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Effect of Bacteria on Chemotaxis in the Cellular Slime Molds

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The effect of chemotactic substances, secreted by *Escherichia coli*, on the cellular slime molds was studied by deposition of bacteria near myxamoebae populations. Droplets of a bacterial suspension and a myxamoebae suspension were placed separately, at predetermined distances from each other, on a hydrophobic agar surface of low rigidity. Myxamoebae remained confined inside the droplets, except when they were activated by the bacterial products. The sphere of attraction increased at higher bacterial concentrations. Myxamoebae could be attracted over distances as great as 5 mm. Myxamoebae in droplets close to dense bacterial populations not only were attracted toward the bacteria but also moved out in an opposite direction from the bacteria. There was a gradual decrease of attraction at increasing distances between amoebae and bacteria. The attraction by bacteria or bacterial products was reduced at lower temperatures. Light did not affect the distance over which attraction could be observed. Myxamoebae close to their aggregation phase were most sensitive to the bacterial attractants. Bacterial attractants at high concentrations could disperse aggregates, even when they were in an advanced stage. At still higher concentrations of the bacterial products, cells stopped moving altogether. The bacterial attractants activated different species of cellular slime molds. They appeared to be present not only in *E. coli* but also in all other bacterial species that were tested. These results are discussed in the light of earlier observations on the attraction of cells by aggregates of myxamoebae.

The acrasieae, also known as acrasina, are characterized by a unicellular amoeboid stage followed by a multicellular pseudoplasmodial stage from which a simple fruiting structure emerges. The cells in the developing fruiting structure differentiate into stalk cells and spores.

Uptake of food is limited to the vegetative stage when myxamoebae engulf bacteria. Raper (11) compared the growth of *Dictyostelium discoideum* when fed with different species of bacteria and found that a large variety of bacteria support the growth of the amoebae. Bacteria not only are the food source of the myxamoebae but also attract myxamoebae (1, 12; T. M. Konijn, Ph.D. thesis, University of Wisconsin, 1961).

A quantitative approach to the attraction of myxamoebae by bacteria is described in this paper. Small bacterial populations were placed on a hydrophobic agar surface at various distances from small amoebae populations. The low rigidity of this specially prepared agar permits the bacteria to attract cells from the neighboring amoebae populations. The effects of various environmental conditions on the attraction of sensitive amoebae by bacteria and bacterial extracts are described. The results are discussed in the light of earlier observations on the attraction of responding amoebae by aggregating amoebae (5, 6, 9).

MATERIALS AND METHODS

Escherichia coli was used as a source of attraction and as a food source to grow the myxamoebae. The attracting bacteria, *E. coli* B/r, were grown on a tryptone (0.5%)-yeast extract (0.5%)-glucose (0.1%)-K_2HPO_4(0.1%)-agar medium at 30 C. After 1 day, the bacteria were suspended in Bonner's salt solution (2), centrifuged, and resuspended in the salt solution. Centrifugation and storage at 5 C gave results similar to those at room temperature. The density of the bacteria was measured in a spectrophotometer at 535 nm and correlated with the number of bacteria counted directly in a hemocytometer. Small drops of the *E. coli* suspension were deposited on a hydrophobic agar surface (5, 6).

Myxamoebae of *D. discoideum* NC-4(H) were grown in darkness on a glucose-peptone medium (2), with *E. coli* 281 used as a food source. The amoebae were harvested after about 40 hr and suspended in Bonner's salt solution diluted 100 times. The cells were centrifuged two or three times to remove excess

bacteria. After the final suspension, the required cell density was reached by dilution with full-strength salt solution. Small droplets of this amoebae suspension were deposited on a hydrophobic agar surface (5, 6). The diameter of the drops containing the amoebae varied from 0.5 to 0.7 mm. The petri dishes, each containing about 120 small populations, were kept in darkness at 22 ± 1 C. Droplets of *E. coli* B/r containing 10^5 to 10^8 bacteria (0.5 to 0.7 mm in diameter) were deposited close to the amoebae populations. The distances between the droplets of bacteria and amoebae were measured. Cell attraction was scored as positive if the myxamoebae moved out of their droplet toward a droplet containing bacteria. The result was scored as negative when all of the amoebae stayed inside their droplet. When amoebae crawled out of the droplet in all directions, the attraction was marked positive if the number of amoebae at the side of the bacterial population was at least three times higher than that at the opposite side.

