and reported the cAMP concentrations necessary to obtain a positive response in the assay at two different stages labeled vegetative and aggregating. The concentration required dropped by 100 times for the later stage (7). Another study, using pulser techniques, reported weak chemotactic response at 2 h and full response at 4 h with first cell contact (streaming?) occurring at 8 h (74). Recent work using the net movement of a dense drop of cells suggested that the chemotactic response remains low until ~6 h of development and then rises dramatically (88).

In our experiments the chemotactic response was measured each hour, from 1 through 15 h after starvation. There was strong and obvious autonomous activity after 15 h and pronounced streaming by 20 h. Each hour, we administered three consecutive 1-min cAMP pulses with 2 min between pulses (3-min period). The pulses did not appear to accelerate development; the unstimulated controls and the stimulated preparations all began aggregation between 17 and 20 h. Depending on the conditions, cAMP may accelerate (15, 34) or delay development (57).

Fig. 6 is an example showing the chemotactic response at 14 h for 13 cells. The response to the first stimulus is clear and strong, reaching CI = 0.6 at its maximum. The response has not died out by the time the second stimulus begins, and the response then reaches CI = 0.7. The second response falls off much more slowly than the first and little effect of the third stimulus can be seen. The response pattern for 14-h cells is typical of all late cells (from 10 h on): (1) strong, (2) stronger and slow to decay, (3) little effect. The sequence of responses in the earlier cells is more complex and all responses varied with the spacing and duration of the stimuli.

We then compared the chemotactic responses of early (1–5 h), middle (6–10 h), and late (11–15 h) cells. This was done by averaging together the responses for the five ages in each group. To reduce variability, the responses to the three stimuli in each group have been merged to yield the average stimulus-related response for the three age groups shown in Fig. 7. In interpreting these data it is necessary to understand that the distinct responses to the individual stimuli have been lost and that some of the “pre-stimulus” behavior contains remnants of the response to the previous stimuli. The data show that the response increases almost linearly during the stimulus, reaching

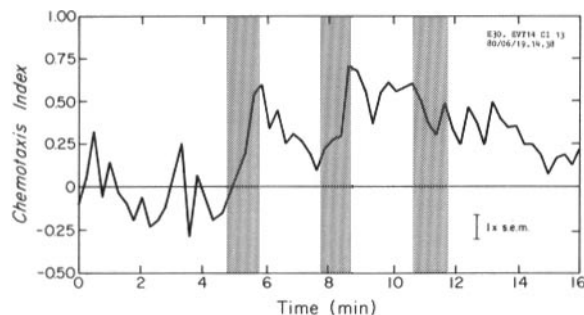


FIGURE 6 The average chemotaxis index of 13 cells during three consecutive 1-min cAMP pulses (gray bars) 2 min apart; 14-h cells, standard conditions. The corresponding average speed is shown in Fig. 13.

its maximum abruptly at from 35 s (early cells) to 45 s (late cells). The maximum CI for late cells (0.48) is about double that for early (0.27). The slower decay of the late response is also obvious from the figure.

The chemotactic response strengthens steadily with age. It appears to be present even in 1-h cells. This is probably an accurate indication of the early cells' competence because we have been careful to harvest growing cells two doublings short of stationary phase in an effort to avoid initiation of development on the growth plates (Materials and Methods). Early cells show no marked change in morphology during chemotaxis. They are not elongate. Chemotaxis in early cells is attributable to a tendency to alter paths taken rather than to extend noticeably specialized processes towards the cAMP source. Still photos of chemotaxing early populations gave no indication that chemotaxis was in progress unless it had been in progress so long that there was a noticeable accumulation of cells near the pipette. When the chemotaxis index was low, say 0.1, as it was in very early cells, it was not even possible to see the response to 1-min pulses by viewing the film. In such a case the net motion toward the source was 1 μm while the random motion was 9 μm in the 1-min interval. Nevertheless, the data analysis could distinguish such small differences.

Late cells appear to maintain their directed motion after stimulus removal for a longer time than early cells (Fig. 7). Analysis of this “persistence” phenomena, including additional data by Futrelle and Hunt (27), suggests that much of the change can be attributed to the fact that the mean cell speed drops with age but that the “step length” before a significant directional change remains about the same (~15 μm). Other data on persistence of motion in *D. discoideum* have shown no significant variation of persistence time with age (69). Those studies were done at extremely low cell densities (10 to 10³ cells/mm²). At all but the highest cell densities they used, *D. discoideum* will not aggregate (50) and, furthermore, at the low densities the cells will not develop—their later performance when reconcentrated is delayed (unpublished experiments, quoted in reference 16, pg. 113). It is therefore not surprising that no developmental changes in persistence time were observed (69). Furthermore, the persistence times were not well-defined, varying by 2:1 for the same raw data depending on whether the data were pooled or broken into 1-h blocks.

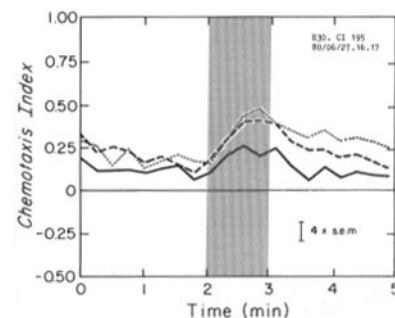


FIGURE 7 Chemotaxis in response to 1-min cAMP pulses (gray bar) for cells in early (—, 1–5 h), middle (---, 6–10 h) and late interphase (····, 11–15 h). For each age group the average chemotaxis index is shown for 195 events (responses of 13 cells to 3 consecutive pulses 3 min apart repeated each hour for 5 h). The corresponding speed plots are shown in Fig. 16.

Cell Polarity and Movement Refractoriness

As *D. discoideum* interphase cells age, they develop an elongate morphology. During aggregation they move unidirec-

tionally towards centers. This suggests that the cells are polar, with a true anterior-posterior distinction. There are no obvious morphological markers of polarity, so evidence was sought by looking at cell behavior. Bonner (4) moved natural (attractant) centers past the distal ends of streams and found that cells balled up and then moved toward the new center or made U-turns, rather than simply reversing with little shape change. Subsequent studies (36, 81) suggest that cells are more labile. As a question of developmental biology it is of interest to know whether cells are spatially differentiated with unique anterior, posterior, and lateral regions. Also, to understand aggregation we have to face the 'back of the wave' problem (66, 67, 80). As a relayed cAMP wave approaches a cell proximally, from the attractant center, the cell sees a gradient (technically, one of negative sign) which attracts the cell towards the center. As the wave passes, the gradient must reverse and the cell, if it responds rapidly enough, could reverse and follow the 'back of the wave' outwards. Cells do not reverse during natural aggregation.

