Selection of Chemotaxis Mutants of *Dictyostelium Discoideum*

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Abstract. A method has been developed for the efficient selection of chemotaxis mutants of *Dictyostelium discoideum*. Mutants defective in the chemotactic response to folate could be enriched up to 30-fold in one round of selection using a chamber in which a compartment that contained the chemoattractant was separated by a sandwich of four nitrocellulose filters from a compartment that contained buffer. Mutagenized cells were placed in the center of the filter layer and exposed to the attractant gradient built up between the compartments for a period of 3–4 h. While wild-type cells moved through the filters in a wave towards the compartment that contained attractant, mutant cells remained in the filter to which they were applied. After several repetitions of the selection procedure, mutants defective in chemotaxis made up 10% of the total cell population retained in that filter.

Mutants exhibiting three types of alterations were collected: (*a*) motility mutants with either reduced speed of movement, or altered rates of turning; (*b*) a single mutant defective in production of the attractant-degrading enzyme, folate deaminase; and (*c*) mutants with normal motility but reduced chemotactic responsiveness. One mutant showed drastically reduced sensitivity in folate-induced cGMP production. Morphogenetic alterations of mutants defective in folate chemotaxis are described.

CHEMOTAXIS is important in several stages of the life cycle of *Dictyostelium discoideum*. Growth phase amebas are led to bacteria, their normal food source, by chemotactic responses to folate secreted by the bacteria (Bonner et al., 1970; Pan et al., 1972). Upon starvation, cells begin to secrete cAMP, and their ability to respond chemotactically to cAMP increases dramatically (for review see Devreotes, 1983). The propagation of waves of cAMP outward from aggregation centers leads to chemotactic movement of amebas into the centers. Responses to cAMP, and, possibly, to folate (McRobbie and Newell, 1983), are thought to play a role in later stages of development in slug behavior (Fisher et al., 1984), sorting out of pre-spore and pre-stalk cells (Matsukuma and Durston, 1979) and fruiting body formation (Merkle et al., 1984).

Chemoresponses to folate and cAMP have been dissected into several behavioral and biochemical changes (for reviews see Gerisch, 1982; Frazier et al., 1985; Klein et al., 1985; Newell, 1986). Addition of folate or cAMP produces a rapid decrease in light scattering in cell suspensions, and cells attached to a surface first round up and then flatten out. Both folate (de Wit et al., 1985) and cAMP bind to cell surface receptors and lead to several biochemical responses including increased association of actin with the detergent-insoluble cytoskeleton, an increase in intracellular levels of cGMP, an influx of Ca^{2+}, and alterations in the phosphorylation of myosin.

Both folate and cAMP have effects on the development of starved cells. Periodic addition of folate or cAMP in the form of submicromolar pulses stimulates development of these cells while continuous application has either no effects or inhibitory effects, suggesting that the response system adapts (Wurster and Schubiger, 1977). Both folate and cAMP induce extracellular folate deaminase and cAMP phosphodiesterase while suppressing phosphodiesterase inhibitor (Bernstein et al., 1981; Klein and Darmon, 1977). These effects may be produced via a common pathway. Although the action of cAMP is a significant factor in development, there is no evidence that responses to folate are essential for development.

For studying the mechanisms of ameboid chemotaxis through mutant analysis, *D. discoideum* is the organism of choice. It is easily cultured in the laboratory and is normally haploid, facilitating the generation and isolation of mutants. Although several mutants defective in responses to cAMP have been reported (Lo et al., 1978; Barclay and Henderson, 1977, 1982), there has been no concentrated effort on isolating and analyzing chemotaxis mutants and correlating their behavioral defects with biochemical alterations.

We have decided to isolate mutants defective in chemotaxis towards folate for two reasons. First, although responses of growth phase cells towards folate are weaker than responses of starved cells to cAMP, strong responses to cAMP require cell development which is induced by starvation (Bonner et al., 1969). Thus, cells defective in development up to the aggregation stage will be defective in cAMP chemotaxis.

Cells defective in folate responses should not have this complication. Second, the growth phase cells of different cellular slime molds, including *Polysphondylium* species, respond to folate while the attractant used in aggregation varies (Pan et al., 1972). Thus, folate chemotaxis represents a general response of cellular slime mold amebas.

Materials and Methods

Cultivation of Strains

D. discoideum strain AX2, clone 214, was used as the parent strain. If not stated otherwise, experiments were performed with cells grown in shaken suspension at 23°C with 10^{10} *Escherichia coli* B/r cells/ml in 17 mM Soerensen phosphate buffer, pH 6.0 (termed phosphate buffer), and harvested at densities of not more than 3×10^6 cells/ml. For axenic growth, cells were cultivated at 23°C in nutrient medium with 1.8% maltose as described by Watts and Ashworth (1970), and harvested at densities of not more than 5×10^6 cells/ml. Cells were washed three times in cold phosphate buffer before further use. For growth on agar, cells were cultivated with *Escherichia coli* B/2 at 23°C on nutrient agar containing 0.1% bacteriological peptone (Oxoid, Basingstoke, UK), 0.1% glucose, and 2% Bacto-agar (Difco, Detroit, MI) in phosphate buffer.

Mutagenesis and Selection of Chemotaxis Mutants

Cells were mutagenized in two separate experiments. Mutants from the first and second experiments were numbered from 5001 to 5114, and 5115 to 5149, respectively. In each experiment, 6×10^8 cells were incubated for 20 min in 65 ml of 1 mg/ml 1-methyl-3-nitro-1-nitrosoguanidine in 17 mM phosphate buffer, pH 7.0, at room temperature in the dark under gentle agitation. The cells were washed, dispensed into five or six flasks containing nutrient medium with penicillin and streptomycin, and shaken at 23°C for 4–6 d before the first selection for chemotaxis mutants.

Cells from each of the flasks were selected in separate chambers in order to isolate independent mutants. 2×10^6 cells were spread in a 3.5-cm diameter area on a nitrocellulose filter (Sartorius no. 11301-50 N, 5-cm diameter, 8-μm pore size), by clamping the filter in a glass 47-mm Millipore filter holder (inner diameter, 3.5 cm), and placing the cell suspension on top with no vacuum attached. The buffer slowly drained out, leaving the cells on the filter. A sandwich consisting of a filter with cells on it and three more filters was pressed together with nylon mesh (monofilament with 850-μm spacing) in the chamber shown in Fig. 1 and the assembled chamber incubated at 23°C in the dark. For the first mutagenesis, the filter with cells on it was oriented with three other filters such that the surface with the cells was the bottom of the second filter from the top. The lower compartment contained 1 mM $MgCl_2$, 1 mM $CaCl_2$ in the phosphate buffer (termed Ca/Mg buffer), and the upper compartment contained 7×10^{-6} M folate, 10^{-7} M cAMP, in Ca/Mg buffer. (cAMP was included to eliminate mutants that lose folate chemotaxis by rapidly developing; such mutants should also become chemotactic to cAMP.) The upper solution was renewed after 2 h. After 4 h, the chambers were disassembled, the upper side of the second filter was scraped with a razor blade, and the second filter was placed in a bacterial suspension to grow up the cells remaining on it. These cells were then subjected to three more such cycles of selection and growth before plating for single colonies. Single colonies with altered colony morphology were further analyzed.