RESULTS

Effect of bacterial density on attraction of myxamoebae. Previously it was shown that small populations with aggregates of only a few hundred amoebae yield a maximal secretion of attracting substance (6). To study the effect of bacterial density on attraction, small droplets of bacterial suspensions with different densities were deposited at various predetermined distances from amoebae populations. Each amoebae population contained about 1,000 cells. The amoebae were deposited 2 or 3 hr earlier than the bacteria.

Within 30 min after the deposition of the bacteria, amoebae started passing the boundaries of their droplets. After 45 min, several myxamoebae were outside the margins of the populations. Earlier it was shown that, when aggregating myxamoebae instead of *E. coli* occupy the attracting drop, responding cells move out only at the margin closest to the attracting population (5, 6). On the other hand, cells attracted by bacteria could move out in all directions (Fig. 2e). Sometimes as many amoebae moved away from the bacterial population as toward it. Particularly at high bacterial concentrations and with a space less than 0.5 mm between the bacterial population and the responding amoebae population, the number of cells outside the droplet often was the same in all directions. The cells that moved out at the side opposite the bacterial colony left the amoebae droplet at the same time or slightly later than the amoebae that passed the boundary closest to the attracting bacteria.

There was considerable variation among different experiments, but within the same experiment the attraction of amoebae by bacteria was stronger as the bacteria concentration increased or when the time of observation was postponed. A few very dense bacterial populations attracted cells out of amoebae populations that were deposited as far as 5 mm from the bacterial populations.

The amoebae inside the population were homogeneously distributed throughout the droplet when the concentration of bacteria in the neighboring droplet was low. At high bacterial concentrations several amoebae tended to group together at the side closest to the bacterial population. A few hours after exposure to high concentrations of the bacterial attractants, small clumps of amoebae could be observed which often dispersed later. Attraction by bacteria continued for several hours. At short distances (less than 200 μm between the droplets) and at high bacterial densities, sometimes all cells in a population migrated into the bacterial population.

All amoebae in control populations stayed inside the boundaries of the droplet. These amoebae aggregated earlier than cells in droplets near bacterial colonies. When the cells were close to dense bacterial populations, aggregation could be inhibited completely. When the bacterial density was lower or the distance greater, aggregation was only delayed. Shaffer (13) observed a delay of aggregation when he separated amoebae and bacteria by a thin layer of agar.

Effect of temperature and light on attraction of myxamoebae by bacteria. The attraction exerted by myxamoebae is stronger at lower than at higher temperatures (5). The attraction of amoebae by *E. coli* B/r was studied at various temperatures by deposition of droplets containing ca. 500 amoebae near *E. coli* B/r populations, each containing ca. 5×10^5 bacteria. The bacteria were placed on the agar 1 hr before the myxamoebae and incubated at 5 C. After the myxamoebae were deposited at various distances from the *E. coli* populations, the petri dishes were incubated at various temperatures and observed after 5 hr.

Above 30 C amoebae lost their ability to respond to attractants secreted by the bacteria. At 25 and at 30 C, more amoebae populations responded positively than at 20 C. At 15 C the distance over which bacteria caused myxamoebae to cross the margins of their populations was reduced still further (Fig. 1). When there were more bacteria, ca. 5×10^7 per population, the attraction of myxamoebae by bacteria again was less at lower temperatures.

The variations among different experiments were considerable (Fig. 1). Different temperatures may have a different effect on the secretion of attractants by the living bacteria and on the response of the myxamoebae. Owing to these two variables, a wide range of values for the dis-