We tested movement refractoriness in a number of experiments. In one experiment we studied 12-h cells, stimulating with the pipette at one position on the agar for a few minutes and then lifting and moving the pipette to a new position 50 to 100 μm away within 3 s. This was done repeatedly. Cells within 50 μm of the pipette reacted strongly, moving toward the pipette and elongating. When the pipette was repositioned, cells that were moving at a large angle (at right angles or away from the new source position) tended to project lateral lamellipods towards the source as described by Gerisch et al. (36). These projections then became the leading edge of the cell. In the most extreme case, cells that were moving directly away from the new source position simply reversed their motion (81). Such a cell is shown in Fig. 8. The chemotactic response (Fig. 9) shows that onset is rapid, 20-s half-time, and reversal even more rapid, 15-s half-time. The speed build-up (Fig. 10) is also faster in response to the second pulse, once the cell is elongate. Though the $\frac{1}{2}$ -times are 15 to 20 s, the figures show that the responses begin rapidly, within a few seconds of the stimulus onset. (Since the filming frame rate was 1 fr/7.5 s, estimates to within ≤ 3 s cannot be made reliably.) Our results complement Bonner's (4), mentioned earlier, because he studied cells attached to streams in more fully developed preparations. Our observations on such later cells showed that they responded to repositioned sources, but their behavior was complicated by cell contact.

Alcantara and Monk (1) attempted to study movement refractoriness by assuming that cells were attracted toward waves moving outwards in streams and by observing cells moving erratically between streams. Their conclusions rest on their (mostly unstated) assumptions about the changes and fluctuations in the attractant gradients—gradients which they could neither control nor measure. In addition, they assumed that the cells which moved erratically or away from the aggregation center were competent and responding to the changing gradients—again, they had no independent way of checking the cells' competency. We think their arguments are circular and therefore inconclusive in establishing any understanding of directed motion refractoriness.

A study similar to ours was done earlier on polymorphonuclear leukocytes, with similar results. On reversing the chemoattractant pipette source position, "the cell reacted within seconds and began actual displacement in the new direction within 40 sec." (73). Zigmond recently reported detailed studies of leukocyte polarity (93). It appears to us that the only notable

difference between *D. discoideum* and leukocytes that affects their behavior is that leukocytes have a pronounced morphological polarity with a knoblike uropod (tail).

"Cringing" and the Speed Response

We have discovered that *D. discoideum* cells exhibit a transient response to cAMP which we call "cringing." Cringing in *D. discoideum* is induced by both short and long cAMP pulses. In the normal cringe response, a cell slows appreciably or stops within 20 to 30 s after the onset of the pulse. This is accompanied by a rounding and contraction of the cell. In phase microscopy the cell appears to brighten centrally as it contracts. In SEM, retraction fibers appear at the periphery and ruffles form on the dorsal surface of the rounded cell or at the leading edge of a more elongated cell (B. Storm and R. P. Futrelle, unpublished observations). After 40 to 50 s the cell respreads and continues normal locomotion. Under some not-well-understood conditions a cringing cell does not round up but instead undergoes a transient slowing or cessation of both translocation and shape changes—the cell appears momentarily paralyzed. If the cAMP signal duration is long enough to induce chemotaxis, the cell will begin to orient during the cringe—the two phenomena can occur simultaneously. Cring-



FIGURE 8 Outlines of successive positions of a single cell (12 h of development) 15 s apart showing the effects of reversing the cAMP source position. Two pulses of ~ 1.5 min duration were applied, the first 45 μm from the cell center in the direction shown by the upwards arrow, the second about 50 μm from the cell center in the other direction shown. About 50 other cells were visible, but the cell shown here did not contact any of them during this sequence.

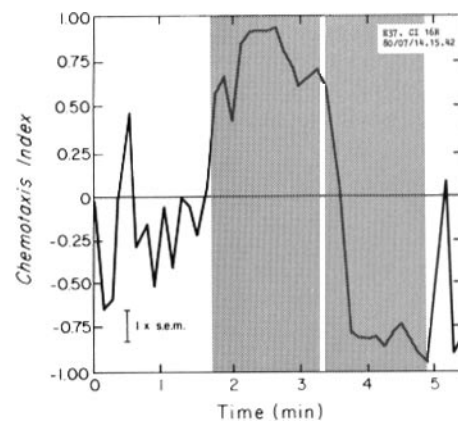


FIGURE 9 The chemotaxis index of the cell shown in Fig. 8. The index is evaluated with respect to the first source position. The gray bands correspond to the cAMP pulses and the light gap between them shows the time at which the source position was reversed. The index becomes large and negative at ~ 4 min when the cell moved away from the first source position and toward the second. In this figure and Fig. 10, the data are averaged over 16 replicate entries of data from the same cell.

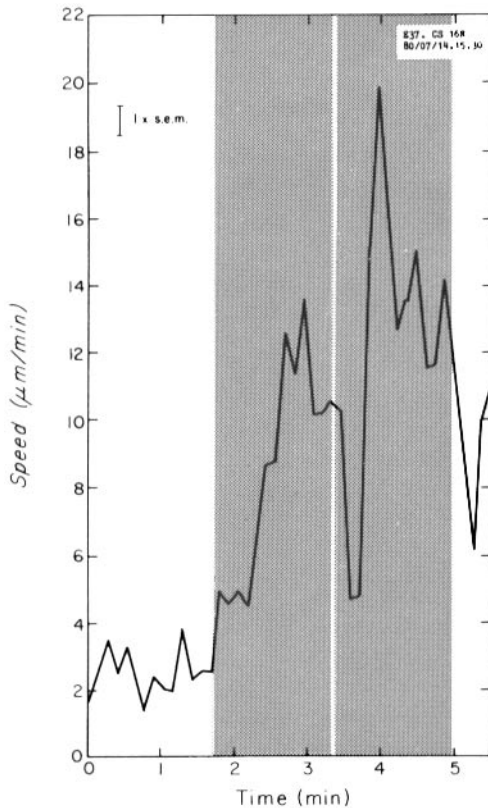


FIGURE 10 Speed of the cell shown in Fig. 8. (The speed is averaged over eight replicates of the upper cell edge and eight replicates of the lower cell edge. Both edges did not stop simultaneously, so the cell speed reported does not fall to zero during reversal.)

ing was earlier described in leukocytes (92) where it was discussed as a process of sensory adaptation.

Rapid transient responses of amoebae were first discovered in 1879 when Engelmann used upshifts in luminous intensity and observed a transient cessation of movement he called a “shock reaction” (Schreckbewegung) (22)—now called a phobic response. Research on these reactions to light was pursued by Mast and his co-workers until the early 1940’s (24, 58, 59). Then in the late 1970’s other workers, apparently unaware of the earlier work, began finding rapid transient responses in cells to chemical upshifts (cf. “Other Systems”).

