P2: A BEHAVIOURAL MUTANT OF
DICTYOSTELIUM DISCOIDEUM

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SUMMARY
P2 is a mutant strain isolated from the cellular slime mould, Dictyostelium discoideum strain NC-4. P2 differs from NC-4 in 3 major respects. P2 lacks the cyclic AMP chemotactic and signalling properties and the aggregative development of NC-4. P2 development consists of slow differentiation of resistant microcysts, which have not previously been reported in D. discoideum. Most important, P2 amoebae display a novel pattern of movement, quite distinct from that of NC-4 amoebae and any other related amoebae for which data are available. P2 amoebae move on agar at a mean velocity of 13.3 μm/min, almost twice as fast as NC-4 amoebae. P2 amoebae have a persistence time, or directional memory 'half-life', of 13.2 min, over 2.5 times the NC-4 value. However, this measure is based on straight-line movement, and actual P2 persistence may be much greater because P2 amoebae move almost always in gradual left turns and left loops interspersed with occasional long, straight segments. When P2 amoebae are plated in drops on agar, they migrate away in all directions, and the expanding drop edge can move at nearly the mean individual cell velocity. Spiral bands of amoebae can be seen in the expanding area of the drop; these invariably unwind from the original drop in a counterclockwise direction. The persistent left-turning behaviour of individual P2 amoebae is probably the major cause of this pattern of movement from drops. It also probably helps explain the similar coordinated banding behaviour observed in fields of cells. Amoebae move long distances in bands of contacting or separated cells, with no apparent organizing centre. Statistical analysis shows that P2 amoebae have some tendency to cluster, and cell-cell contact interactions may be strong enough to promote band formation.

INTRODUCTION
Dictyostelium discoideum is a cellular slime mould widely studied as a model developmental system. The organism progresses through its life cycle from single, free-living amoebae to an integrated, multicellular slug and finally to a fruiting body with 3 differentiated cell types (Bonner, 1967). Formation of the multicellular body is accomplished by aggregation of the cells, which is triggered by starvation and mediated by the chemotactic agent, cyclic AMP (adenosine-3',5'-cyclic monophosphate, cAMP), and an array of enzymes and receptors that make up an efficient intercellular communication system (Newell, 1978).

Some closely related species of cellular slime moulds such as Dictyostelium mucoroides and Polysphondylium pallidum possess an option for development in addition to aggregation: microcyst formation (Blaskovics & Raper, 1957; Bonner, 1967). Microcysts are individual amoebae which have secreted a rigid cellulose-containing wall and are resistant to drying and other adverse conditions (Toama & Raper, 1967a,
b). Microcyst formation has not to my knowledge been reported in \textit{D. discoideum}.

\textbf{P2 is a mutant strain isolated from \textit{D. discoideum} strain NC-4.} Developmental and behavioural alterations from the parental strain make P2 interesting and of potential value in studies of the control of development and of amoeboid movement. P2 does not aggregate; instead its development after starvation consists of a slow formation of resistant microcysts. P2 cells show a pattern of amoeboid movement quite distinct from that of NC-4 cells, moving at a higher mean velocity and in much straighter paths. Recently developed methods of data collection and the use of statistical analysis have made it possible to study quantitatively the movement of P2 and other cell types. These methods could become the basis of studies relating the biochemical and behavioural aspects of cell locomotion.

**MATERIALS AND METHODS**

Strains P2 and NC-4 were grown in association with \textit{Klebsiella aerogenes} on nutrient agar containing, in g per litre, glucose (5), peptone (5), yeast extract (0.5), agar (20), and KK a buffer, pH 6.5. KK a buffer consisted of potassium phosphate (20 mM) with MgSO$_4$·7H$_2$O (2 mM). For developmental studies, vegetative amoebae were separated from bacteria by centrifugation 4–5 times in KK a buffer for 2 min at 433 g, then plated at various densities on \% Difco purified agar and incubated at 21–23 °C.

Time-lapse films at various magnifications were taken with Bolex M16 cameras and Nikon Cine Autotimer motor drives mounted on Nikon SKE microscopes. Films were digitized and analysed with an interactive computer graphics system, Galatea (Potel & Sayre, 1976). This system is built around the Digital Equipment Corp. GT44 Graphics System, based on the PDP-11/40 computer and VT-11 display processor. It uses an acoustic digitizing pen (Science Accessories Corp. Graf/Pen GP-2) to record objects and events from the projected film image. A 16-mm variable-speed, stop-motion projector (L-W International Data Analyzer model 224 A, Mark IV) is controlled by the operator and/or by the computer. A projection kinescope (based upon an RCA 4862 Display Tube and Schmidt reflective optics) superimposes a computer-generated image of the digitized data over the film image while the film is running. A variety of data collection modes are available, for example, simple markers, moving point traces, and animations. Galatea thus greatly facilitates data collection from films, and since these data are automatically placed in computer files they are readily available for analysis with the PDP-11/40.

Cells and microcysts were counted with a haemocytometer, and volume measurements were done with Wintrobe Macrohematocrit Tubes and a Coulter Counter model Zs with a Channelizer C1000. Karyotyping was done according to the method of Brody & Williams (1974). The cAMP amplification properties of the cells were assayed by the method of Devreotes, Derstine & Steck (1979). cAMP phosphodiesterase (PDE) activities were assayed by the method of Thompson, Brooker & Appleman (1974). Binding of cAMP to the cells was assayed by the method of Green & Newell (1975).

**RESULTS**

**Growth and development**

\textbf{History.} P2 was derived from \textit{D. discoideum} strain NC-4 (originally obtained from Dr K. B. Raper) after treatment with the frameshift mutagen picrolonic acid (Rosenkranz & Stein, 1975). The original isolate of P2 (P23B) was able to form small chemotactic mounds which often had small tips, but was usually incapable of completing development to fruiting bodies. Drops of P23B cells on agar containing 10$^{-8}$ M cAMP formed outward-moving spikes, and on cAMP they formed expanding
Behavioural mutant of *D. discoideum*

rings as does NC-4. These reactions indicate chemotactic ability. The original isolate was lost when the present P2 arose spontaneously. P2 has been stable through weekly clonal passages for over 2 years, except for one occasion in which a new spontaneous mutant, P2a, was isolated from P2. P2a resembles the original P23B but never develops past mounds with small tips. Its morphological responses to cAMP are roughly similar to those of the original P23B isolate. P2 thus appears to be a variant which arose from one or more spontaneous mutations after an original picrolonic acid-induced mutation(s).