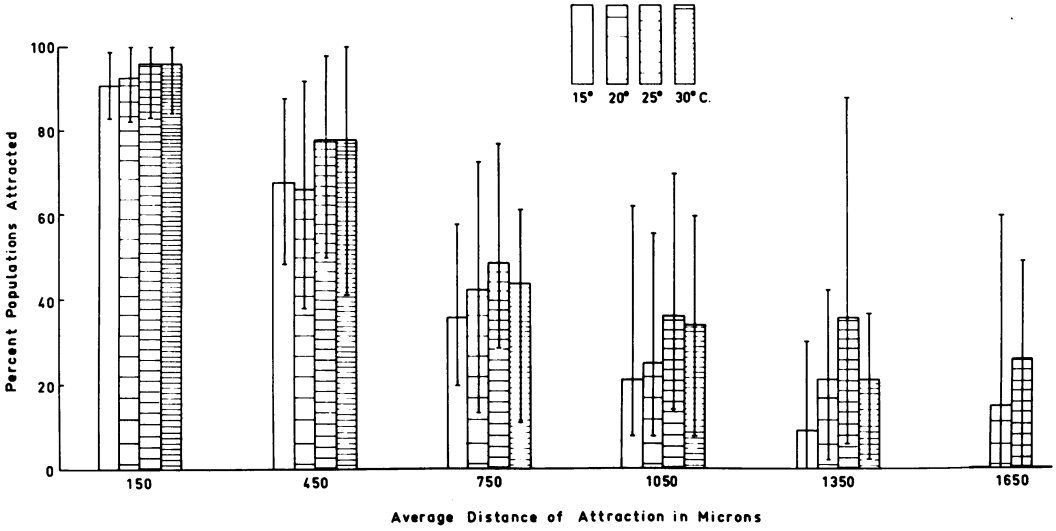

FIG. 1. *Effect of temperature on the percentage of myxamoebae populations that respond to E. coli B/r colonies, and the distance between both populations. Each column represents the average of five experiments. Vertical bars represent range of values.*

tances of attraction may be expected. To exclude one variable, the *E. coli* attractant was tested without the bacteria. *E. coli* was grown on agar, harvested with distilled water, and centrifuged (10). The water extract of the bacteria was concentrated in a vacuum evaporator at 50 C and deposited as small droplets six to eight times at 10-min intervals near the myxamoebae populations. These had already been incubated at the desired temperature for 1 hr. The petri dishes were kept in an incubator except during the time that the bacterial extract was deposited, which took place at 22 ± 1 C. At 0.5 hr after the last deposition of the extract, myxamoebae incubated at 30 C were attracted more strongly than those incubated at 20 C. Both young and physiologically more advanced myxamoebae were less responsive at low temperatures. The activity was due to the bacterial products and not to the medium, since droplets of the latter deposited as a control exerted no attraction.

When the extract of the bacteria was applied only once, cells moved out as well, but were less clearly directed toward the extract than when it was applied several times.

When responding cells are attracted by aggregating myxamoebae in darkness, the attraction extends over a larger distance than in light (9). The effect of light on attraction by bacteria was examined by deposition of small droplets containing about 5×10^5 *E. coli* cells at various distances from myxamoebae populations (500 cells) that had been deposited 1 hr before. The influence of light ("cool white" fluorescent tubes with an intensity of ca. 60 ft-c at the level of the agar surface) on the attraction of amoebae by *E. coli* was measured after 5 hr. The distance over which amoebae responded to bacteria in light was similar to that of cells kept in darkness. Both in light and in darkness there was a gradual decrease of attraction with greater distances.

Bacteria attracting myxamoebae of various developmental stages. Starvation induces myxamoebae to attract each other and to aggregate. Do myxamoebae after feeding become increasingly sensitive to the bacterial attractant or do the morphological, physiological, and biochemical changes coincide with a decreased sensitivity to bacteria? To answer this question, *E. coli* populations were deposited on a hydrophobic agar surface at various times before and after implantation of the myxamoebae populations. Small droplets of a bacterial suspension, deposited 20 or 10 hr before the myxamoebae populations were deposited near them, attracted cells across the margins of the myxamoebae populations. Myxamoebae which were about to aggregate were more sensitive to a bacterial population than were cells which had been starving for a shorter period or had been deposited at the same time as the bacteria. After aggregation had started in the responding populations, bacteria in the neighboring droplet still attracted some of the myxamoebae that had not yet been incorporated into the aggregate. Even when bacterial populations were several hundred micrometers away from the myxamoebae populations in which cells had began to aggregate, myxamoebae at the periphery of these droplets moved out.

Time-lapse films taken at 8-sec intervals showed clearly the increased sensitivity of starving myxamoebae to bacterial attractants. *E. coli* populations with 5×10^7 bacteria per drop were photographed in the same field with myxamoebae populations of about 500 cells. The distance between bacteria and the myxamoebae was ca. 160 μm.