A transient dip in the speed is a clear indicator of cringing. Fig. 11 shows the speed variation after a 5-s pulse of a pipette with $10^3 \times$ the standard cAMP concentration, giving an estimated concentration $<2 \times 10^{-7}$ M at a cell 100 μm from the pipette. The average speed dropped from 10 $\mu\text{m}/\text{min}$ to 2 $\mu\text{m}/\text{min}$ within 15 s. (Due to inherent statistical bias the minimum speed is probably $\sim 0.5 \mu\text{m}/\text{min}$; see Appendix 3.) The speed returned to its pre-stimulus (baseline) value in 50 s and then increased an additional 50%, peaking at 15 $\mu\text{m}/\text{min}$. It then decayed to the baseline with a half-time of ~ 1 min. In Fig. 11 the response appears to precede the stimulus, but this is because a straight line has been drawn between the speed data point before the stimulus and the next speed data point 7.5 s later.

Detailed analysis of the data shows that the response is quite rapid, with most of the speed drop occurring within <3 s after the stimulus onset. The cells move only $\sim \frac{1}{3} \mu\text{m}$ in this 3-s interval before they essentially stop.

In spite of the clarity of the response shown in Fig. 11, the cringe phenomenon is easily missed when viewed through the microscope or on film. It is most clearly seen in films when

they are run backwards so that the cringe is seen before the visually disturbing appearance of the pipette. Only Gerisch appears to have noticed cringing in the cellular slime molds. He refers to it in passing as a “slight contraction” of the cell within 5 s of the pipette stimulus (38).

The cringe response for a single cell is shown in Fig. 12. The cell was one in a field of cells developing under liquid (BSS) in a glass chamber. At 16 h of development it was subjected to a rapid cAMP upshift. No substantial gradients of appreciable duration persisted for long in the chamber, as was evident from examining the rapidity of dye mixing. The lack of coordinated directional movement of the cells also indicated that no appreciable gradients existed. The final concentration was precisely known in this experiment, 7×10^{-8} M cAMP. This was near the reported dissociation constant K_d of $\sim 10^{-7}$ M for the most numerous (low-affinity) cAMP binding sites on *D. discoideum* (40, 44, 49) and close to the maximum dose response level for cAMP relaying as measured in concentration-clamp experiments (18). It is less than the peak concentration measured in waves *in situ* by using isotope dilution-fluorography (87).

In Fig. 12 the anterior, leading edge of the cell is moving to the right while the posterior, trailing edge is moving up at the time of the unshift. The leading edge stops abruptly and is seen to brighten in the film (phase-contrast image) when the upshift occurs, while the motion of the trailing edge continues a bit longer. Leukocytes which are locomoting and subjected to an upshift in chemoattractant concentration behave in essentially

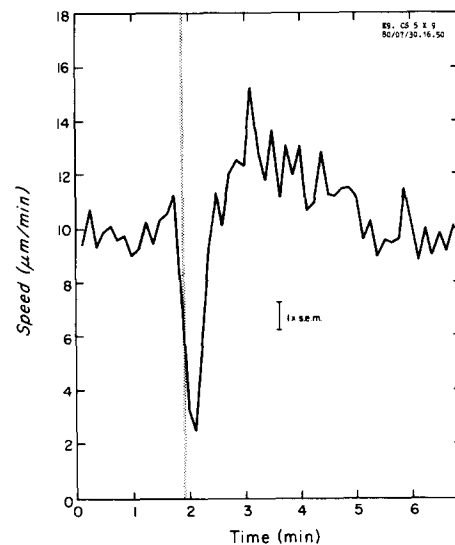


FIGURE 11 The “cringe” response to 5-s cAMP pulse (gray bar). Interphase cells at 500 cells/ mm^2 were stimulated with a standard pipette containing $10^3 \times$ the standard cAMP concentration. The peak concentration at a cell 100 μm away was $<2 \times 10^{-7}$ M. The average speed during 45 events is shown (responses of nine cells to five consecutive pulses 5 min apart). The speed drop appears to precede the cAMP pulse because the average pre- and post-pulse speeds are connected with a straight line.

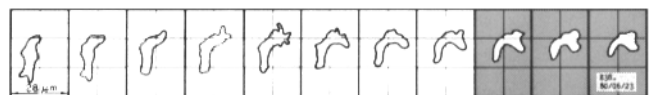


FIGURE 12 Outlines of a late interphase (16 h) cell cringing (slowing and contracting) in response to an upshift from 0 to 7×10^{-8} M cAMP in a glass filming chamber. Frames are 7.5 s apart; gray frames indicate presence of 7×10^{-8} M cAMP. The cell was fixed for SEM in the final frame, after 15 s of exposure to cAMP.

the same way (92). *D. discoideum* cells in glass chamber experiments subjected to later fixation or no fixation appeared to resume normal locomotion within 60 s. SEM study of the cell in Fig. 12 showed ruffling at the leading edge along with numerous retraction fibers around the periphery (figure not included). The ruffling and retraction fibers were less common on control cells for which BSS was injected into the chamber.

Early cells and cells subjected to strong upshifts, e.g. 1 mM cAMP, show more extreme rounding and ruffling over their entire dorsal surface when studied in SEM (see also [78]). In phase-contrast microscopy such cells appear as bright round discs, similar to the leukocytes in Fig. 5 F and D of reference 92.

Cells subjected to 1-min pipette-produced pulses recover and resume locomotion before the stimulus ends (Fig. 13). This figure shows the average speed calculated from the same cell position data used for the chemotaxis index plot of Fig. 6. By comparing Figs. 6 and 13, it is clear that the cells were orienting at the same time their speed was depressed. In Fig. 13 the cells appear to have slowed again, reaching a second minimum just after the cessation of each 1-min pulse. This later reaction appears to be independent of the pulse duration as is the primary cringe response itself. It is not a reaction to the downshift at the end of the 1-min pulse.

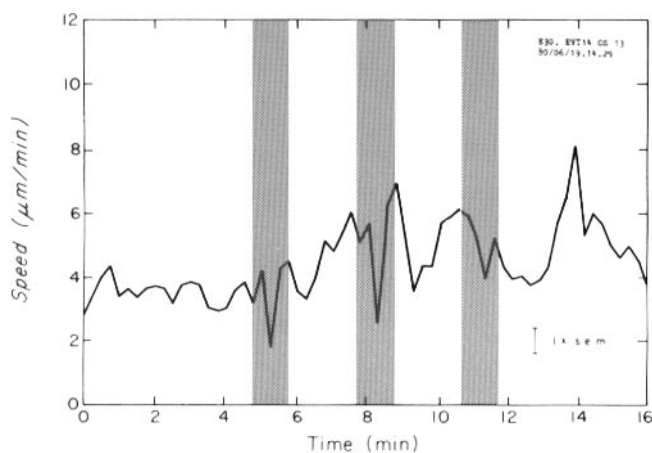


FIGURE 13 The average speed of 13 cells during three consecutive 1-min cAMP pulses (gray bars) 2 min apart, standard conditions. The corresponding chemotaxis index is shown in Fig. 6.

The primary cringe is most easily seen in a confluent cell population (Fig. 14). The pulse was 5-s long (Fig. 14a) and the cells showed maximal contraction 22.5 s (3 frames) later (Fig. 14b). In viewing the film it was possible to see the later event, a slight contraction of the cells, about a minute after the first, but the event is not particularly evident in individual stills from the film. The later event is discussed in the next section.