For the second mutagenesis, 2×10^6 cells were placed on the top side of the third filter, and incubated in the dark at 23°C with 7×10^{-6} M folate in Ca/Mg buffer in the upper compartment and Ca/Mg buffer in the lower compartment. The upper solution was renewed after 1.5 h. After 3 h, the central 3.5-cm diameter section of the third filter was cut out and placed in a bacterial suspension to grow up the cells. After five or six more such cycles, the cells were plated for single colonies. These were screened using the chemotactic assay on agar described by Varnum and Soll (1981) to identify chemotaxis mutants.

The other filters were stained as described to determine the distribution of the rest of the cells. For both mutageneses, the movement of cells during the first round of selection was quite poor, possibly due to aftereffects of the mutagenesis. For the following rounds of selection, good chemotactic movement was seen, somewhat reduced for the final round.

According to the following criteria, the 10 mutants which were studied in more detail are all derivatives of AX2 and not contaminants picked up during the selection procedure. Colony blotting (Gerisch et al., 1985) with mAb 41-71-21 showed that all except HG5115 produce the contact sites A glycoprotein of *D. discoideum*. With the exception of HG5104, all mutants could still be grown on nutrient medium like their parent, AX2.

Staining of Filters and Cell Counting

The distribution of cells in the filter layer was determined after fixing and staining. For fixation, the filters were incubated in a mixture of 60% ethanol, 11% formaldehyde, and 10% acetic acid for 30 min. After rinsing briefly in water, the cells were stained in a 0.1% Nuclear Fast Red (Aldrich 22,911-3), 5% aluminum sulfate solution for 30 min. The filters were dehydrated by bathing them for 1 min each in 70% ethanol, 1:1 ethanol/chloroform, and chloroform, and prepared for viewing by incubation for 30 min in 1:1 chloroform/immersion oil (Merck No. 4699), then in immersion oil alone.

Cell distributions were determined by counting the cells in 5 fields of 1-mm diameter to a depth of 45 μm from the surface per filter side, using a Zeiss Optophot microscope with 16× objective, and a green filter. For each side, the average value was calculated, and then the fraction of the total number of cells counted for all four filters was determined. By focusing through the filter, it was ascertained that the counts on the surfaces were consistent with cell distributions inside the filters. The average distance traveled was then calculated as the sum of the distances of successive filter surfaces from the center of the chamber (where the cells were deposited), weighted by the fraction of total number of cells counted that was on that surface. Filters were assumed to be 140 μm thick, as specified by the producer.

Chemotaxis Assay on Agar

For easy screening of potential chemotaxis mutants, the following assay, based on the one described by Varnum and Soll (1981), was used. 35-mm diameter petri dishes were filled with 3 ml of 1% Bacto-agar in Ca/Mg buffer. A 6-mm diameter well in the center of each dish was filled with 1 mM folate in phosphate buffer. Cells from the feeding zone of a colony were placed in several drops 4 mm from the edge of the well. The dishes were stored at 23°C in the dark for 12–18 h, and the distributions of cells were observed.

Measurement of Motility

Motility parameters were measured using a computer-controlled time-lapse video analysis sytem (Fisher, P., manuscript in preparation). Briefly, 35-mm petri dishes were filled with 1 ml of 1% Bacto-agar in Ca/Mg buffer, and 1 ml of Ca/Mg buffer put on top. About 4×10^4 cells were spread over the agar and allowed to settle and start moving. The speed and turning rate were calculated using the change in cell position occurring during a time-lapse interval of 90 s. Speed is the average distance between successive cell centers divided by the time-lapse interval. The turning rate is an estimate of the rotational diffusion coefficient and is calculated as the variance of direction changes between successive time-lapse intervals divided by the time-lapse interval (Fisher et al., 1983). To compensate for the fact that measurements of the magnitudes of the direction changes are limited to the range between 0 and 180 degrees, the variance is calculated as $-2\times \ln C$, where C is the average cosine of the direction changes (Mardia, 1972). The average speed and turning rate over a 30-min period (20 intervals) was determined for a field of at least 50 cells. Typically, cell speed values peaked at ~2 h after deposition on the agar while turning rates remained relatively constant. For each petri dish, the 30-min interval with the highest speed value and its associated turning value were used for calculating the averages in Table III.

Measurement of Extracellular Deaminase Activity

Bacterially or axenically grown cells were washed and shaken for 4 h at a density of 10^7 cells/ml in phosphate buffer. Cells were removed by centrifugation and the activity in the supernatant was measured following Bernstein and van Driel (1980). The rate of change in optical density at 325 nm was followed in a 10^{-4} M folate in phosphate buffer solution after addition of supernatant. In phosphate buffer, conversion of 10^{-4} M folate to product causes a 0.298 increase in the absorbance at 325 nm. Axenically grown AX2 cells released ~20–30% as much activity as bacterially grown AX2.

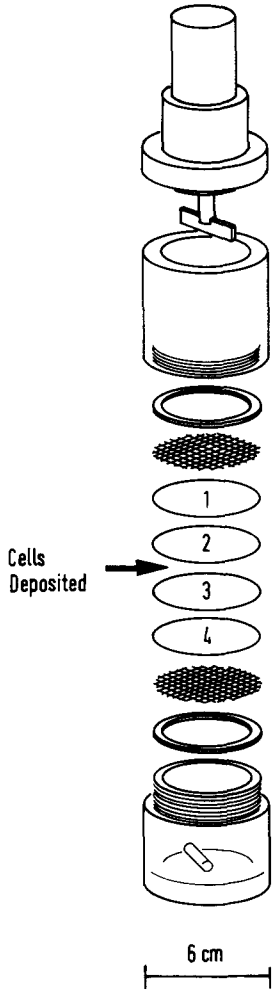

Figure 1. Schematic drawing of the selection chamber. The plexiglas chamber consists of two compartments of 4.5-cm inner diameter that are screwed together, clamping the filters between them. The filters are pressed together by nylon mesh with rubber rings serving as washers. The lower compartment is stirred by a magnetic stirrer and the upper compartment by a blade spun by an electric motor. The filters are counted from top to bottom.