When the bacteria were deposited 150 min after the deposition of the amoebae, the first myxamoebae moved out of the population after 30 min. The first cell arrived at the bacterial colony after 150 min. At that time, 30 cells had moved out of the myxamoebae population. The next three myxamoebae reached the bacterial colony within 30 min. Bacteria that were deposited near myxamoebae which already were on the agar surface for 6.5 hr attracted myxamoebae toward the margin of the bacterial colony in half the time that was needed for the younger amoebae. The number of cells counted outside the myxamoebae population when the first cell reached the bacterial population was nearly double that of the younger cells. Still older myxamoebae had moved out in even greater number when the first cells reached the bacterial population. Also the speed with which myxamoebae moved over the agar surface within the older populations was enhanced. Samuel (12) observed this increased cell movement shortly before aggregation in *D. mucoroides*. Myxamoebae which were about to aggregate lined up in parallel streams directed toward the bacteria (Fig. 2d).

Cells that already had begun to aggregate dispersed when a dense population of bacteria was deposited near them. A concentrated extract of *E. coli*, deposited near an aggregate into which nearly all cells had entered, caused disintegration of the aggregate even at this late stage (Fig. 2 a-f). Within 10 min after deposition of the extract near the advanced aggregate, the myxamoebae started to disperse. They did not leave the aggregate as single cells but departed as streams (Fig. 2c). The outflowing streams and single myxamoebae moved not only toward the source of attraction, but also in other directions. The outward movement was not pulsating, as is the case when cells crawl inward to form an aggregate. Amoebae moved in parallel streams to the source of attraction (Fig. 2d). After 80 min, the aggregate dispersed completely (Fig. 2e) and the cells were free again. Some of them crawled out of the droplet into the agar, at the side of the concentrated extract as well as at the opposite side (Fig. 2e); others started to form a new aggregate, competing for myxamoebae situated between the two sources of attraction. The majority of the cells returned to the new aggregate (Fig. 2f). This is not surprising, since the new aggregate constantly secretes the attractant in pulses, whereas the droplet with the bacterial attractant had been deposited only once, more than 1 hr earlier.

When very concentrated *E. coli* extract was deposited near myxamoebae, no cells moved out of the population. Cells stopped crawling or heaped together in small clusters.

Myxamoebae of various species attracted by different bacteria. Various strains of *D. discoideum*, including strain Acr. 12 which differs morphologically and physiologically from the others (8), were attracted by *E. coli* and *Aerobacter aerogenes*. *E. coli* 281 and B/r attracted *D. purpureum*, *D. mucoroides*, *Polysphondylium pallidum*, and *P. violaceum*. Dense *E. coli* populations attracted the various species of myxamoebae over longer distances than less concentrated bacterial populations.

The attraction exerted by different strains of the same bacterial species may differ. The attraction sphere of *E. coli* B/r extended over a larger distance than that of strain 281.

Other gram-negative bacteria which attracted myxamoebae of *D. discoideum* were *Serratia marcescens* and *Sarcina lutea*. The gram-positive bacteria *Bacillus subtilis* and *B. megaterium* also secreted a myxamoebae attractant. The effect of density and distance that had been found in *E. coli* also applied to these species.

DISCUSSION

Myxamoebae of *D. discoideum* crawl across the margins of the responding populations if the distance between the centers of the aggregates and the closest side of the responding populations is less than 700 μm. Cells are not attracted if this distance exceeds 1,500 μm (5). At lower temperatures the sphere of attraction increases, but the difference between the distance leading to response in all populations and that leading to no response at all does not exceed 800 μm.

In situations where bacterial populations generally attracted myxamoebae across the boundaries of the responding droplets, a few myxamoebae populations failed to respond even at close distances. At distances far exceeding that between aggregates and neighboring myxamoebae droplets, some populations reacted positively to the bacteria. An obvious difference between a bacterial population and an aggregate as a source of attraction is the time during which they secrete attractants. Bacteria attract myxamoebae all the time, whereas aggregates attract cells out of responding populations predominantly from early till late aggregation. When bacterial populations and myxamoebae aggregates attracted responding myxamoebae during the same period, the percentage of positively responding populations

plotted against their distance from the source of attraction showed a much steeper slope in the case of aggregates than when bacteria secreted the attractant (*unpublished results*). This difference could be explained on the basis of a difference in the mode of attractant secretion. The attractant produced by myxamoebae of *D. discoideum* is secreted in pulses. Bacteria, as observed in a time-lapse film, attracted amoebae continuously. A repeated orientation of the myxamoebae by a pulsating aggregate results in a more directed movement toward the source of attraction than a continuous exposure to the bacterial attractant. The observed slow decrement of the attraction by