**Growth and cytology.** P2 grows more slowly than NC-4 on agar with *K. aerogenes*; the generation time for P2 is 6.1 h compared to 3.2 h for NC-4. The mean volume of rounded P2 cells in suspension is 1420 μm³ which corresponds to a diameter of 14 μm. P2 microcysts have a mean volume of 899 μm³ and a diameter of 12 μm. Both volume distributions are broad. P2 cells are thus intermediate in size between NC-4 cells (10.5 μm diameter) and axenically grown cells of the axenic strain, AX-3 (16.2 μm diameter; Dr R. Clark, personal communication). No multinucleate cells were observed among the several thousand stained and unstained P2 cells studied, and the normal haploid complement of seven chromosomes is present. P2 microcysts are larger than either NC-4 spores (6.9 μm long; Bonner, 1967) or *P. pallidum* microcysts (4.6 μm diameter; Toama & Raper, 1967a).

The typical flattened, motile P2 cell is 18.5 μm (s.d. 2.18; s.e. 0.260; n = 70) in length and 8.79 μm (s.d. 1.61; s.e. 0.177; n = 83) in width at the level of the nucleus (Fig. 1). On agar, glass, or plastic the cells possess a single broad, flat, leading pseudopod devoid of granular cytoplasm. Behind the clear area is the granular cytoplasm containing the nucleus and contractile vacuole. The vacuole is usually behind the nucleus but may move alongside and occasionally has been observed in front of the nucleus. At the rear end is a semi-permanent ‘tail’. While the broad pseudopod is the major organ of locomotion and indicates the direction of motion, the cells may have filopods extending from all edges. Rounded cells not attached to a surface have many filopods, but quickly extend a pseudopod upon settling and beginning motion.

**Development.** The normal development of *D. discoideum* involves chemotactic aggregation toward cAMP sources and development of the associated stage-specific proteins. The small drop test of Konijn (1965) was used to test attraction of P2 cells to *K. aerogenes* and to cAMP. Little or no attraction towards adjacent drops of bacteria or 10⁻² M cAMP was visible (Fig. 2), as P2 cells tended to move away from the original drop area in all directions equally (see below). When P2 cells are mixed with NC-4 cells in ratios of P2/NC-4 from 45 to 0.0045, the P2 cells do not participate in aggregation, and no spores of the P2 phenotype are produced. The NC-4 cells are able to aggregate and culminate in these mixtures, but a slight delay occurs, particularly when P2 cells are in excess, perhaps because of physical interference by the P2 cells.

In suspension cultures, starved NC-4 cells form compact agglomerates which are able to develop into fruiting bodies directly when plated on agar (Gerisch, 1968). P2 cells fail to agglomerate, forming only small, loose clumps which quickly disperse upon plating. NC-4 cells are able to amplify and secrete a suitable cAMP stimulus,
C. E. Frantz

Fig. 1. Washed P2 amoebae on buffered agar. The amoebae have the same morphology on plastic or glass. Dimensions of motile amoebae were measured with Galatea from a film of a similar population on plastic. Length and width were measured along lines through the nuclei of amoebae that assumed the typical shape exhibited by the cell in the centre of the photograph. Bar = 10 μm.

Fig. 2. Lack of attraction of P2 amoebae (drop at left) to food bacteria, *K. aerogenes* (drop at right). Amoebae starved 1 h 40 min were plated in a drop, and a drop of bacteria was added 1.2 mm away. P2 amoebae moved out of their original drop area in all directions immediately after plating, and movement is not biased toward the drop of bacteria. A dotted line marks the approximate original drop area. The response of P2 amoebae to drops of cAMP at various times of starvation is identical. Bar = 200 μm.
this phenomenon being the basis of the relayed waves of chemotactic aggregation (Newell, 1978; Devreotes et al. 1979). When \(10^{-8} - 10^{-6}\) M cAMP pulses are applied to NC-4 cells labelled with \([\text{H}]\)adenosine and starved for 8 h or longer, the response rate of cAMP secretion increases so that the typical peak of \([\text{H}]\)cAMP reaches 0.1–0.2% of the total cellular label (Devreotes et al. 1979; P. Devreotes, personal communication). P2 cells are unable to relay; their responses to cAMP pulses after 10–11 h starvation are negligible (0.003% or less of total label). The cell-bound PDE activity of P2 cells after 10 h of starvation is 0.0084 nmol cAMP/min/10^7 cells, only 0.58% of the NC-4 activity (1.53 nmol cAMP/min/10^7 cells). P2 cells at 12–14 h of development do not bind cAMP when exposed to concentrations from 0.048 to 100 nM cAMP, whereas a binding curve similar to that reported by Green & Newell (1975) was obtained for NC-4 cells. Thus, P2 does not develop the competences (chemotaxis to cAMP, intercellular contacts, and cAMP relaying, hydrolysis, and binding) that characterize NC-4 aggregation.

Encystment. When P2 exhausts the bacterial food supply or is centrifuged free of bacteria, the amoebae wander over the substrate and eventually stop, round up, and transform into microcysts. Encystment requires several days on agar (Fig. 3). The most favourable conditions for encystment were found to be starvation of washed vegetative amoebae on KK2-buffered or unbuffered agar plates without excess surface fluid, incubated in a humidified box to prevent complete drying (Fig. 4). This is the standard condition used for efficient development of NC-4 to aggregation and fruiting. Standing fluid inhibits encystment (Fig. 3); the fluid need not cover the entire agar surface. On wet plates microcysts are dispersed, while on dry plates and on growth plates they form clusters (Figs. 4B, 5).
Figs. 4, 5. P2 microcysts on agar. Cells were treated as in Fig. 3.