Measurement of cGMP

Bacterially grown cells were washed and resuspended in a volume of 10 ml at a density of 3×10^7 cells/ml. After incubation for 1 h at 23°C with oxygen bubbled through the suspension, increasing amounts of folate were added at 5-10-min intervals. 50 µl of the cell suspension was removed 10 s before, and 10, 20, 30, and 60 s after addition of each folate stimulus, and then mixed with 50 µl of 2N $HClO_4$. The mixture was neutralized with 3 M K_2CO_3 and assayed for cGMP using a radioimmunoassay kit supplied by New England Nuclear (Boston, MA).

Determination of the Selection Ratio

To determine the relative enrichment of the mutant strains compared to the parent strain in the selection chambers, 2×10^6 cells from a suspension containing 5% mutant and 95% parent AX2 cells were placed on either the bottom of the second filter or the top of the third filter. An aliquot of the cell suspension was immediately plated for single colonies to determine the fraction of mutant cells that gave rise to progeny. The chambers were incubated in the dark at 23°C for 3–4 h with 7×10^{-6} M folate in Ca/Mg buffer or the buffer alone in the top compartment and Ca/Mg buffer in the bottom one. The top solution was renewed halfway through. After the incubation, cells remaining on the filter to which they were applied were subjected to a bacteria suspension to grow them up. For the second filter, the top was scraped and the filter then placed in the bacteria, while for the third filter the region where the cells were applied was cut out and placed in the bacteria. After growth, the cells were plated for single colonies and the fraction that was mutant cells determined by morphology.

To compensate for the fact that the fraction of mutant cells cannot go above 1, the following formula was used to determine what the relative enrichment would be when the ending fraction of mutant cells was much smaller than 1. The selection ratio is $a/b \times (1\text{-}b)/(1\text{-}a)$ where b is the fraction of mutant colonies before selection and a is the fraction after selection. This is the enrichment produced by a single selection cycle under these conditions.

Results

A Chamber for Selecting Chemotaxis Mutants

The selection method is based on the Boyden chamber assay, which was developed for measuring the chemotactic responses of white blood cells (Boyden, 1962). It relies on the movement of cells through filters in the presence of a chemoattractant gradient. Two compartments, the top one that contains attractant and the bottom one that contains buffer, are separated by four filters pressed together by nylon mesh (Fig. 1). Cells are placed on the center two-thirds of the bottom of the second filter or the top of the third, within the gradient generated by the diffusion of attractant from the top compartment to the bottom. The assembled chambers are incubated for 3–4 h at room temperature, with the solution in the attractant compartment being renewed halfway through. Wild-type cells should migrate onto the top filter, while chemotaxis and motility mutants should remain behind.

Experiments were done with the parent strain, AX2, to determine how well growth-phase amebas performed chemotaxis under these conditions. After incubation in the chamber, the cells in the filters were fixed and stained to reveal their distributions in the filters. With 7×10^{-6} M folate in the upper compartment, >70% of the total number of cells counted were found on the upper surface of the top filter (Fig. 2, solid line). These cells have moved a distance of ~280 µm (two filter thicknesses) in 4 h. Around 50% of the original number of cells applied was accounted for by the distributions of stained cells. Cells at the edge of the circle within which the cells had been deposited (a region corresponding to <10% of the total area over which cells were deposited) did not show as strong movement towards the top, possibly reflecting a chemotactic repulsion from the main cell mass (Kakebeeke et al., 1979). With only buffer in both compartments, there was some movement towards the upper compartment, possibly due to the change of buffer after 2 h (Fig. 2, dashed line). The dose–response curve (Fig. 3) showed a broad peak between 10^{-7} and 10^{-5} M folate. Time course experiments showed continuous movement towards the attractant compartment (Fig. 4). In other experiments, it was found that aggregation-competent cells moved toward the top compartment when there was 1×10^{-7} M cAMP in it, showing that the method can also be used for selecting mutants defective in chemotaxis to other attractants.

Although the chamber was designed to produce a gradient of chemoattractant to orient cell movement, it is likely that the cells themselves steepen this gradient. In the absence of cells, the concentration difference between the upper and lower compartments decayed through diffusion of the attractant with time constants in the range of 3 to 6 h. When cells were present on the filters, examination of the spectrum of the compounds in the lower compartment indicated that the folate had been deaminated as it diffused past the cells (Table I). Cells are much less sensitive to the deaminated product (van Haastert et al., 1982). The net effect of this deamination might be to steepen the gradient that the cells perceive, and improve the chemotactic response that is observed.

To test whether mutant selection in the chamber might be

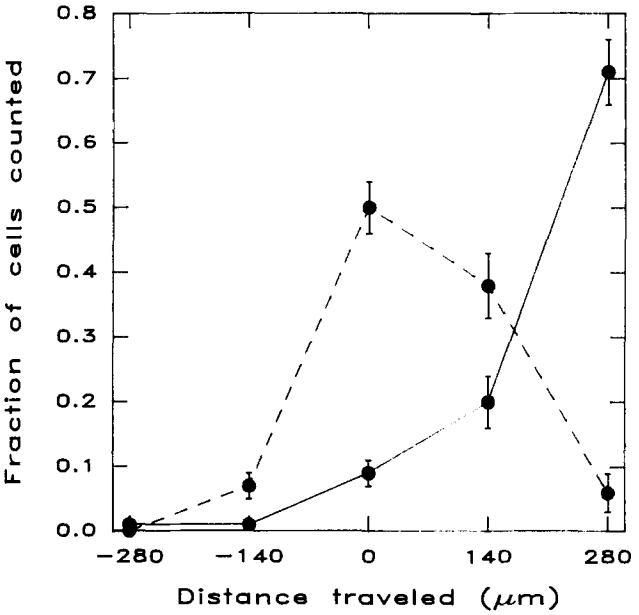

Figure 2. Movement of wild-type AX2 cells in the selection chamber. 2×10^6 cells were deposited on the lower side of the second filter and incubated in the selection chamber for 4 h at 23°C in the dark. 7×10^{-6} M folate in Ca/Mg buffer (*solid line*) or Ca/Mg buffer alone (*dashed line*) was in the upper compartment and renewed after 2 h. Ca/Mg buffer alone was in the lower compartment in all cases. The filters were stained and counted as described in Materials and Methods. Values for adjacent filter surfaces were summed and plotted according to the distance from the center of the chamber where the cells were deposited, assuming a filter thickness of 140 μm. The filter surface next to the attractant solution is at 280 μm, and that next to the buffer solution is at −280 μm. Plotted are the means and standard errors calculated from nine experiments with folate and six experiments with buffer.