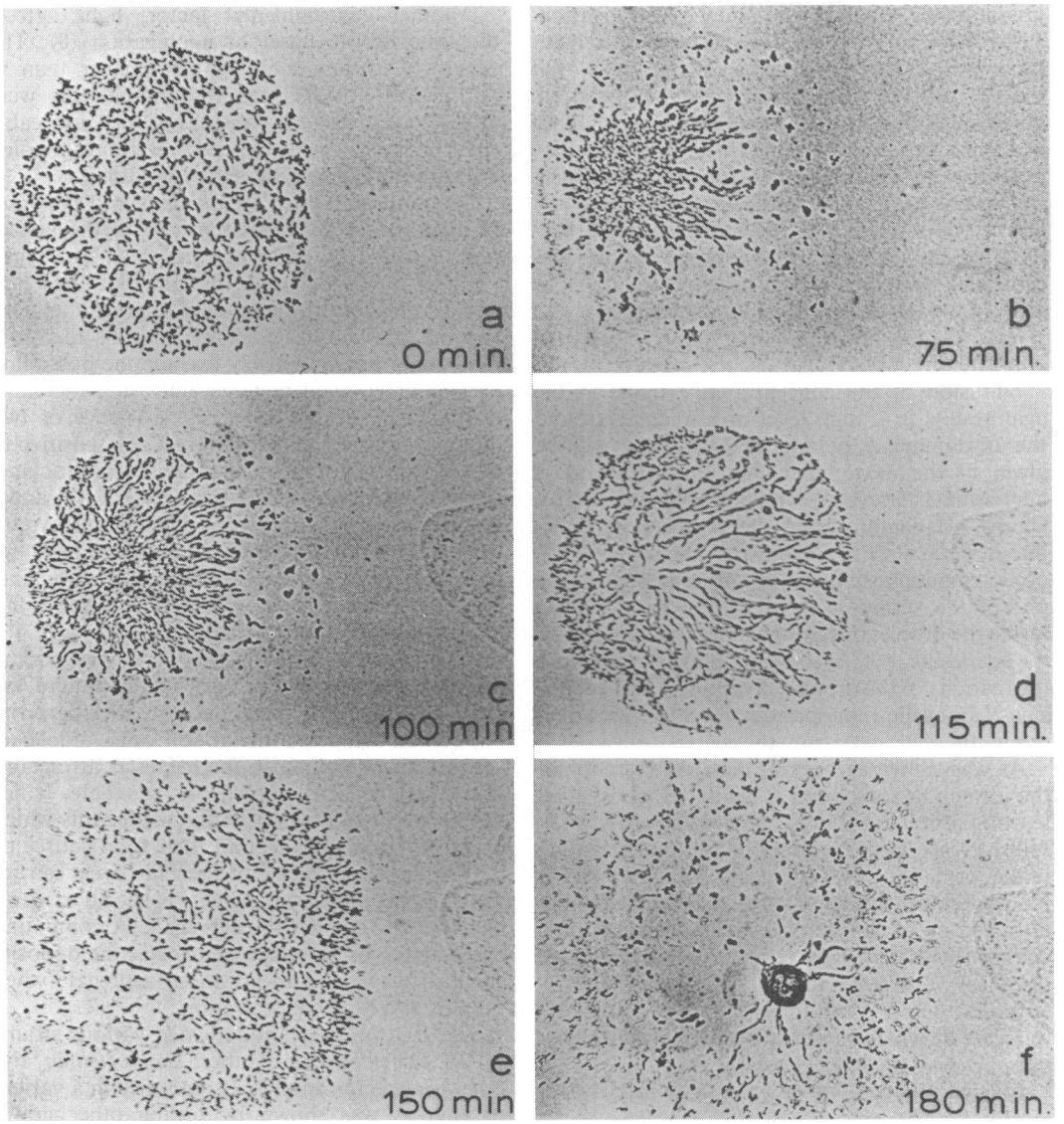

FIG. 2. *Aggregate of D. discoideum dispersed by bacterial products. The population of myxamoebae was placed on a hydrophobic agar of low rigidity. Frames from a time-lapse film.* × 60. *(a) Myxamoebae close to aggregation. The cells are confined inside the boundaries of the droplet. (b) The aggregate is in an advanced stage; immediately before deposition of the bacterial extract. (c) Dispersal of the aggregate 25 min after deposition of a concentrated E. coli extract at the right of the myxamoebae population. (d) Myxamoebae line up in streams directed toward the bacterial attractant. (e) The aggregate is dispersed completely. Myxamoebae cross the margins of the droplet in all directions. Outside the droplet cells do not move on the agar surface but crawl through the agar. (f) The myxamoebae reaggregate.*

bacterial populations over increasing distances may be due to this lack of pulsating waves.