Fig. 4. Dry KK$_2$-buffered agar at 4-8 days (A) and 11 days (B).

Fig. 5. Wet KK$_2$-buffered agar at 6-0 days (A). Growth plate (nutrient agar) at 6-0 days with a few motile cells between the microcyst clumps (B). Bar = 100 μm.

Media of high osmotic strength, for example 0.114 M sucrose or 0.08 M KCl, promote encystment of *P. pallidum* amoebae in suspension; on agar, a higher concentration of KCl (0.12 M) is needed for optimal encystment (Toama & Raper, 1967a). P2 amoebae encyst very poorly in agitated suspension in a variety of media (KK$_2$, 0.08 M KCl, or 0.144 M sucrose, for example), apparently due to cell damage.
and lysis. P2 encystment is not promoted by 0.12 M KCl in the agar substrate, in either wet or dry agar conditions (Fig. 3).

Excystment. When P2 microcysts are seeded on nutrient agar with K. aerogenes, they release amoebae which feed on the bacteria and grow. Clones of cells originating from single microcysts are visible 5–6 days after plating, but these are quite diffuse and difficult to see until they are about 20 mm in diameter. Under these conditions about half of 1-week-old P2 microcysts germinate and give rise to colonies, and this plating efficiency does not decline for microcysts over 7 weeks old. The microcysts are resistant to desiccation and can be stored indefinitely on dry silica gel (Perkins, 1962). Growth plates allowed to dry and left at room temperature for over 2 years have many viable microcysts. A heat shock of 45 °C for 30 min, which activates NC-4 spores (Cotter & Raper, 1966), does not affect the plating efficiency, except perhaps in older microcysts (Table 1).

Table 1. Plating efficiency of P2 microcysts

<table>
<thead>
<tr>
<th>Cyst age</th>
<th>Plating efficiency, % ± S.D. (n*)</th>
<th>Plating efficiency after 30 min at 45 °C, % ± S.D. (n)</th>
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<tbody>
<tr>
<td>1 wk</td>
<td>47.9 ± 6.37 (4)</td>
<td>51.6 ± 2.98 (3)</td>
</tr>
<tr>
<td>2 wk 2d</td>
<td>66.6 (1)</td>
<td></td>
</tr>
<tr>
<td>3 wk 1d</td>
<td>73.5 (1)</td>
<td>69.7 (1)</td>
</tr>
<tr>
<td>7 wk 4d</td>
<td>58.8 (1)</td>
<td>86.8 (1)</td>
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</table>

* n is number of experiments.

The effects of various conditions on P2 excystment in the absence of bacteria were studied. Nine- to 19-day microcysts were agitated in suspension with the test substance (2 x 10^6 microcysts/ml), plated on agar containing the test substance, or plated on plastic under the test substance (2 x 10^6 microcysts/cm²) for at least 48 h. In none of the conditions used did more than one third of the microcysts germinate, including low-strength buffer (10 mM potassium phosphate, pH 6.5), which promotes excystment in P. pallidum (Cotter & Raper, 1968; O'Day, 1974), and 2% glutamic acid, which promotes excystment in some hartmannellid and related amoebae (Singh, 1975) (results not shown). In both stationary and agitated cultures, microcysts usually clumped, making counting of ungerminated microcysts difficult. Maximal excystment (30%) was observed when 16-day microcysts were plated on plastic under KK₅ and incubated for about 43 h. Microcysts could be removed from these stationary cultures by gentle agitation, leaving the amoebae attached to the dishes for counting. Little or no cell division occurred in this period, and amoebae did not re-encyst.

Most, but perhaps not all, microcysts germinate by swelling, with the appearance of a vacuole, and subsequent emergence of the amoebae through a small hole. The microcyst case is left as a hyaline 'ghost' (Fig. 6). In stationary or agitated bacteria-free cultures the number of these ghosts was very small compared to the number of
cells observed; thus excystment might also occur by other means. Empty microcysts were occasionally observed, not swollen but having a thick, wrinkled wall as in mature microcysts, but amoebae were never observed emerging from these shells; they may represent microcysts whose amoebae have died and degenerated.

Fig. 6. A cluster of germinating P2 microcysts. The microcysts are 12 days old, suspended in KK₁ on a haemocytometer. Of the 5 microcysts in the clump, 4 have swollen; one of these has fully germinated (a), another is releasing an amoeba out of the plane of focus (b), and a third is just beginning to excyst (c). Twenty minutes after this photograph was taken, all 4 of the swollen cysts had germinated. The unswollen cyst, and others in the field, failed to germinate. Bar = 10 μm.

Cell movement

Velocity. The most striking feature of P2 behaviour is the motion of individual cells. Washed P2 cells on buffered 2% agar move with a mean velocity of 13·7 μm/min (s.D. 2·90; s.E. 0·179; n = 263). While velocities of individual P2 cells during 15-min segments range from 0·318 to 24·6 μm/min in the first 17 h of starvation, the distribution of cell velocities is fairly symmetrical, distinct from the highly skewed

Table 2. Cell movement parameters of P2 and NC-4

<table>
<thead>
<tr>
<th></th>
<th>P2 amoebae</th>
<th>NC-4 amoebae*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cell velocity (V), μm/min ± s.d.</td>
<td>13·3 ± 2·90</td>
<td>7·19 ± 4·41</td>
</tr>
<tr>
<td>Skewness of mean velocity distribution</td>
<td>-0·703</td>
<td>+1·13</td>
</tr>
<tr>
<td>Median cell velocity, μm/min</td>
<td>13·3</td>
<td>6·27</td>
</tr>
<tr>
<td>Modal cell velocity, μm/min</td>
<td>13·14</td>
<td>6·6</td>
</tr>
<tr>
<td>Persistence time (t*), min ± s.d.</td>
<td>13·2 ± 5·03</td>
<td>4·89 ± 2·76</td>
</tr>
<tr>
<td>Diffusion constant (D*), μm²/min ± s.d.</td>
<td>984 ± 827</td>
<td>1356 ± 991</td>
</tr>
</tbody>
</table>

* NC-4 data, for preaggregative cells up to 10 h of development, are taken from Potel & MacKay (1979). P2 data are for cells up to 17 h of development.
Fig. 7. Velocity ($\mu$m/min) distribution among P2 cells (solid line). Five films and 263 cells were analysed, the fields ranging in density from $2.4 \times 10^8$ cells/cm$^2$ and in age from 1 to 14 h after harvest. Velocities (in $\mu$m/min) are the means for each cell, obtained by averaging instantaneous velocities from 15-min (60-frame) intervals. The velocity distribution of NC-4 cells (dotted line) is from Potel & MacKay (1979).