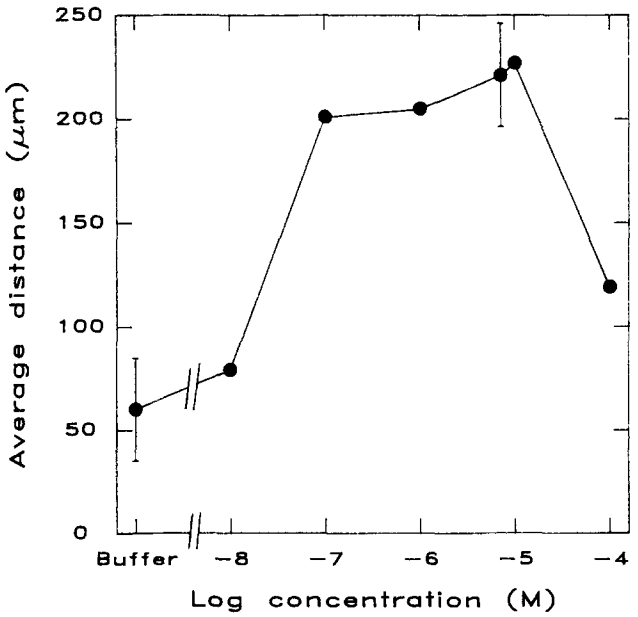

Figure 3. Response of AX2 cells as a function of folate concentration in the upper compartment. Experiments were performed as described in Fig. 2 with varying concentrations of folate in the upper compartment and buffer in the lower compartment. The average distance traveled was calculated as described in Materials and Methods. Data are from single measurements or represent means of duplicates, except for buffer and 7×10^{-6} M folate, where means and standard deviations from, respectively, six and nine experiments were calculated.

influenced by chemokinesis, the effect of folate on the motility of wild-type AX2 cells was tested. In the presence of 1×10^{-5} M folate, a concentration optimal for chemotaxis, only an increase of 20% in the speed of movement of the cells on agar was observed.

Selection for Folate Chemotaxis Mutants

Cells were mutagenized with nitrosoguanidine with a survival rate of 1% in two separate experiments. Each time, the cells were immediately separated into five or six flasks in order that a number of independent mutants would be assured. In the first experiment, cells were deposited on the bottom of the second filter and incubated for 4 h with 7×10^{-6} M folate and 1×10^{-7} M cAMP in the upper compartment. In the second experiment, cells were placed on the top of the third filter and incubated for 3 h with 7×10^{-6} M folate in the upper compartment. In both experiments, the solutions in the upper compartment were renewed halfway through. The cells that remained on the original filter after incubation in the chamber were grown up on bacteria. Such selection cycles were repeated four to seven times, and the cells plated for single colonies.

Morphologically altered and normal colonies from both selected and unselected mutagenized cells were screened using a modification of the assay described by Varnum and Soll (1981). Cells from the growing zone of a colony were placed on agar 4 mm away from the edge of a well containing 1×10^{-3} M folate, and incubated at 23°C for 12–18 h. Diffusion of the attractant from the well created a gradient that caused wild-type AX2 cells to form an outward moving ring, with the highest cell density on the side near the well (Fig. 5). If the well contained only buffer, wild-type cells did not move out but instead aggregated. Mutants that had substantially reduced movement toward the well (less than one-third of wild-type) or altered distributions were classified as potentially defective in chemotaxis to folate. Several types of distributions were observed as shown in Fig. 5. One mutant, HG5104, moved towards the attractant in clumps. Some mutants, e.g., HG5142, had reduced movement towards the attractant, often without formation of a clear ring. Others, such as HG5079, showed almost no spreading. Quantitation of the results from the second mutagenesis showed that in this assay ~10% of the single colony isolates from cultures subjected to the selection procedure were identified as defective compared to ~0.1% of clones from control cells grown for an equal number of generations without selection (Table II).

There was also a clear increase in the fraction of colonies with altered morphology for selected cells compared with unselected cells, as quantified in the second mutagenesis (Table II). Before the first selection cycle, ~4% of the cells formed colonies with some alteration in morphology: small colonies, changed pattern of aggregation, no or aberrant fruiting bodies. This value increased about fivefold after selection, while for control cells grown for the same number of generations it remained about the same.

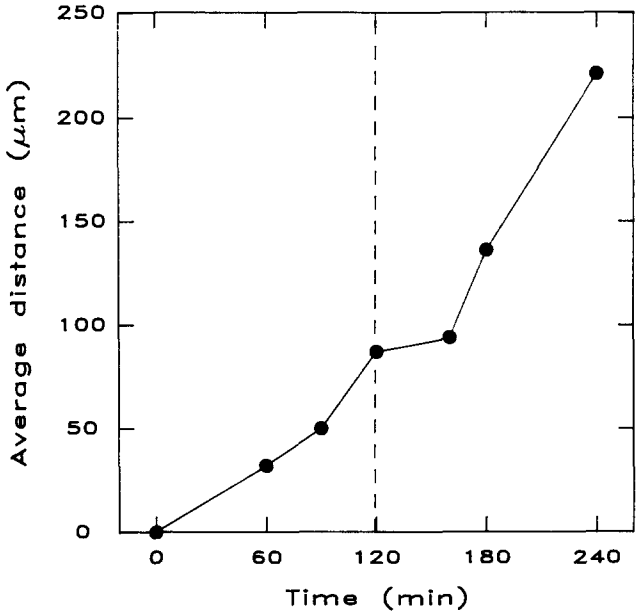

Figure 4. Time course of movement of AX2 cells in the selection chamber. Experiments were performed as described in Fig. 2, with 7×10^{-6} M folate in the upper compartment, and buffer in the lower compartment. At the indicated times, the filters were stained and the average distance traveled was calculated as described in Materials and Methods. The vertical dashed line marks the time when the attractant solution was renewed. The point at 240 min is the average of nine experiments.

Characterization of Mutants

From the two mutageneses, 10 independent mutants were chosen for further analysis (Table III). Nine of the mutants were isolated using the selection chamber, and the tenth was the single unselected mutant that was identified as defective using the agar assay (from flask III in Table II). All these mutants grew in suspension cultures with *Escherichia coli* as food bacterium with the normal generation time of 3 h.

The selection chambers were used to determine the extent of the defect present in each mutant (Table III). The cell distributions on the four filters were used to calculate the average distance traveled towards the top compartment. The average distance traveled towards the top compartment when it contained 7×10^{-6} M folate (labeled "folate response") less the distance traveled in the absence of attractant (labeled "buffer response") was chosen as a measure of the response to folate (labeled "net response"). The mutant net response divided by the wild-type net response then reflects the fraction of wild-type response remaining in the mutant.

A reduced response could be due to several factors. Low folate deaminase activity might alter the gradient from what is present with normal deaminase activity and lead to a reduced response. This is a possibility only with HG5104: this mutant secreted low levels of deaminase, had a low net response, and normal motility (Table III). Also, in mixtures with wild-type cells, the selection ratio for HG5104 was low, as would be expected if deaminase from wild-type cells could improve its response. A second possible reason for a reduced response is a defect in motility. HG5125 had the strongest motility defect of the group since its cells showed

Table I. Deamination of Folate

	Absorbance ratio* 280 nm/325 nm
Folate (10^{-5} M)	3.78 ± 0.01
Deaminated product‡	1.82 ± 0.01
Lower compartment solution§	1.79 ± 0.05

* This ratio (buffer absorption subtracted) reflects the relative amounts of folate and its product (spectra shown by Pan and Wurster, 1978). Data are means and standard errors from measurement of seven samples for folate and deaminated product, and of 10 samples from different experiments for the lower compartment solution.