Particularly at high bacterial densities, cells moved not only across the boundaries of the droplet at the bacterial side but also at the opposite side. Cells activated by the bacterial attractants move faster; they pass the boundaries of the droplet but the shallowness of the attractant gradient may obscure the source of activation.

To test the effect of the bacterial attractant gradient on the distribution of the cells that moved out of the myxamoebae population, bacterial extract was deposited near myxamoebae not once but repeatedly. When this was done five times at 5-min intervals, imitating the pulses characteristic of aggregates, nearly all cells that left the population crawled out toward the bacterial colony. A single deposition of a more concentrated bacterial extract resulted in amoebae passing the droplet margins in all directions.

Why do myxamoebae also move outside in opposite directions?

Diffusion of the concentrated extracts in the agar results in a high level of attractant around the myxamoebae population. Evidence will be given in the next paper that the attractant is adenosine-3′,5′-cyclic monophosphate (10). Chang (4) demonstrated the presence of phosphodiesterase, which inactivates adenosine-3′,5′-cyclic monophosphate, in myxamoebae. The phosphodiesterase secreted by the myxamoebae inside the droplet diffuses outward and inactivates the adenosine-3′,5′-cyclic monophosphate around the margins of the droplet, creating a steep adenosine-3′,5′-cyclic monophosphate gradients which orient, the cells away from the center.

As was shown earlier (6), maximal secretion of the attracting compound is exhibited by as small a group as a few hundred aggregating cells. A 10-fold increase in the number of cells participating in the formation of an aggregate does not increase its attraction sphere. Dense bacterial colonies attracted myxamoebae over larger distances than colonies with fewer bacteria, especially at large distances. If there is a feedback mechanism in bacteria that controls the total output of attractant, the level at which it works must be much higher than in myxamoebae aggregates.

When aggregates of myxamoebae were found to attract responding cells more strongly at lower temperatures (5), it was difficult to distinguish between two alternative explanations, namely, whether the aggregates exerted more attraction at lower temperatures, or whether the responding cells became more sensitive to the attractant. The attraction of myxamoebae by bacteria decreased at lower temperatures. The diminished attraction was due not only to a reduced production of the attractant at lower temperatures, since a concentrated bacterial extract also was less active at lower temperatures. Therefore, the stronger attraction of aggregates at lower temperatures probably is not the result of a higher sensitivity of the responding cells.

Another environmental factor, light, affects the time of the onset of aggregation (8). The sphere of attraction in light is smaller than in darkness (9). When the responding cells were attracted by bacteria, there was no noticeable effect of light, which suggests that the responding cells are not affected by light conditions.

Myxamoebae which are themselves at the onset of aggregation are most sensitive to the attractants secreted by other myxamoebae. The sensitivity of myxamoebae to the bacterial attractant was also highest at the onset of aggregation. This suggests that both attractants may be identical, but it does not completely exclude the possibility of two different attractants activating the same responding mechanism, or the existence of two different responding mechanisms, one sensitive to bacterial attractants and the other to attractants secreted by myxamoebae. Bonner et al. (3) and I started independently with different assays to isolate bacterial products. In other papers, we presented evidence that indeed both attractants are one and the same compound: adenosine-3′,5′-cyclic monophosphate (7, 10).

After aggregation had started in the responding myxamoebae population, the aggregate and the attractants secreted by the neighboring bacterial population competed for the still single amoebae; some of them yielded to the bacterial attractant, whereas other cells entered the aggregate. It has not been shown previously that attractants which enable myxamoebae to find their food source or initiate the social phase of their life cycle can reverse aggregation even when nearly all streams have already entered the aggregate. A compound necessary to bring cells together has the opposite effect of dispersing an advanced aggregate when present at a high concentration.

The *E. coli* attractant activated various strains of *D. discoideum* and other *Dictyostelium* and *Polysphondylium* species. Its presence in a variety of sources was shown by testing other gram-positive and gram-negative bacteria which all attracted myxamoebae. It remains to be shown that the attractant is identical in different species of bacteria. The production of one attractant common to all bacteria would secure a very effective food-seeking mechanism for myxamoebae. The fact that this bacterial attractant seems to

activate all species of slime molds, even though they themselves do not come together into common aggregates, remains to be explained.

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