Fig. 8. Time course of mean P2 cell velocity. Mean velocity ($\mu$m/min) for a total of 263 cells is plotted vs. time after plating (min). Vertical bars represent ± S.E. at representative points.
NC-4 cell velocity distribution (Fig. 7 and Table 2). The mean P2 cell velocity is almost constant after a gradual rise in the first 6 h of starvation (Fig. 8), and at 46-48:25 h the minimum velocity observed in one film was still 9:30 μm/min (s.d. 2:14; s.e. 0:334; n = 41). Preaggregative NC-4 cells at comparable plating densities have a much lower overall mean velocity (Table 2); the highest NC-4 cell velocity among over 495 cells studied by Potel & MacKay (1979) was 40:8 μm/min. During aggregation, NC-4 cells move in steps of about 20 μm at 12 μm/min (Cohen & Robertson, 1971).

In time-lapse films P2 cells appeared to stop abruptly when they finally rounded up and apparently encysted. This impression was confirmed by using Galatea to track filmed cells in an encysting population backward from the point at which they first became rounded and stationary. The number of cells contributing to each time point is indicated above the point; vertical bars represent ± s.e.

Persistence. In addition to moving quickly, P2 cells tend to move in straight lines— that is, the cells make infrequent and shallow turns compared to NC-4 cells. A tendency to move in straight lines is called persistence (Gail & Boone, 1970; MacNab & Koshland, 1973) and can be described quantitatively with 2 parameters: \( D^* \), a modified diffusion constant, and \( t^* \), the persistence time (Gail & Boone, 1970). \( D^* \) is a measure of net progress made by the cells, and \( t^* \) is a measure of how long
the cells move in the same direction. Two related methods were used to calculate these parameters, the graphical method of Gail & Boone (1970) and the velocity autocovariance method (Potel & MacKay, 1979). Both are based on a modified random walk model of cell motion. The instantaneous values of persistence are distributed exponentially, so that the calculated $t^*$ is equivalent to the half-life of the cells' memory of their direction of movement. For P2, $t^*$ is over 2.5 times longer than that for NC-4, and $D^*$ for P2 is over 7 times the NC-4 value (Table 2).

The high velocity and persistence of P2 cells produce cell tracks that are much longer and straighter than those of NC-4 cells (Fig. 10; cf. Potel & MacKay, 1979). The average flattened, motile P2 cell (18.5 μm long) moves almost 9.5 cell lengths in one $t^*$ period, during which 50% of the cells in a population remain within 90° of their original direction of motion. Further observations described below suggest that P2 cells actually have much longer 'persistence', if this term is extended beyond the present straight-line definition.

**Travelling bands.** P2 cell motion is further characterized by the presence of large travelling bands of cells in fields which were random when first plated (Fig. 11). These bands are visible to the naked eye in fields of washed cells at sufficient density, but occur in some form at lower densities (see below). Bands occur at all times during starvation of washed cells, until microcysts form; they also occur in some areas of growth plates, but the amoebae and bacteria are often too dense to permit distinct bands. These bands consist of monolayers of dense or contacting cells and move without apparent organizing centres, generally changing direction only when they collide with other bands (Fig. 11B). The bands are evidently not caused by a propagated signal, as are the waves of chemotactic activity in NC-4, but reflect the movement of their constituent cells, since they move through very sparse areas of a field without loss of size or thickness.

**Drop expansion.** When P2 cells are plated in drops on agar, they move out of the original drop area (Figs. 2, 12). All or most of the cells participate in this drop expansion, which begins immediately after plating, although some may be left behind as microcysts. Drops of 10^5, 10^6, or 2.5 × 10^6 cells on KK_2-buffered agar expand at constant rates for over 12 h (Fig. 13) with outer boundaries visible to the naked eye. At comparable densities, NC-4 cells show little tendency to spread from a drop, and most of these cells are eventually attracted into aggregates in the drop area.

P2 drop expansion is somewhat dependent on the number of cells in the drop and on the composition of the agar. On KK_2-buffered 2% agar, drops of 2.5 × 10^6 cells expand significantly faster than drops of 10^6 cells, and the latter expand faster than drops of 10^5 cells (Table 3A). The rate of drop expansion is significantly lower when the cells are plated on 2% agar made in deionized water for all 3 cell concentrations (Table 3A), and these drops have a better defined ridge of cells at the outer edge of the expanding area. Drops of NC-4 cells also behave somewhat differently on buffered and water agars, as these cells spread slightly further and with a less well defined outer edge on buffered agar. These phenomena may be related to the ionic strength of these agars. On water agar, the outer ridge of cells suggests that the leading cells are restrained, rather than that all the cells are slower. P2 drops also expand signifi-
Fig. 10. Representative cell tracks of P2 cells. Arrowheads indicate the ends of tracks, which are 9.37 to 11.1 h long. Tracks are digitized data plotted by computer with the Galatea system.
Fig. 11. Travelling bands of P2 amoebae moving on agar. A, One frame of a film showing a band moving to the right through a sparse field; 108 h of starvation, $10^6$ cells/cm$^2$. Frame width = 3.7 mm; B, Two sets of bands colliding, one moving from the top, the other from the left of the photograph; 22 h of starvation, $7.5 \times 10^4$ cells/cm$^2$; C, Bands moving down in the photograph; 22 h of starvation, $5 \times 10^4$ cells/cm$^2$. Bar for B, C = 100 $\mu$m.
cantly faster on 1% agar (buffered or water) than on 2% agar (Table 3). If cAMP is added to 1% buffered or water agar, the P2 drop expansion rate does not differ significantly from that of controls (Table 3B).