‡ Bacterially grown AX2 cells were harvested, washed, and starved for 4 h at 10^7 cells/ml. 0.1 ml supernatant was added to 0.9 ml 10^{-5} M folate and the ratio determined when the absorbance at 280 nm was stable, indicating that the reaction was complete. The absorbance due to the supernatant is subtracted in calculating the ratio.

§ Experiments were done as described in Fig. 2, and the absorbance ratio of the solution in the lower compartment was determined.

the lowest speed of all mutants tested (Table III). HG5079 and HG5135 showed both small buffer responses and reduced speed on agar and are, therefore, along with HG5125, the strongest candidates for mutants having defects in the motility system. HG5115 and HG5048 had low buffer responses, normal speed, and increased rates of turning (Table III). They could have alterations either in the motility system or in the signal transduction system (see Discussion). HG5142 is of interest because it showed a reduced net response to folate and a high selection ratio but had a normal buffer response. It was the only mutant tested which had a significantly lower rate of turning than the wild type.

The remaining mutants, HG5013, HG5145, and HG5043, had close to normal buffer responses, speeds similar to wild-type or greater, and roughly normal turning rates, making them the strongest candidates for mutants that have defects in the signal transduction system itself. The high selection ratios of HG5013 and HG5145 indicate that these mutants are drastically defective in the chemotactic response (Table III). Measurements of changes in the total cGMP of cells stimulated by the addition of folate provide clear evidence that HG5145 has a drastic defect in signal transduction. Compared to AX2, HG5145 required roughly 1,000-fold higher concentrations of folate to produce equivalent changes in cGMP levels (Fig. 6). The time course of the response is also altered in comparison to AX2, and the basal cGMP concentration of unstimulated cells is probably higher than in AX2.

There is no single feature in their colony morphologies that can serve to identify the mutants as folate chemotaxis mutants. Morphogenesis of the 10 mutants analyzed varied from almost normal development to complete lack of cell aggregation (Fig. 7). Wild-type colonies growing on bacteria on a nutrient-agar plate have a feeding zone where the edge of the colony contacts the bacterial lawn and forms a ridge, then a preaggregation zone where cells are starving, followed by an aggregation zone with streams of cells moving into centers. Multicellular slugs and fruiting bodies are found in the central portion of the colony. HG5104 aggregated without streams, and did not appear to proceed past the aggregate stage, leaving large aggregates of cells in the center of the colony. HG5142, HG5013, and HG5043 had morphologies as shown in Fig. 7 for HG5142. These mutants formed extremely small aggregates with no streams and a few tiny

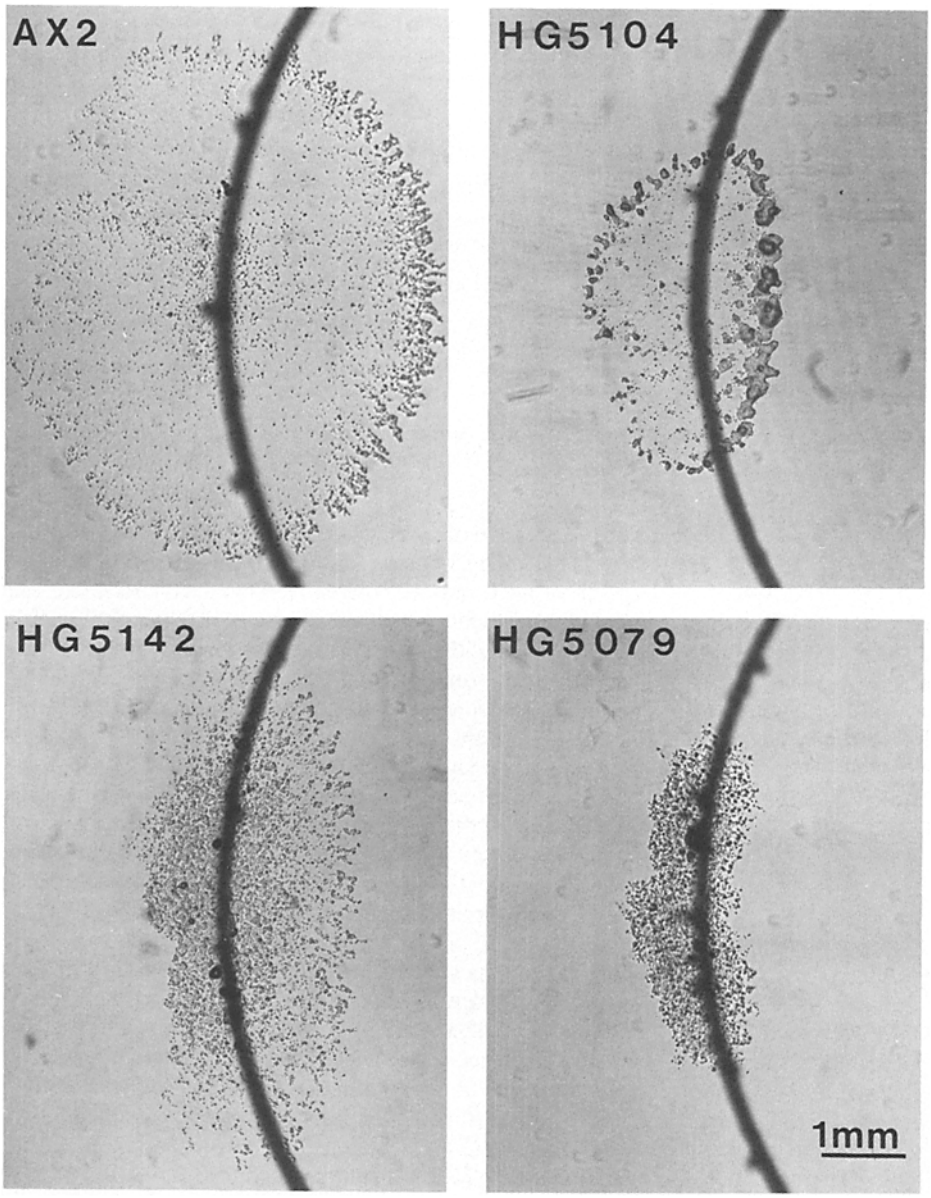

Figure 5. Response of wild-type AX2 and three mutants in chemotaxis assay on the agar. Cells were spotted on agar with a toothpick 4 mm from the edge of a well of 6-mm diameter containing 1 mM folate (to the right of the area shown). Each dark arc is a ring inscribed on the bottom of the petri dish, marking a distance of 4 mm from the edge of the well. The responses of mutants HG5115, HG5048, and HG5043 were between that of HG5142 and that of HG5079. The responses of HG5145, HG5013, HG5135, and HG5125 were similar to HG5079.