We have seen autonomous cringe phenomena in natural populations which never received external cAMP stimulation. This assures us that cringing is a normal cellular process in *D. discoideum* and not merely a result of the particular stimulation protocols we used. One film showed two events in which various groups of cells in the field slowed, rounded, and brightened simultaneously. The data for one event are shown in Fig. 15. Only some cells exhibited clearly visible cringing, but over 90% showed an increase in average speed from the 0- to 2-min interval to the 5.5- to 7.5-min interval. The cells showed oriented movement (towards the east). When the chemotaxis index was plotted for this direction it was seen to increase

from 0 to 0.5 during the interval 2.5–3.5 min and then to decay with a half-time of ~1.5 min.

Development of the Cringe Response and Its Relation to Light-scattering Experiments

Two types of rapid responses to cAMP have been observed in stirred suspensions of *D. discoideum* cells: a light-scattering decrease with a half-width of ~25 s (33, 55) and a pulse of intracellular cyclic GMP which reaches a maximum at 10–20 s after stimulation (61, 89). We suggest that the change in the scattered light intensity is a manifestation of the cringe response. When cells cringe, they round. The theory of light scattering applied to cells in suspension shows that rounded cells scatter less light than more irregular cells of the same volume (54). Making the simple assumption that cell volume does not change during cringing, there should be a decrease in scattering and thus a decrease in extinction or optical density. This is what is observed (33, 35). We found that light scattering and cringing develop in the same way as the cells age. Fig. 16 is an average over a large number of events designed to show some of the more subtle details of the response. In the early cells, the response starts quickly and then recovers smoothly. In the middle cells, a small dip appears at ~1.5 min after stimulus onset. In the late cells, the dip is a pronounced response, another minimum in the speed, though not so deep as the primary cringe response. (Recall the discussion, in the previous section, of this later response in connection with Fig. 14.) For the late cells the second minimum is significantly lower than the intermediate peak at time 2.9 min, $p < 0.03$ (one-sided test, paired comparison). A detailed study of the development of the light-scattering response was made by Lax (55). He showed that a second rapid peak develops as the cells age and the second peak occurs from 0.8 to 1.3 min after the primary rapid response.

Sussman's "I-Cells"

In some of our films we saw a few very large, well-spread, and rapidly moving cells. They varied in area from $380 \mu\text{m}^2$ to $580 \mu\text{m}^2$, which is 2.5–4 times the area of a typical cell. The average speed (higher for larger cells) varied from 211 to 44 $\mu\text{m}/\text{min}$, 3 to 8 times the speed of a typical cell. These large cells were presumably the "I-cells" reported by Sussman and co-workers (23). Because the large cells were a minor and distinct subpopulation, data from them were excluded.

DISCUSSION

An Integrated View of Signal Propagation and Cell Response in Natural Aggregation

Cells move inwards in $20 \mu\text{m}$ "steps" lasting 100 s or longer (1, 20). The steps occur as often as every 2.5 min as waves of inward movement activity propagating outwards. Shaffer realized that when an acrasin wave passes over cells, the back of the wave, closer to the center, would present cells with a reversed gradient. This might cause them to reverse their motion. But cells do not reverse after their forward step. He suggested reasons for the nonreversal including cell shape, spacing, velocity, and polarity (81), as well as, "... the cells might be less responsive because of adaptation or fatigue" (80). Some of these ideas were formulated mathematically by Cohen and Robertson (12, 13). Shaffer gave no estimate of the acrasin "pulse" duration; Cohen and Robertson assumed it was 1 s or

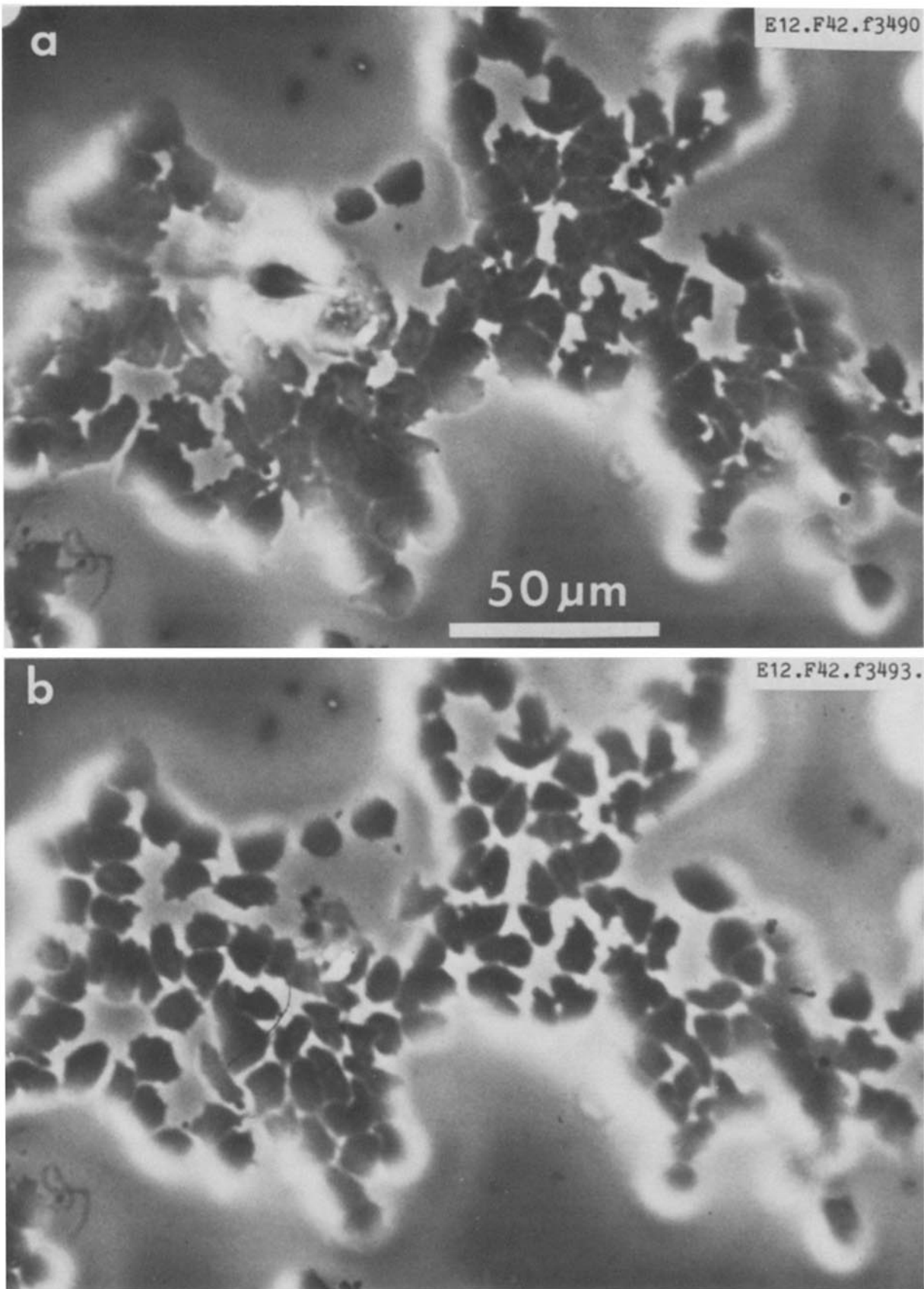


FIGURE 14 Phase micrographs showing the response of a field of late interphase cells to a 5-s cAMP pulse. (a) Beginning of the stimulus. The cells are tightly packed. A standard pipette, seen touching the agar surface to the left of center, was filled with $10^3 \times$ standard cAMP concentration. (b) 22.5 s later the cells have contracted, forming a bright reticulum of gaps, which disappeared after about another 30 s. (Micrographs reproduced from 16-mm movie film.)