Further observations make it unlikely that P2 drop expansion is due to the action of a repellent or enzyme-attractant (as in the PDE-cAMP system). Drops plated near each other expand over one another, and either the outer edges maintain their integrity after intersecting so as to give overlapping circles of cells, or the cells push each other out of the way (Fig. 14). If a repellent (or attractant) were responsible for drop expansion, cells would avoid the area between the drops, and drop edges would not cross.

Table 3. P2 drop expansion

<table>
<thead>
<tr>
<th>A. P2 drop radius expansion rate on 2% agar (10-μl drops).</th>
<th>μm/min ± S.E. (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells/drop</td>
<td>KK 2% agar</td>
</tr>
<tr>
<td>2.5 x 10⁶</td>
<td>12 ± 0.384 (3)</td>
</tr>
<tr>
<td>10⁷</td>
<td>11.4 ± 0.167 (3)</td>
</tr>
<tr>
<td>10⁸</td>
<td>10.0 ± 0.589 (3)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. P2 drop radius expansion rate on 1% agar with and without cAMP (10-μl drops; 10⁷ cells/drop).</th>
<th>μm/min ± S.E. (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP, M</td>
<td>KK 1% agar</td>
</tr>
<tr>
<td>0</td>
<td>13.6 ± 0.400 (2)</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>12.6 ± 0.318 (3)</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>13.6 ± 0.233 (3)</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>12.8 ± 0.693 (3)</td>
</tr>
</tbody>
</table>

Rates were obtained by fitting straight lines by linear regression to data from 4 to 13 h; correlation coefficients were 0.994 or greater and most were 0.999. Significance of rate differences was assessed at the 5% level of significance by a one-tailed t test, using appropriate degrees of freedom. n is number of experiments.

In photographs, spiral or tangential bands of P2 cells may be seen in the expanding area of drops of sufficient density (Fig. 12), similar to the bands observed in fields. In time-lapse films these bands appear to 'unwind' from the drop. Between bands most of the cells move in the same direction as the bands, although there are always some cells moving in other directions, a few even moving back toward the drop. Thus, general cell movement in expanding P2 drops is not straight out from the drop centre, but follows a curved path. This is confirmed by analysis of a film of a drop of 8.4 x 10⁶ cells, in which the outer drop edge moved at an overall radial rate (for 356 min) of 10.8 μm/min. When a small clump of cells at the outer edge was tracked separately, it took a curved path, moving at 13.5 μm/min, very close to the mean velocity of single P2 cells. The spiral paths of cells and bands in these drops result in
a lower rate of overall drop expansion than would be seen if the cells moved straight out from the drop.

*Left-turning.* The cause of this spiral movement was unclear until it was noted that, among the hundreds of expanding drops examined in various experiments, none had spirals unwinding clockwise; all drops in which spiral bands could be distinguished unwound counterclockwise as viewed from above (Figs. 2, 12, 14), and in films cells and clumps of cells moved counterclockwise. Gravity is not the orienting factor, as drops incubated upside down immediately after plating also generated counterclockwise spirals. These observations suggested that individual P2 cells might have a left-turning bias in their motion.
Fig. 13. Rates of P2 drop expansion on various agar backgrounds. A representative experiment is shown. Ten-microlitre drops containing $10^6$ cells were plated 1-5 h after harvest of vegetative amoebae. P2 amoebae: KK$_2$-buffered 2% agar (○); H$_2$O 2% agar (●); KK$_2$-buffered 1% agar (□); H$_2$O 1% agar (■). NC-4 amoebae: KK$_2$-buffered 2% agar (△); other agar backgrounds similar.

Fig. 14. Intersection of 2 expanding P2 drops ($2.1 \times 10^4$ cells/drop) on KK$_2$-buffered 2% agar, 5 h after plating. The drops can be seen winding into each other with a counterclockwise tendency. Approximate original drop areas are marked by dotted lines. Bar = 500 μm.
Such a property was best examined in films of cell fields in which the cell densities were low and cell-cell collisions, which could affect cell direction, were minimized. In 2 films averaging $5.5 \times 10^3$ cells/cm$^2$, the angles between successive 30-min cell motion vectors show a clear leftward bias; 229 cells turned left 82% of the time (Fig. 15). To obtain comparable data for NC-4 cells, angles between successive 10-min vectors were examined from data collected by Potel & MacKay (1979); 10 and 30 min are approximately double the persistence times for NC-4 and P2, respectively. NC-4 cells have no turning bias: 256 cells in 7 films turned left 48.5% of the time (range 46.6–51.7%).

![Figure 15](image)

Fig. 15. Histogram of differences between successive 30-min P2 cell motion vector angles. The number of vectors is plotted vs. degrees, with positive angles corresponding to right turns. Number of cells, 229. Of 752 angles, 617 (82%) are left turns, and 135 are right turns (18%).

The minority of right turns by P2 cells might be accounted for by direction changes due to collisions, which in the 2 films examined occurred at about 2 contact events/cell/h (see below). A film of P2 cells at very low density, about 240 cells/cm$^2$, in which very few contacts occurred, was also examined. Among 20 cells which made no contacts during the period of observation (11 h), all except 2 cells (which were only briefly in the field of view) turned predominantly left, moving in counterclockwise loops of various sizes, ranging upward from about 100 µm in diameter. Left turns and loops were separated by occasional long straight stretches (Fig. 10).

Cell interactions

Contactless bands. In time-lapse films of P2 cell fields, the cells often seem to move in the same general direction even when low cell density prevents formation of visible
Fig. 16. Analysis of contactless bands. A, Cell motion vectors digitized with Galatea from a 2.5-min interval (10 frames). Vector heads are the final cell positions; B, Histogram of absolute angles of cell motion vectors in A. In panel A, 0° points to the right and 90° points to the top of the page.
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travelling bands. To test this visual impression, a film segment was chosen in which such an orientation seemed to occur. All 153 cells in the 2.5-min interval (10 frames; 2.39 x 10^4 cells/cm^2 and 29 h of development) were digitized through the interval, and their motion vectors were plotted (Fig. 16A). In the interval, the cells moved on average 33 μm, or about 1.8 cell lengths. The vectors were then grouped according to their absolute orientation, and the resulting histogram (Fig. 16B) shows that the cells were highly oriented within a 60-degree range.