fruiting bodies in which stalks and spore heads were still distinguishable. HG5135 was similar but produced fewer small aggregates and fruiting bodies. HG5145 showed a broad preaggregation zone indicating that the onset of aggregation was delayed. This mutant formed long streams of aggregating cells and fruiting bodies with short stalks (Fig. 7). HG5079 had a reduced rate of colony growth. It formed small aggregates with few or no streams and fruiting bodies smaller than wild-type but definitely larger than HG5142 (Fig. 7). HG5048 developed normally except that the rate of colony growth was reduced and, accordingly, the preaggregation zone was short (Fig. 7). HG5115 showed no development at all, and HG5125 formed a mesh of cells packed into a monolayer (Fig. 7). HG5115 was the only mutant analyzed that did not express the contact site A glycoprotein, a marker of cell development to the aggregation-competent stage. This was shown by colony blotting and labeling with mAb 41-71-21, an antibody specific for this membrane protein (Bertholdt et al., 1985).

Optimization of Selection Conditions

Mutant HG5013 has been used to test and optimize the selection conditions. If mutant cells were applied together with wild-type AX2 cells to the bottom of the second filter (Fig. 1) and incubated for a 4-h period in the selection chamber with 7×10^{-6} M folate in the upper compartment, there was a sixfold enrichment (Table III). Applying the cells to the top of the third filter and incubating them under the same conditions resulted in a 24-fold enrichment (Table III). If only a 3-h incubation period was used there was a 13-fold enrichment. With buffer in both compartments, the enrichment was only twofold. The results with other mutants were consistent; high selection ratios were obtained by applying cells to the top of the third filter and using a 4-h incubation period. HG5142 or HG5145 cells applied together with wild-type AX2 cells yielded ~30-fold enrichment of the mutant cells under these conditions.

The reduced enrichment that was attained when cells were

*Table II. Enrichment of Mutants by Selection with Folate**

	Colonies from unselected cells						Colonies from cells selected in the chamber					
	I	II	III	IV	V	Total	I	II	III	IV	V	Total
Colonies with altered cell responses on agar‡												
Colonies with normal morphology	0/16	0/19	0/12	0/21	0/12	0/80 (0%)	1/13	0/18	0/11	1/3	0/8	2/53 (4%)
Colonies with altered morphology	0/8	0/9	1/13	0/7	0/9	1/46 (2%)	6/18	4/14	4/18	11/26	2/12	27/88 (31%)
Colonies with altered morphology												
Before growth	5/229	13/248	4/87	5/121	1/97	28/782 (4%)	–	–	–	–	–	–
After growth‡	9/161	10/237	20/267	8/199	11/256	58/1120 (5%)	29/197	15/179	30/128	95/230	24/173	193/907 (21%)

* Results are from the second mutagenesis. Roman numerals refer to separate flasks of mutagenized cells. The number of mutant colonies vs. the total number tested is given. Ratios of colonies with normal and altered morphology tested for cell responses do not reflect their frequencies of occurrence.
‡ Determined after 6–7 rounds of selection for selected cells. Unselected mutagenized cells were grown as many generations as selected cells.

applied to the bottom of the second filter may have been due to worse contact between the top and second filters compared to the contact between the second and third filters. There were often regions on the top of the second filter where there were high densities of cells and no cells in the corresponding regions of the top filter. The cells may not have been able to cross to the top filter in these regions. Such regions were never seen on the top of the third filter when cells were applied to it.

Discussion

Efficiency of Selection for Chemotaxis Mutants

We know of three previous studies of the movement of *D. discoideum* cells through filters. The first (Bonner et al., 1971) reported that amebas could migrate through filters in a Boyden chamber in a cAMP or bacterial extract gradient. The second study found that cells only responded to the initial establishment of the gradient and became evenly distributed afterwards over a layer of filters (Vicker et al., 1984). We found that the cells clearly continued to move up the gradient for hours. In the third study, three potential temperature-sensitive cAMP receptor mutants were isolated (Barclay and Henderson, 1977, 1982).

We have developed an improved Boyden chamber for the efficient selection of chemotaxis mutants. This method used the ability of cells to migrate in a sandwich of four filters in response to a chemoattractant gradient and allowed up to 30-fold enrichment of mutants per round (Table III). Although

Table III. Characteristics of Mutants as Compared to Wild-type AX2

Strain	Buffer response	Folate response	Net response	Fraction AX2 response	Speed‡	Turning rate	Deaminase§	Selection ratio‖ II	Selection ratio‖ III
	*μm**	*μm*	*μm*		*μm/min*	*rad²/min*	*U/ml*		
AX2	60 ± 25	221 ± 25	161	–	6.6 ± 1.2	1.12 ± 0.22	42 ± 14	ND¶	
HG5104	27	35	8	0.05	7.1	0.95	6	2	ND
HG5142	64	85	21	0.13	5.7	0.74	27	ND	29
HG5013	53	88	25	0.16	6.9	0.99	46	6	24
HG5145	38	65	27	0.17	8.8	1.04	58	ND	30
HG5079	11	41	30	0.19	4.4	1.50	22	7	ND
HG5043	43	102	59	0.37	5.5	1.23	40	ND	
HG5135	7	67	60	0.37	4.1	1.13	13	ND	
HG5048	29	94	65	0.40	6.5	1.56	34	ND	
HG5115	4	73	67	0.42	5.7	1.57	61	ND	
HG5125	ND	10	10	<0.1	1.5	ND**	10	ND	4

For AX2, means and standard deviations of 6–10 independent measurements are given. Mutants with numbers up to 5114 are from the first mutagenesis while those with larger numbers are from the second.
* Experiments were performed as described in the legend to Fig. 2. Filters were stained and cell numbers on each filter determined. The average distance traveled was then calculated. For the mutants, the buffer response is a single measurement while the folate response is the mean of at least two independent measurements.
‡ Speed and turning rates were determined as described in Materials and Methods. Values for the mutants are means of at least two independent measurements covering at least 50 cells each. Speed values for HG5145, HG5079, and HG5135, and turning values for HG5142, HG5079, HG5048, and HG5115 are significantly different from wild-type (three measurements each, $P < 0.05$ in t test).
§ Units of extracellular deaminase released from bacterially grown cells are nmol/product per min. Values are means of at least two independent measurements.
‖ Selection ratio determined as described in Materials and Methods. II, cells were deposited on the bottom of the second filter; III, cells were deposited on the top of the third filter. The total incubation time was 4 h.
¶ ND, not determined.
** Distances between cell centers for successive time-lapse intervals were too small for enough data to be collected.