less, giving no justification. They also assumed a delay in the relay response; this had been suggested earlier by Jaffe using estimates of diffusion times (45). To prevent reversal, Cohen and Robertson assumed that the cells became refractory for a period of time during which their motion and relaying could not be altered. This left the impression that an aggregation wave propagated outwards as a series of narrow pulses, illustrated in Fig. 17a. When the model was stimulated by Parnas and Segel (66), they discovered that the waves were a few hundred micrometers broad (Fig. 17b). The waves propagated outwards with little change in shape once well-removed from the center, rather than propagating as a series of pulses, each of which built up and decayed before the next occurred. Another difference was that on the back of the wave where the gradient was reversed, the acrasin concentration decreased with time in the simulation. Later simulations using a longer secretion, 60 s, showed even more symmetric waves (Fig. 17c).

The long secretion model of Fig. 17c is consistent with our new results for the duration of chemotaxis and with *in situ*

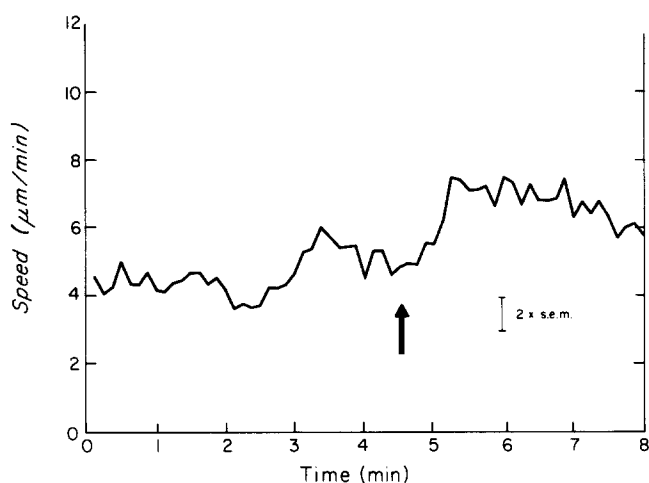


FIGURE 15 The average speed of 81 cells during an autonomous cringe 5 h after plating at 2,400 cells/mm². The arrow indicates the time at which peak brightening of cells was observed.

measurements of cAMP concentrations (87).

Since the cells are exposed to a reversed gradient after the wave peak has passed but don't reverse, we must explain the cell motion reversal results discussed earlier. The difference between our reversal experiment and the gradient reversal in a propagating wave (Fig. 17b and c) is that in the reversal experiment the concentration at the cell rises quickly after the gradient reversal, but in the propagating wave the concentration falls after reversal. This suggests that cells may not be chemotactically sensitive to a gradient whenever the concentration is falling with time. There is a strong precedent for this type of behavior from Devreotes' results (discussed in an earlier section) that the cAMP relay response is only produced by cAMP upshifts. Cringing behaves similarly; it is a reaction to upshifts. We hypothesize that the chemotactic response can occur only in a gradient if the concentration is constant or

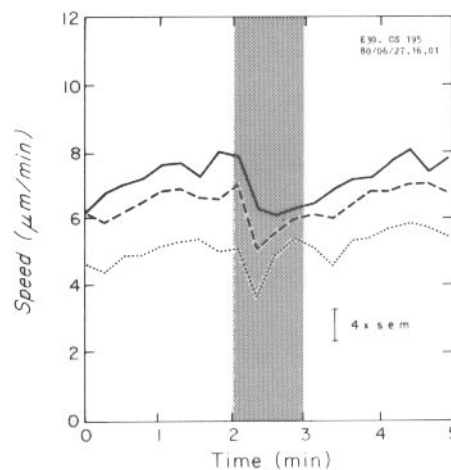


FIGURE 16 Cringing in response to 1-min cAMP pulses (gray bar) for cells in early (—, 1–5 h), middle (---, 6–10 h) and late interphase (····, 11–15 h). The primary speed response peaks at ~20 s after the stimulus onset. A later speed drop, occurring ~1 min after the first, develops as the cells age. The chemotaxis indices for the same data are presented in Fig. 7.

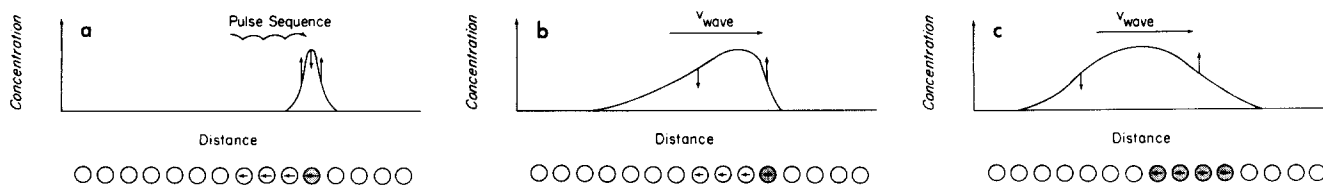


FIGURE 17 Schematics of three models of wave propagation. The vertical coordinate is the acrasin or cAMP concentration and the horizontal coordinate is the distance from the attractant center, assumed to be at the left. The circles represent cells, with arrows to indicate the cells that are moving inward and cross hatching to indicate cAMP secretion. (a) Pattern of activity in the "brief pulse" model. The crosshatched cell has just pulsed, producing a sharp peak in the acrasin concentration. The acrasin diffuses so that the concentration near the cell falls (down arrow) while the concentration on both sides rises (up arrows). The cells on either side see identical concentrations but reversed gradients. The "backwards wave" problem exists because this model presents cells closer to the center with a gradient which would tend to attract them outwards. This would contradict what is seen in natural aggregation where cells only move in and never reverse their motion. In this model, the "wave" moves out as a series of pulses, each of which dissipates before the next one occurs. (b) Schematic of the results obtained by a simulation of acrasin wave propagation (66, 67). Acrasin from earlier cell signals is still present to the left of the signaling cell. Due to acrasin degradation and lack of secretion, the concentration in that region falls (down arrow) rather than rises. The concentration can only rise where a source is active (up arrow). The cells to the left of the signaller still see a reversed gradient on the "back of the wave." These simulations show that the wave moves steadily outwards with little change in shape—the sharp wave front, the peak, and the back of the wave all move outwards together at the same speed v_{wave} , just as a water wave does. (c) Wave propagation based on the assumption that the acrasin wave duration is comparable to the movement step duration in *D. discoideum* (100 s or more.) This view is supported by our chemotaxis measurements and by Tomchik and Devreotes' measurements of cAMP waves *in situ* (87). Here the wave continues to build throughout the entire movement-secretion step (up arrow). The back of the wave has a reversed gradient. The wave moves steadily outwards with no shape change. The cells move only while the concentration is rising.

increasing with time. This leads to further questions about whether *D. discoideum* cells sense gradients by spatial mechanisms or, like bacteria, by temporal mechanisms (29, 35, 93).