In the film segment analysed few cells were in contact, mostly in pairs, and many isolated cells moved in the same direction. This example does not prove that all film sequences in which cells appeared to be moving in the same direction actually contained oriented cells; such visual impressions are sometimes misleading. However, its confirmation in this case suggests that P2 cells may orient one another without maintaining contact. P2 cell bands are clearly a result of coordinated movements, in the sense that many cells in a population move in the same direction due to some initial organizing event and/or some continuing intercellular interaction, which is superimposed on the left-turning behaviour of individual cells. However, it is not clear that these bands result from cell stickiness or from chemotactic behaviour. It is more likely that the bands in dense fields and the oriented movement seen in sparse fields are the result of highly persistent movement, perhaps with some contribution from side-to-side adhesion. The cells may bump into and glance off one another to such an extent that their paths eventually line up in parallel and in dense fields they may remain crowded together in bands. Whatever the cause, it must be effective very rapidly, as travelling bands can be seen within an hour of plating the cells.

To examine this aspect of P2 cell motion, quantitative estimates of cell spacing or clustering and of cell contact properties (frequency and duration of contact) were made.

**Spacing.** Randomness or clustering of P2 cells was assessed using a method developed by Clark & Evans (1954). In this method a ratio \( Q = r_A/r_E \) is formed from the actual mean distance from each cell to its nearest neighbour \( r_A \) and the expected mean distance for a random (Poisson) spatial distribution \( r_E = 1/(2\sqrt{N}) \), where \( N \) is cell density. Thus \( Q = 1 \) for a random distribution, \( Q > 1 \) indicates a more uniform distribution (up to a theoretical maximum of 2.1491), and \( Q < 1 \) indicates a clustered distribution (Clark & Evans, 1954; Potel & MacKay, 1979).

To assess \( Q \), all the cells in 7 or 8 frames of each film, 400–500 frames (1.7–2.1 h) apart, were digitized with Galatea. Four P2 films, in which cell densities were low enough to permit each cell to be distinguished, were used for this study. At these densities, pre-aggregation NC-4 cells are randomly distributed (Potel & MacKay, 1979). Three of the 4 P2 films gave \( Q \) values not significantly different from 1 (Table 4), although 5 out of the 24 individual frames examined showed significant clustering. The fourth film, which had an intermediate cell density, gave a \( Q \) of 0.874, indicating significant clustering; 5 of the 7 frames studied showed clustering. In this film several travelling bands of contacting cells were present, but since individual cells could not be distinguished in these bands, only interband regions with dispersed cells were analysed, before or after a band passed through the field. In the films with random
cell distributions no bands of contacting cells were present, even in the highest-density film, although intervals with contactless bands were discernible in each. Of all the frames studied, only 2 had significantly uniform cell spacing. Thus, transient clustering may occur in fields of P2 amoebae, and in one case clustering was a significant, long-term feature of the field. Small groups of 2 or 3 cells are frequently observed in P2 films, and these are probably responsible for the measured clustering rather than fewer, large clusters.

Since P2 microcysts may form clumps and networks on agar (Figs. 4B, 5B), a film of an encysting population was analysed for any significant clustering as the number of microcysts increased. When motile cells and non-motile microcysts were digitized separately and their $Q$ ratios calculated, neither population showed significant clustering (Table 4). This result indicates that, at the total density studied ($2.73 \times 10^4$

<table>
<thead>
<tr>
<th>Film</th>
<th>Mean density</th>
<th>Mean $r_B$</th>
<th>Mean $r_A$</th>
<th>$Q$</th>
<th>$C^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1843†</td>
<td>$1.04 \times 10^8$</td>
<td>49.1</td>
<td>42.9</td>
<td>0.874</td>
<td>-3.43</td>
</tr>
<tr>
<td>2047†</td>
<td>$4.45 \times 10^8$</td>
<td>74.9</td>
<td>71.0</td>
<td>0.947</td>
<td>-0.587</td>
</tr>
<tr>
<td>2048†</td>
<td>$6.48 \times 10^8$</td>
<td>62.1</td>
<td>59.6</td>
<td>0.959</td>
<td>-0.567</td>
</tr>
<tr>
<td>2049†</td>
<td>$2.39 \times 10^8$</td>
<td>32.4</td>
<td>30.9</td>
<td>0.954</td>
<td>-1.26</td>
</tr>
<tr>
<td>2049</td>
<td>$6.68 \times 10^4$</td>
<td>61.2</td>
<td>55.4</td>
<td>0.905</td>
<td>-1.36</td>
</tr>
<tr>
<td>2049</td>
<td>$2.60 \times 10^4$</td>
<td>34.9</td>
<td>34.4</td>
<td>0.987</td>
<td>-0.338</td>
</tr>
</tbody>
</table>

$C$ is a normally distributed statistic used to evaluate the significance of a deviation of $r_A$ from $r_B$. $C = (r_A - r_B)/\sigma_{r_B}$. An absolute value of $C$ greater than 1.96 (2.58) would occur by chance less than 5% (1%) of the time (Clark & Evans, 1954).

† Values are the means of data from 7 or 8 frames 400–500 frames apart in each film.
‡ Values are the means of data from 4 frames 500 frames apart. These measurements were made only in the second half of the film, while cells were encysting, accounting for the higher total density.

| Cells/cm²), microcysts do not increase in number by pre-existing microcysts trapping motile cells and that prolonged contact with microcysts is not needed for differentiation, although contact might serve as a trigger. Motile cells appeared to be somewhat affected by the microcysts with which they came in contact; most cells paused slightly when in contact with a microcyst but their paths were not always altered by the contact. At higher densities the microcysts are obviously clustered (Figs. 4B, 5B), suggesting that this slight effect may be stronger in these cases simply because there are more microcysts.