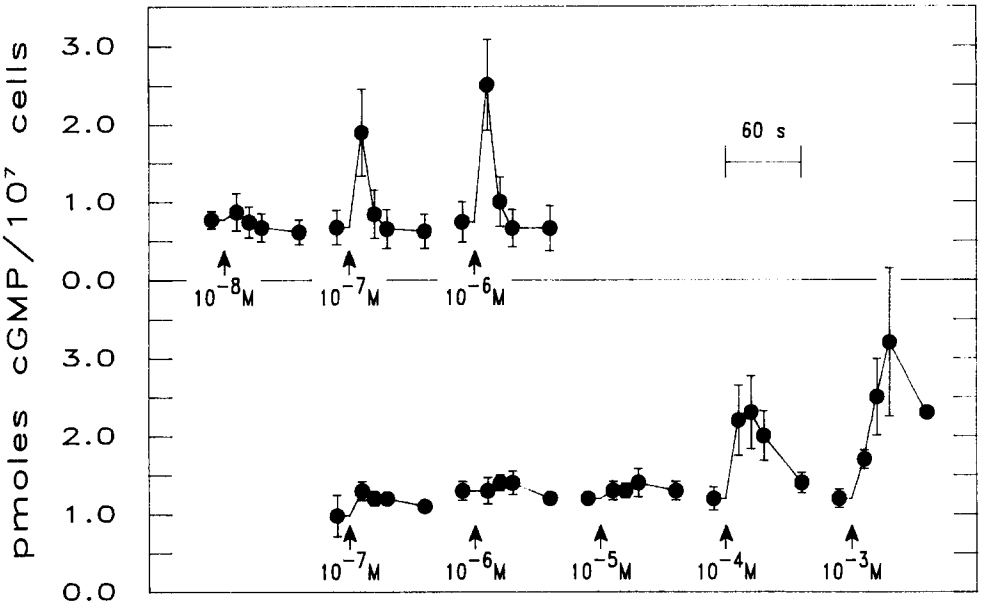

Figure 6. Changes in total cGMP in AX2 (*top curves*) and HG5145 (*bottom curves*) as a function of folate stimulus concentration. Folate was added to give the indicated final concentrations at the times marked by the arrows. Samples were taken 10 s before and 10, 20, 30, and 60 s after addition of folate. Successively higher folate stimuli were delivered at 5–10-min intervals. Plotted are the means and standard errors of three independent experiments. In a fourth experiment, HG5145 cells had a very high basal concentration (~3 pmol/10^7 cells) which did not change in response to folate stimuli.

the method was established for the selection of *D. discoideum* mutants defective in chemotaxis to folate, it should be applicable to the selection of cAMP chemotaxis mutants and adaptable to the selection of nonchemotactic strains from leukocyte or other cell lines.

The cell population moved through the filters as a wave, and after 4 h over half the cells had reached the filter surface nearest to the attractant solution. Such movement is evidence that the cells were responding not only chemokinetically but also chemotactically. Only chemotactic movement will result in the cells moving in a wave up the attractant gradient (Wilkinson et al., 1982). *D. discoideum* does show a slight chemokinetic response to folate. Such a chemokinetic response will produce, in the absence of a chemotactic response, a tail in the cell distribution extending towards higher concentrations of attractant. However, the final distribution will still have more cells at lower concentrations of attractant than at higher concentrations if only chemokinetic responses are involved (Lapidus, 1981; Rohlf and Davenport, 1969; Vicker et al., 1984).

Nonchemotactic mutants should not move in the wave of attracted cells. They should be enriched relative to wild-type cells in the filter to which the cells were applied. However, not only chemotactic mutants will be selected in this way. First, mutants defective in chemokinesis may also be enriched, although this effect should be very weak for folate. Second, nonmotile mutants will be co-selected in the chamber with those defective in chemotactic orientation. There are several possible reasons why we have not selected any so far. The cells remaining on the original filter were always grown before the next round of selection. Growth requires either phagocytosis of bacteria or pinocytosis of liquid nutrients. Thus, if there is a defect in endocytosis associated with the defect in motility, the selection ratio will be low because the cells are counterselected during growth. Indeed, all the mutants isolated grew as quickly on bacteria in suspension cultures as the parent strain. Nevertheless, it is possible that some of the selected mutants, particularly mutants HG5079, HG5135, HG5142, HG5048, HG5135, and HG5115 with either reduced speed of cell locomotion or altered turning rates (Table III), have defects in cytoskeletal proteins. HG5125, an almost nonmotile mutant isolated by screening unselected cells, had a low selection ratio in the chamber (Table III). Cells of this mutant may not detach well from the filters and would then have little chance of being selected.

A third type of mutant which might be selected is one altered in the production of attractant-degrading enzymes. *D. discoideum* produces cell surface-bound as well as extracellular folate deaminases and cAMP phosphodiesterases. Degradation of folate during its diffusion through the filter layer appears to improve chemotactic movement of the cells as suggested by the deaminase defective mutant HG5104 (Table III). However, as long as the cell density in the filters is reasonably high, mutants defective in the production of extracellular attractant degrading enzymes should not be selected because they are supplemented by the wild-type cells surrounding them. This is not necessarily true for cell surface-bound folate deaminase or cAMP phosphodiesterase which might improve the response of an individual cell to the respective attractant.

HG5104 showed substantially reduced production of extracellular folate deaminase (Table III). When mixed with wild-type, the selection ratio was low for this mutant, as expected from supplementation by wild-type cells. Severe alterations in development (Fig. 7) suggest that the mutation may be pleiotropic rather than specific for folate deaminase production.

Analysis of Behavioral Defects in the Selected Mutants

Analysis of the mutants is not only required for the sorting out of chemotaxis mutants from those selected for other reasons, but also for the classification of mutants actually defective in the chemotactic response. The mutants may be classified according to the nature of the alteration and the step in the signal transduction system that is altered. The nature of the alteration could simply be a block in signal transduction. Mutants with normal motility in the absence of attractant, such as HG5013 and HG5043, may contain such a defect. Or the alteration could be the continuous activation (or inhibi-