“Quivering”

During normal aggregation, cells essentially stop between movement steps. The duration of stopping can be substantial, from 100 to 500 s (1). During these periods the cells do not translocate but they do actively form pseudopods; hence they appear to “quiver” in place when viewed in time-lapse. They remain chemotactically responsive. The cell in Figs. 8 and 10 is quivering in the period before the first pipette stimulus. In experiments on other late cells in which the pipette was moved at intervals, cells farther than 100 μm or so showed no translocation, with net displacements of 2 μm or less in 40 min, an average speed of $<1/20$ $\mu\text{m}/\text{min}$, $<1\%$ of normal.

We hypothesize that quivering serves to increase the efficiency of pattern formation during aggregation. To understand this, consider a cell that has just “stepped” and is close to but not yet in contact with a cell at the end of a stream. If the cells quiver, then the positions attained by the step would be maintained. If the cells wander off randomly they would lose their advantageous positions. If we think of the field of cells as a “tissue”, then quivering stabilizes the pattern developing in this tissue by suppressing random cell motion or “mixing.”

Other Systems

Chemotaxis has been discovered in a number of “crawling” cell types besides *D. discoideum*. Only leukocyte chemotaxis has been examined in comparable detail (91). Systems needing detailed study include negative chemotaxis (47) and folic acid chemotaxis (65) in *D. discoideum* as well as chemotaxis in vascular endothelial cells (8), palate mesenchymal cells (11), nerve fibers (42), fibroblasts (68), and tumor cells (76).

Our measurements suggest that cringing is the morphological and locomotory correlate of the rapid light-scattering response in *D. discoideum* cell suspensions. Mato et al. come to similar conclusions by more indirect arguments (60, 61, 62, 89). They suggest that the response is made up of two components: a rapid, transient, generalized contraction of the cell and an increase of cGMP in a localized region of the cell leading to pseudopod extension and therefore directed movement (62). A shape change alone could explain the light-scattering results but the change in cell volume that they suggest is an equally plausible mechanism.

The cringe response in *D. discoideum* is similar to transient responses in a number of other cells types. Early studies examined the responses of *Amoeba proteus* to upshifts in illumination. If a brief flash of light was given, the cell ceased locomotion within a few seconds (24, 58). After a permanent upshift in illumination, the cell ceased locomotion quickly but then resumed moving after 3 min or so (59).

Monolayers of A-431 human carcinoma cells show ruffling within 60 s of exposure to EGF and cells at the periphery of a colony begin to retract after 10 min (10). When NGF is applied to PC12 pheochromocytoma cells they show extensive ruffling on their dorsal surface within 1 min (14). When NGF is applied to growth cones of chick dorsal root ganglia in vitro, the neurites begin retraction within 2 min. This is a lengthy but ultimately transient response, because outgrowth resumes some 2 h later (41). Membrane hyperpolarization occurs in macrophages within 5 s of exposure to chemoattractants. The re-

sponses are 10 to 30 s in duration and show desensitization to repeated stimuli (30, 31).

Studies of early chick development show organized waves of cell movement analogous to cellular slime mold waves (84). It might be possible to demonstrate this behavior in the dissociated system, allowing it to be studied by our methods.

CONCLUSIONS

It is clear from our results that no reaction of a cell to a stimulus can be viewed in isolation. A stimulus alters morphology and locomotion and, presumably, receptor function and internal pools. These in turn modulate the response to the next stimulus, leading to adaptation and facilitation. If we try to avoid these “memory” effects by waiting a few hours before applying another stimulus, then *D. discoideum* cells show developmental changes in response.

In chemotaxis, adaptation and facilitation are clearly evident in the three successive responses in Fig. 6. The second response is enhanced (facilitated). This could be a true increase in the response of each cell or simply a summation as additional cells are recruited into the well-oriented population, adding to the cells recruited by the first stimulus which persist in their motion. The third stimulus produces a weaker response. This is adaptation.

In the cell reversal experiment, the speed response (Fig. 10) shows “priming”; the rate of increase of speed (acceleration) is larger in response to the second stimulus. Again, this could be due to a shift in the rate parameters of early transduction events or due to a change in morphology. Once elongate and actively moving, the motor apparatus and membrane structures at the two ends of the cell may respond more rapidly.

The computerized approach has allowed us to see these differences in response. The approach has allowed us to analyze responses which are too weak or subtle to be picked up reliably by manual pencil and ruler methods. Compared to our approach, the classical chemotactic assays typically give far less information and are restricted in the conditions and configurations allowed (13, 51, 52, 88, 90). For *D. discoideum*, the cell populations which are used in most of these assays develop autonomous, coordinated activity as they age, and this interferes with the assays. A micropipette can locally override autonomous signals and extend the assay to aggregating populations (75).

Using the computerized approach, a weak but unambiguous chemotactic response was detected in *D. discoideum* which probably begins as early as 1 h of development. This early weak chemotaxis occurs by biased random walk (klinokinesis). As the cells age, this changes smoothly into true directed motion. Thus we see no reason to make a sharp distinction between klinokinesis and directed chemotaxis as some have suggested (48). Organized wave activity occurs by 8 h in our populations which don’t aggregate for another 7 h. (Fig. 15 shows this behavior at higher than the standard cell density.) The existence of the waves shows that relaying occurs. Aggregation and streaming are probably prevented at these early stages by the fact that chemotaxis is weak and that the cells do not stop (quiver) between waves to stabilize nascent patterns.

Cringing is a process of rapid adaptation because cell locomotion resumes within a minute even if the stimulus is maintained. Cringing is more rapid than the 2- to 3-min transient cAMP relay response (21). A series of strong cAMP pulses a few minutes apart will gradually extinguish cringing—a long term adaptation (R. P. Futrelle, unpublished observations)—

and high cAMP levels, ~1 mM, can prolong the response indefinitely (78).

Confluent cells separate from one another when cringing (Fig. 14). This may be related to a phenomenon we see during aggregation: quivering (stopped) cells sometimes separate from their proximal or distal neighbors and then rejoin as the next wave begins. This process is particularly noticeable during aggregation on glass under BSS. But any hypothetical relation between cringing and quivering is not strong if cAMP is considered to be the only controlling agent, because cringing is a transient response to high cAMP levels (upshifts) and quivering is a long-lived response to low cAMP levels. It is easier to imagine that quivering is under the positive control of an additional "stopping agent."