Contacts. The data for P2 cell spacing are inconclusive but suggest some occasional clustering. Meanwhile, large numbers of P2 cells exhibit bands of coordinated behaviour, visible whether or not the cells are in contact. If contacts among P2 cells are more frequent or longer than expected for randomly moving objects, then some type of long-range or contact interaction might be responsible for the travelling bands of cells. Cell contact events were digitized using Galatea and analysed as in Potel & MacKay (1979).
Most contacts are very short, and the histogram of contact duration has a sharp modal peak at 1.5-1.75 min (Fig. 17). However, 34.7% of the contacts are longer than 4 min. For strain NC-4 the duration frequency distribution is also highly skewed to the right, but the modal contact duration for NC-4 (Potel & MacKay, 1979) is almost twice as long as that for P2 (Table 5). The longer P2 contacts may reflect the effects of high persistence in P2 cells; cells going in the same general direction that collide at shallow angles may travel together for very long times simply because P2 cells change direction relatively rarely. On the other hand, in all P2 films many very brief contacts were observed in which cell directions were totally unchanged. Indeed, several single cells which collided head-on with broad advancing bands could be observed passing through the band from front to back, emerging with their paths apparently unaffected.

In the 2 films studied, P2 cells averaged 1.65 periods of contact/cell/h; this value corresponds to periods of continuous contact of one cell with one or more different cells at a time. The slightly higher mean total contact event frequency – that is,
pairwise contact events per unit time – was estimated by dividing the total number of contact periods by the amount of time the cells were not in contact (Table 5). P2 cell contact frequency rises over the first several hours of development to a maximum at 5–6 h of about 2-9 contact periods/cell/h and declines irregularly thereafter, a pattern similar to the time course of P2 cell velocity.

Contact event frequencies observed for P2 cells are 2-2- and 2-5-fold higher than those predicted for randomly colliding particles by the formula used to study NC-4 by Potel & MacKay (1979). Thus both P2 and NC-4 cells appear to have some affinity for contacts, insofar as frequency of contacts is concerned. However, the short duration of the most frequent P2 cell contacts suggests that most contacts last only while the cells happen to overlap, as the cells move about 2 cell diameters in 2 min.

**DISCUSSION**

P2 differs in 3 important respects from its original parental strain, *D. discoideum* NC-4: P2 amoebae do not aggregate, they differentiate into individual microcysts, and the cell movement characteristics of P2 are quite different from those of NC-4. The aggregateless phenotype is relatively frequent among *D. discoideum* developmental mutants (Newell, 1978). Other mutants have been isolated which probably lack the aggregation components studied here, but none has been reported to form microcysts or to have aberrant cell motion or altered cell morphology.

Several cellular slime mould species closely related to *D. discoideum* form microcysts, and this behaviour in P2 may indicate that *D. discoideum* NC-4 possesses the genetic capacity for microcyst formation but does not express it under any known environ-

### Table 5. Cell contact properties of P2 and NC-4

<table>
<thead>
<tr>
<th></th>
<th>P2</th>
<th>NC-4*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell density, cells/cm²</td>
<td>4.45–6.48 x 10³</td>
<td>10³–10⁶</td>
</tr>
<tr>
<td>Total no. of cells</td>
<td>165</td>
<td>222</td>
</tr>
<tr>
<td>Total no. of contacts</td>
<td>319</td>
<td>1322</td>
</tr>
<tr>
<td>Contact duration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean contact period duration, min ± S.D.</td>
<td>5.37 ± 6.75</td>
<td>7.17 ± 8.40</td>
</tr>
<tr>
<td>Modal contact duration, min</td>
<td>1.5–1.75</td>
<td>2–3</td>
</tr>
<tr>
<td>Median contact duration, min</td>
<td>2.28</td>
<td>—</td>
</tr>
<tr>
<td>Skewness of contact duration</td>
<td>3.06</td>
<td>3.28</td>
</tr>
<tr>
<td>% time spent in contact</td>
<td>14.8</td>
<td>20.8</td>
</tr>
<tr>
<td>Longest contact, h</td>
<td>3.75</td>
<td>0.38</td>
</tr>
<tr>
<td>Contact frequency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contact periods/cell/h</td>
<td>1.65</td>
<td>1.74</td>
</tr>
<tr>
<td>Contact events/cell/h</td>
<td>1.97</td>
<td>2.20</td>
</tr>
</tbody>
</table>

* NC-4 data, for preaggregative cells up to 10 h of development, are taken from Potel & MacKay (1979). P2 data are for cells up to 17 h of development.
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P2 amoebae are morphologically similar to some species of small, free-living soil amoebae such as some hartmannellids, which also form cysts (Singh, 1975). Cyst formation by slime moulds and free-living amoebae emphasizes their close relation and probable common evolutionary origin (Olive, 1975).

However, P2 microcysts differ in some ways from both of these types of cysts. P2 encystment is much slower than encystment in the cellular slime mould, *P. pallidum*, and is unaffected or inhibited by conditions that promote *P. pallidum* encystment. P2 microcysts are resistant to heat, in contrast to *P. pallidum* microcysts which are killed by heat treatment, or NC-4 spores which are activated by heat (Cotter & Raper, 1966). Furthermore, P2 excystment is not promoted by factors effective in promoting excystment in *P. pallidum* or in hartmannellid amoebae.

A genetic analysis of P2, to determine the extent and type of its genetic alteration from NC-4, might shed some light on the evolutionary relations of the cellular slime moulds, as P2 resembles the acrasid amoebae rather than the dictyostellid type (Olive, 1975). The existence of a partial revertant, P2a, suggests that the mutations responsible for the P2 phenotype are not large, irreversible changes such as large deletions.