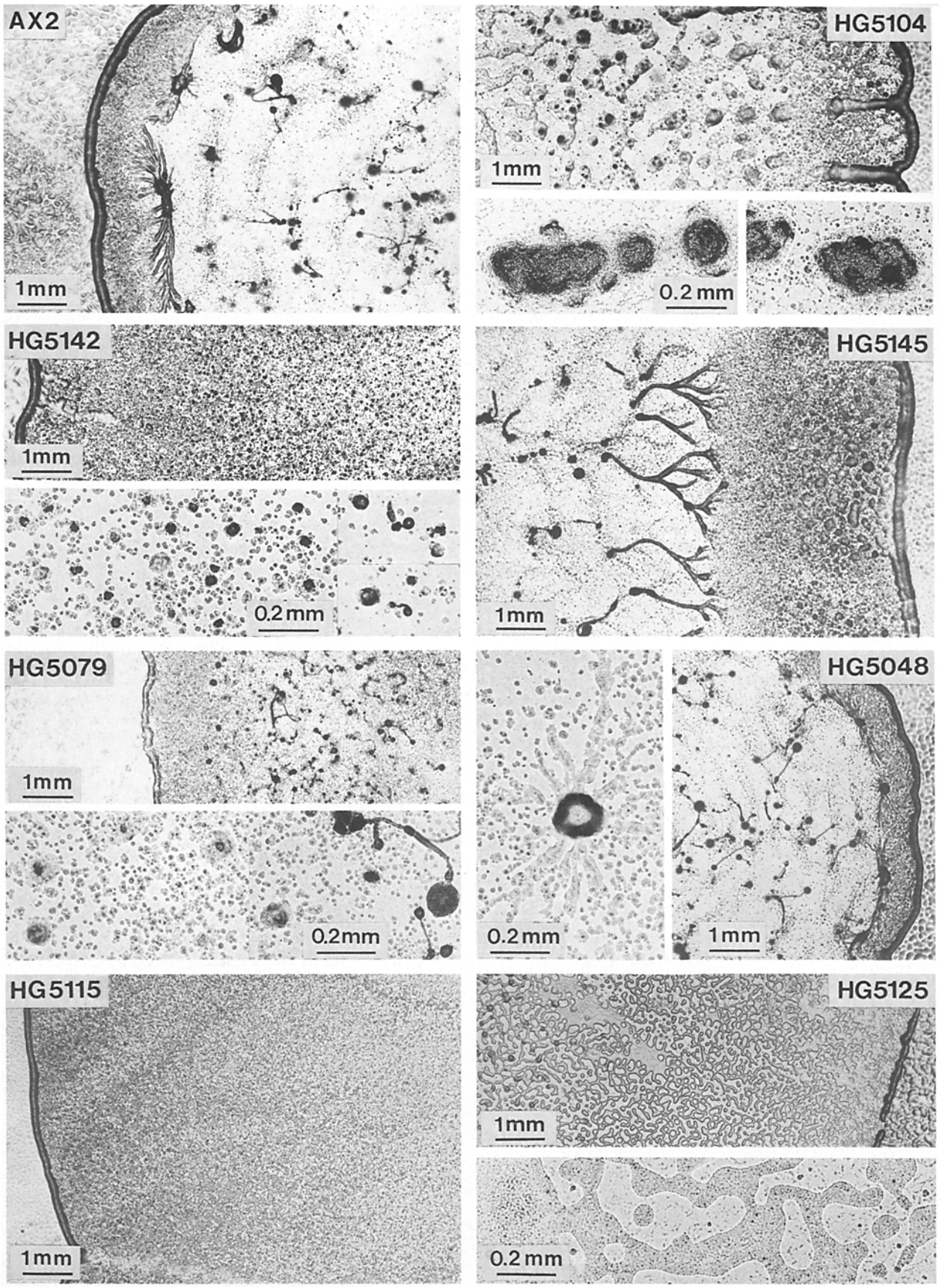

Figure 7. Colony morphology of wild-type AX2 and of mutants grown on *E. coli* on nutrient agar plates.

tion) of a component of the transduction system even in the absence of an external stimulus. Since a chemotactic response of *D. discoideum* and other ameboid cells implies turning into the direction of higher attractant concentrations, mutants with an altered spontaneous turning rate might fall into this category. In bacteria, it has been found that all generally nonchemotactic mutants have strongly altered tumbling rates (Parkinson, 1981). Tumbling in bacteria serves a similar function as spontaneous turning in amebas, although the mechanism of movement is totally different. Among the mutants analyzed, HG5079, HG5048, HG5115, and HG5142 showed reduced chemotaxis to folate along with significantly altered turning rates.

A response common to all known chemotactic stimuli for *D. discoideum* and other cellular slime molds is an increase in the cellular cGMP content, which under optimal conditions reaches a peak 10 s after the addition of attractant. The cGMP response was measured in mutant HG5145, a chemotaxis-defective mutant with normal extracellular folate deaminase production, normal turning rate, and somewhat increased speed. The reduced sensitivity of cGMP production to folate stimuli observed for HG5145 indicates that in this mutant either the receptor for folate has an altered affinity or the cell transduction system is deranged in such a way that stimuli less than 10^{-4} M folate produce no response. The higher speed of the mutant compared to wild-type raises the possibility that the transduction system is to some extent continuously activated.

The mutants need to be further analyzed in order to identify the step in signal transduction or the component of the contractile system that has been altered. The transduction system may involve components common to folate and cAMP responses. If so, it should be possible to divide the mutants into those defective in the peripheral part of the transduction system or its central portion, depending on whether the response to only one attractant or to both folate and cAMP is affected. However, a comparison of the chemotactic responsiveness of a mutant to folate and cAMP is difficult since cAMP acts not only as an attractant but also as a regulator of preaggregative development. The cAMP-mediated chemotaxis of a mutant will be dependent on the stimulation of its development by cAMP, while the sensitivity to folate has its maximum before cAMP becomes involved.

There is a great variety of phenotypes among the mutants defective in folate chemotaxis, as shown in Table III and Fig. 7. Most of these phenotypes are represented only by a single mutant, indicating that we are far from having collected mutations in all the genes that code for proteins involved in folate chemotaxis. The variety of phenotypes may reflect the different functions of components involved in chemotaxis.

None of the mutants that we have analyzed proved to be absolutely devoid of a response to folate (Table III). This may be due to leakiness caused by the incomplete abolishment of activity of the particular component affected in a mutant. But it may also mean that chemotaxis is brought about by the concerted action of a number of components of which most improve the response without being absolutely essential. For instance, mutants defective in adaptation may still be capable of responding within a certain range of concentrations.

The finding that a high proportion of the mutants selected in the chemotaxis chamber with folate as an attractant are also morphologically defective deserves further investigation. A straightforward explanation for this finding would be that folate stimuli are required for normal development. However, *D. discoideum* cells are folate auxotrophs (Franke and Kessin, 1977) and are therefore unlikely to release pulses of folate in the same fashion that cAMP is produced. Some of the selected mutants with morphogenetic defects are altered in cell functions other than chemotaxis (e.g., are altered in motility), and this alteration may be responsible for the morphogenetic defect. Finally, folate chemotaxis mutants might be altered in a portion of a response system common to folate and cAMP, the latter being known to serve multiple functions in development.

The results reported here show that the selection method used yields a great variety of mutants. Further biochemical characterization of the mutants should help to reveal the mechanisms by which ameboid cells use an external concentration gradient to orient their movement.

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