Many of the results of this paper are presented as averaged stimulus-related responses (stimulus-locked), in the spirit of electrophysiology. The data are presented nonparametrically, e.g. the chemotaxis index is simply displayed in detail as a function of time. A parametric approach would have forced us to choose arbitrary orientation, step size, and turn angle "bins" and to report the fraction of cells whose behavior fell within them.

Our new approach to the analysis of cell behavior can be used to analyze the results of biochemical and genetic studies on *D. discoideum* and other organisms. Such studies, coupled to computerized analysis of cell shape changes, cell contact, and aggregation, should give insight into the complex, self-regulated process of development.

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APPENDIX 1

DIFFUSION: A conical micropipette (constant taper near the tip) delivers a constant diffusive flux. This sets up a stable concentration distribution in the agar. The diffusion flux from the pipette (71) is,

$$J_d = \pi a \phi D C_p, \quad (1.1)$$

where a is the tip inner radius, ϕ is the $\frac{1}{2}$ -angle (taper of the fluid-filled capillary bore), D the diffusion constant, and C_p the concentration of the diffusing species in the pipette. Hydrostatic pressure produces bulk flow (53) giving a molecular flux,

$$J_b = 3\pi\phi a^3 p C_p / 8\eta, \quad (1.2)$$

where p is the net pressure and η the viscosity. Typical values were $a = 0.3 \mu\text{m}$ and $\phi = 0.05$ radians (by SEM), $D = 10^{-5} \text{cm}^2/\text{s}$ for cAMP, $p = 10^3 \text{dynes/cm}^2$ for each 1 cm head, C_p the concentration of cAMP in the pipette, and $\eta = 10^{-2}$ poise (for water). This gives:

$$J_d = 4.7 \times 10^{-11} \text{ml/s} \times C_p; \quad (1.3)$$

$$J_b = 1.6 \times 10^{-10} \text{ml/s} \times C_p. \quad (1.4)$$

Capillary pressure must be included. It has been ignored in past theories. The glass capillaries gave a capillary rise of 5 cm of water. Usually the pipettes were not completely filled so they exerted a negative capillary pressure at the tip of ~1 to 2 cm of water, giving a net bulk influx. The theory of iontophoretic retaining currents (71) can be applied, predicting a net diffusive efflux J_{net} in the presence of a bulk influx J_b ,

$$J_{\text{net}} = J_b / (\exp[J_b/J_d] - 1) \quad (1.5)$$

Using values from equations (1.3) and (1.4) gives,

$$J_{\text{net}} = 5.5 \times 10^{-12} \text{ml/s} \times C_p. \quad (1.6)$$

Measurements of efflux under a net positive hydrostatic pressure were nearly an order of magnitude less than equation 1.4, so we can only have modest confidence in the theory—not unusual in dealing with micropipettes! We calculated the pipette resistance R ,

$$R = 1/(\pi\sigma\phi a), \quad (1.7)$$

using a conductivity of BSS calculated to be $\sigma = 2.7 \text{mmho/cm}$. This predicted $R = 80 \text{M}\Omega$, within our measured range of 20 to 100 $\text{M}\Omega$.

Ultimately, we relied on calibration of the pipettes by measuring efflux of ^3H -cAMP. When the pipettes were partly filled and allowed to perfuse agar for several hours, no counts above background were recorded. When filled to more than a 5 cm head, significant counts were recorded. After all this, we could only place an upper bound on the efflux from the standard pipette,

$$J_{\text{exp}} \leq 10^{-12} \text{ml/s} \times C_p, \quad (1.8)$$

which for $C_p = 10^{-4} \text{M}$ cAMP (10^{-7}mol/ml) gives the standard flux $J_s \leq 10^{-19} \text{mol/s}$ or $\leq 6 \times 10^4$ molecules/s.

The concentration (C_a) and the gradient (∇C_a) in the agar reach a steady state when the agar is subjected to a constant flux at a point,

$$C_a = J/(2\pi Dr), \quad (1.9)$$

$$\nabla C_a = C_a/r, \quad (1.10)$$

with r the distance from the pipette tip. The cAMP diffuses through the agar—it is not confined to the liquid surface layer. Inserting J_s into equation (1.9) gives upper bounds for typical concentrations and gradients, for $r = 30 \mu\text{m}$: $C_a = 5.3 \times 10^{-10} \text{M}$ and $\nabla C_a = 1.8 \times 10^{-7} \text{M/cm}$; for $r = 100 \mu\text{m}$: $C_a = 1.6 \times 10^{-10} \text{M}$ and $\nabla C_a = 1.6 \times 10^{-8} \text{M/cm}$ for $r = 180 \mu\text{m}$: $C_a = 8.9 \times 10^{-11} \text{M}$ and $\nabla C_a = 4.9 \times 10^{-9} \text{M/cm}$. The latter distance, 180 μm , was the typical maximum distance between the pipette and cells visible in the field of view.

When touched to the agar, the pipette momentarily delivers more diffusive flux than given by equation (1.1). This drops to within a factor of 2 of the steady-state value in $t_2 = a^2/D\pi\phi^2 \approx 0.01 \text{s}$, a negligible time. Then the concentration in the agar obeys,

$$C_a(r, t) = (J/2\pi Dr) \times \text{erfc}(r/\sqrt{4Dt}), \quad (1.11)$$

where erfc is the error function complement. Therefore C_a reaches one half its final steady-state value in a time,

$$t_{1/2} = 1.08 r^2/D, \quad (1.12)$$

giving typical values of:

$$t_{1/2} = 1.0 \text{ s for } r = 30 \text{ } \mu\text{m},$$

$$t_{1/2} = 10.8 \text{ s for } r = 100 \text{ } \mu\text{m},$$

$$\text{and } t_{1/2} = 35 \text{ s for } r = 180 \text{ } \mu\text{m}.$$

If the pipette is touched down briefly, for t_d seconds, the concentration and gradient seen by distant cells never reach the steady-state values. For $t_d = 5$ s, cells at $r = 30 \text{ } \mu\text{m}$ see a signal well-described by equations (1.9) through (1.12). Distant cells, $r = 180 \text{ } \mu\text{m}$, see a concentration which peaks 9 s after the pulse begins, reaching a value of $C_a = 7.4 \times 10^{-12}$ M, more than an order of magnitude below the steady-state value. The maximum gradient is also reduced.

In the Konijn assay a drop of attractant is placed a few millimeters from a small population of cells. This corresponds to applying a brief pulse. For a cell-to-source separation of 2 mm, the attractant concentration seen by the cells takes several hundred seconds to build up and decay, ~ 100 times slower than the reaction time of *D. discoideum* cells.