The most striking aspect of the P2 phenotype is cell movement. Time-lapse films reveal that P2 amoebae move much faster and in much straighter lines than do NC-4 amoebae. Persistence time, as calculated in Results, is a measure of the directional memory of motile cells (Gail & Boone, 1970; MacNab & Koshland, 1973). Since this measure of memory is based on straight-line motion, it is probably an underestimate of true persistence of P2 amoebae, the motion of which seems to be a gradual left turn broken at times by straight stretches. Thus P2 cell motion, instead of being a random walk with some degree of persistence as is NC-4 cell motion, may be virtually all persistence with only an occasional random element. This left-turning behaviour is manifested in individual cell tracks, which show amoebae moving for many hours in large counterclockwise loops (Fig. 10) and in the counterclockwise movement of amoebae away from drops plated on agar (Fig. 12). Further study of isolated P2 cells could establish whether there are any cells that turn right most of the time. In the small population of P2 amoebae that made no contacts, a few made some right turns, but these cells moved out of the frame too soon to tell which direction predominated.

This type of persistent turning behaviour has not to my knowledge been reported in amoebae. Hartmannellid amoebae typically pursue a sinuous path (Bovee, 1964). Among higher organisms, goldfish neural retinal explants on a polylysine substrate produce neurites that have been reported to grow out invariably in a clockwise direction, and this has been interpreted as indicating an inherent right-hand helicity of growing neurites (Heacock & Agranoff, 1977).

P2 amoebae have relatively stable front and rear ends, and this, along with a possible dorso-ventral orientation by the substrate, could provide the information on which P2 left-turning is based. Perhaps microtubules or microfilaments are responsible for left-turning; one or both of these components could be locked in place and prevent direction change after the amoebae settle on a substrate and determine an orientation. The relative homogeneity of P2 cell velocities as compared to those of NC-4 (Table 2, Fig. 7) supports the idea that P2 amoebae are somehow structurally
locked into an altered and more restricted type of movement: high velocity and left turning.

An analogous situation may exist in leukocyte chemotaxis, where cellular structures reflect direction of motion. During chemotaxis, neutrophils have an internal polarization similar to that of P2 amoebae: the leading pseudopod with its clear cytoplasm is followed by granular cytoplasm containing the nucleus. Experiments with colchicine suggest that microtubules are responsible for this polarity and thus for the direction of neutrophil motion (Malech, Root, & Gallin, 1977). Similarly, microtubule structure could determine P2 left turning, or the orientation of the centriole or primary cilium (Albrecht-Buehler, 1977) might restrict choice of direction. Other possibilities remain, for example, actomyosin function could cause faster movement on the right side of the P2 amoeba, or the left side of the amoeba surface could have stronger adhesion to the substrate; either of these conditions would pull P2 amoebae to the left. Some of these possibilities will be explored with microfilament and microtubule inhibitors in experiments similar to those on neutrophils. A fundamental problem is more difficult to approach: why do all the amoebae choose the same direction? Even with individual cell directions locked in, a controlling process is required to establish in all cells the same leftward direction with respect to the substrate. Genetic, biochemical, and behavioural methods must be combined to identify this control.

P2 amoebae exhibit a striking and apparently coordinated movement which appears as travelling bands or sheets of cells in contact (Fig. 11) or as contactless bands, large areas of widely separated cells moving in the same direction (Fig. 16). Since these movements have no apparent organizing centre and since P2 lacks cAMP signalling system properties, the bands are probably not a manifestation of altered chemotactic aggregation. However, many cells move together in these bands, and some means of organizing them must exist. Therefore, the interactions of P2 amoebae, as reflected in cell-cell spacing and contacts, were studied.

If some cell–cell interaction produced affinity or repulsion, the nearest-neighbour test (Clark & Evans, 1954) applied to cell spacing measurements would show clustered or uniform cell distributions, respectively. While uniform spacing was very rare in fields of P2 amoebae, clustering was more frequent and predominated in one field. Thus, P2 amoebae have some transient mutual affinity, probably due to groups of 2 or 3 cells which are often seen in films. It would be necessary to analyse the spacing of more distant neighbours in order to detect larger, less obvious grouping of amoebae (M. Potel, personal communication). Small, transient clusters of motile amoebae and permanent clusters of microcysts could result from directional persistence, cell–cell adhesion, and/or tendency for the cells to share the local moisture around an amoeba or microcyst. In low-density fields, trapping effects by microcysts on amoebae were observed to be slight, but these effects could be cumulative in more dense fields where microcyst patches are seen. Alternatively, amoebae in travelling bands might stay together to form the microcyst patches, although this would require simultaneous differentiation among the band amoebae to keep the patch intact.

The contact behaviour of P2 cells (cell–cell contact frequency and duration) is similar to that of pre-aggregative NC-4 cells (Potel & MacKay, 1979), with a few
exceptions. P2 cells make contact more frequently than comparable randomly moving particles but slightly less frequently than the slower moving, smaller NC-4 cells. The most frequent P2 cell–cell contacts are short, but some are very long; contacts among P2 amoebae in travelling bands apparently last many hours. This wide range of contact durations may result from the high directional persistence of P2 cell motion and from the angles at which moving cells make contact. For cells meeting at wide angles or head-on, persistence may override any tendency for the cells to stay together, and the participants are in contact only while they overlap; such contacts are frequently observed. For cells moving in the same general direction and colliding at shallow angles, persistence may keep them together, although lateral adhesion may play a role here. For cells meeting at any angle, a change of path may result for one or both participants. All these types of contact are observed in films. A quantitative study of the relation of contact angle to contact duration would be necessary to confirm the impression that wide angles generally lead to brief contacts and shallow angles to long contacts. In a field of P2 amoebae successive shallow angle contacts may orient the amoebae in parallel, and with persistence and possible lateral adhesion, travelling bands and contactless bands would be the visible result. Since the factors contributing to bands are best described as probabilistic tendencies, computer simulations of cell motions, analogous to those of MacKay (1978) and incorporating these P2 properties, could show whether bands are a likely consequence.

Quantitative measures of cell movement such as velocity and persistence have already proved valuable in studies of genetic variation in and extrinsic effects on bacterial movement (MacNab & Koshland, 1973). These measures, and others used in this study, will find more use as genetic variants of eukaryotic cells and of types of behaviour such as chemotaxis are studied in more detail. P2 gives a hint of the range of distinctive cell motion behaviour possible in eukaryotic cells.

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