APPENDIX 2

CHEMOTAXIS INDEX (CI): Our experiments required an index of directed locomotion that could show rapid changes in cell behavior. Fig. 3 shows how we defined the chemotaxis index (CI). It is closely related to the McCutcheon index MI, (his chemotropism index [63]). McCutcheon considered motion over an extended period of time, say 0 to T, using the net approach A and the total path P over the interval,

$$MI = A/P. \quad (2.1)$$

This can be written in terms of the average speeds over the interval, since $A = T\langle v_s(t) \rangle_{0,T}$ and $P = T\langle v(t) \rangle_{0,T}$,

$$MI_{0,T} = \langle v_s(t) \rangle_{0,T} / \langle v(t) \rangle_{0,T}. \quad (2.2)$$

The averaged CI is different from MI because the ratio is taken before the averaging is done,

$$\langle CI \rangle_{0,T} = \langle v_s(t)/v(t) \rangle_{0,T}. \quad (2.3)$$

If the cell speed is a constant, the two quantities agree

$$MI_{0,T} = \langle CI \rangle_{0,T}, \text{ if } v(t) = \text{const}. \quad (2.4)$$

If the speeds vary, MI may be greater or less than $\langle CI \rangle$ and may even differ in sign. For example, if a cell moved for 1 min at $18 \text{ } \mu\text{m}/\text{min}$ directly towards a source and then for 9 min at $1 \text{ } \mu\text{m}/\text{min}$ directly away, $\langle CI \rangle_{0,10} = -\frac{1}{2}$ and $MI_{0,10} = +\frac{1}{3}$. MI can be calculated from

$$MI_{0,T} = \langle v(t) \times CI(t) \rangle_{0,T} / \langle v(t) \rangle_{0,T}. \quad (2.5)$$

The $v(t)$ term in the numerator shows that MI is weighted towards higher speeds.

The CI is convenient for analyses because it can be averaged over time and over a set of cells and the order of the averaging does not affect the results. Averaged CI's from different intervals can be further averaged, e.g.

$$\langle CI \rangle_{0,T} = (\langle CI \rangle_{0,T/2} + \langle CI \rangle_{T/2,T})/2 \quad (2.6)$$

An equation such as (2.6) does not hold for MI because, in general, the average of two quotients is not the quotient of the averages of the numerator and denominator.

APPENDIX 3

DATA AND ERROR ANALYSIS: The primary raw data were streams of x,y coordinate pairs, the cell "centers" as estimated by a human. The average cell area was that of a $15 \text{ } \mu\text{m}$ diameter disk (30 mm projected onto the data tablet). The human tracker could consistently estimate a center for a cell to within 3%, which is $\sim \frac{1}{2} \text{ } \mu\text{m}$.

Standard errors of the mean (SEM) were computed from the variation between different cells at each fixed time. The SEM include true cell-to-cell differences as well as random errors of filming, digitization, etc. The standard deviation of the speeds σ_v , fell with age as the mean speed did, e.g. early cells: $\langle v \rangle = 8.0 \text{ } \mu\text{m}/\text{min}$, $\sigma_v = 4.3 \text{ } \mu\text{m}/\text{min}$; middle cells: $\langle v \rangle = 6.5 \text{ } \mu\text{m}/\text{min}$, $\sigma_v = 3.3 \text{ } \mu\text{m}/\text{min}$; late cells: $\langle v \rangle = 5.0 \text{ } \mu\text{m}/\text{min}$, $\sigma_v = 1.5 \text{ } \mu\text{m}/\text{min}$ (all, $n = 195$).

Random errors were analyzed by comparing replicate entries of a single cell's track, giving $\sigma_v = 1.5 \text{ } \mu\text{m}/\text{min}$. At 1 fr/s, the human tracking errors in successive frames were essentially independent, so we estimated the error in tracking the center position by $\sigma_r = \sigma_v \times \Delta t$, or $\sigma_r = 0.8 \text{ mm}$ on the data tablet.

One systematic error was a bias in the cell speeds. Even if a cell doesn't move, errors in estimating its center in each successive frame would lead to an apparent jiggling motion, giving a positive and therefore biased speed. If the true speed is zero, the bias or error is

$$v_b \approx 1.15 \sigma_v = 1.9 \text{ } \mu\text{m}/\text{min}, \quad (3.1)$$

(assuming normally distributed, independent position errors). If v_a is much greater than σ_v , it can be shown that the reported average speed $\langle v \rangle$ is

$$\langle v \rangle \approx v_a + \sigma_v^2/2v_a. \quad (3.2)$$

Equation (3.2) shows that the bias $\sigma_v^2/2v_a$ is a relatively small effect for large v_a . The bias increases as the speed falls, reaching the limit of equation (3.1) as $v_a \rightarrow 0$. Bias effects are important in studies of cringing because cringing cells slow or stop.

To estimate the bias from the data, a number of replicates of a single cell track were again used but the average position in each frame was first computed over all the replicates and then the speed was calculated from the average positions (reversing the usual procedure). The bias term in equation (3.2) then drops from $\sigma_v^2/2v_a$ to $\sigma_v^2/2nv_a$ where n is the number of replicates. The bias can be further extrapolated to very large n (unbiased limit) using the jackknife statistic (72). This gave $v_b = 0.2 \text{ } \mu\text{m}/\text{min}$ for $v_a = 10 \text{ } \mu\text{m}/\text{min}$ and $v_b = 0.7 \text{ } \mu\text{m}/\text{min}$ for $v_a = 2.6 \text{ } \mu\text{m}/\text{min}$.

There was also bias due to film misregistration, e.g. if there was a random error in the position of each frame of film in the camera gate of 1 part in 3,200 ($5 \text{ } \mu\text{m}$), all the objects in the image will have an erroneous speed of $1 \text{ } \mu\text{m}/\text{min}$ superimposed on their motion. Replicate tracking of an inert piece of debris, actual speed zero, gave an average speed of $1.6 \text{ } \mu\text{m}/\text{min}$. Removing tracking bias with the jackknife left a residual of $1 \text{ } \mu\text{m}/\text{min}$ attributable to the camera errors (or related systematic errors).

In summary: The cell-to-cell speed differences, $\sigma_v = 3.5 \text{ } \mu\text{m}/\text{min}$, were the primary source of variability; true random errors (hand digitization, projector registration) contributed only σ_v

= 1.5 $\mu\text{m}/\text{min}$ to the variability. A known motionless object gave an average speed of 1.6 $\mu\text{m}/\text{min}$, of which 0.6 $\mu\text{m}/\text{min}$ was due to bias introduced by random errors and the remaining 1.0 $\mu\text{m}/\text{min}$ was due to systematic "camera" errors. If we apply this to the speed data in Appendix 3, it suggests that observed minimum average speeds of 2 $\mu\text{m}/\text{min}$ are actually $\sim 0.5 \mu\text{m}/\text{min}$, so the cringing response is more extreme than the plots suggest.

Because a cell center follows an irregular trajectory the measured speed will always decrease when computed for longer intervals. When the speed was recomputed from the same data using such 1-min intervals, it dropped to $\sim 85\%$ of the 4 fr/min value. This shows that the speeds in this range of frame rates are stable and not overly sensitive to their method of definition.

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