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Evidence for a Second Chemotactic System in the Cellular Slime Mold, *Dictyostelium discoideum*

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An unknown substance found in bacteria (*Escherichia coli*) is especially effective in attracting the vegetative amoebae of the cellular slime mold, *Dictyostelium discoideum*. However, the aggregating amoebae are not attracted to it at all. On the other hand, the vegetative amoebae show very little chemotactic response to cyclic adenosine monophosphate (cyclic AMP), whereas the aggregating amoebae are exceptionally responsive to it. It is suggested that the new factor may be used in food seeking, whereas cyclic AMP, the chemotactic substance responsible for aggregation, is the acrasin of this species. The important point is that the amoebae are differentially stage-specific in their responses to these two chemotactic agents.

In some recent studies, we have shown that in one species of cellular slime mold, *Dictyostelium discoideum*, the chemotactic agent or acrasin responsible for bringing the amoebae together in aggregation is cyclic-3′,5′-adenosine monophosphate (cyclic AMP; 7-9). Furthermore, this acrasin is apparently produced in large quantities only during the aggregation stage (2), and the cells become especially sensitive to it at that stage (2, 5, 6).

It has also been known for some time that bacteria and bacterial extracts will attract amoebae (3, 6, 9, 13; T. M. Konijn, Ph.D. Thesis, University of Wisconsin, 1961). Since bacteria produce cyclic AMP (10) and cyclic AMP has some ability to attract vegetative amoebae (and also has a strong effect on aggregating amoebae), it was assumed that the amoebae used this substance, first for food location during the vegetative stage and then for aggregation during its developmental stage (2, 7-9).

The new results to be reported here show that there is an additional substance present in bacteria which is very much more effective in attracting vegetative amoebae than cyclic AMP. Furthermore, the amoebae completely lose their ability to respond to this second substance as they enter the aggregation stage, the very moment at which their sensitivity to cyclic AMP increases rapidly. Therefore we shall present evidence not only for a new chemotatic agent present in bacteria, but also for stage-specific responses to the two chemotactic agents.

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MATERIALS AND METHODS

These experiments were done with a haploid strain of stock number NC-4 of *D. discoideum*, kindly supplied by K. B. Raper. The bacterium used for the growth of the slime mold was *Escherichia coli* strain 281, and the extracts were obtained from *E. coli* strain B/r.

Cellophane square test. This test for chemotaxis was devised by Bonner, Kelso, and Gillmor (3). Amoebae were grown on buffered 1% peptone-dextrose agar with *E. coli* at 21 C in the dark. After approximately 40 hr of incubation, the vegetative amoebae were washed free of the surface and gently centrifuged three times in 1% salt solution (1). Washed cellophane squares (5 by 5 mm) were placed in the bottom of a small dish covered with 1% salt solution. The amoebae were allowed to settle on the cellophane squares to form a dense population (20 to 30 min). The squares were lifted with forceps, excess water was removed with filter paper, and the squares were placed on 2% agar (containing 0.5% salt solution) in small plastic petri dishes (50 by 12 mm). The test substance was mixed in with the molten agar, and the test was scored by recording the rate at which the cells moved off and away from the cellophane squares.

Time-lapse cinematography. A Wild-Nachet time-lapse instrument was used. Two speeds were employed, four frames/min and two frames/min. The light source was on only at the instant of exposure. All photography was done at 23 C, and control petri plates were placed on the camera stage to simulate the conditions of the filmed plate. The films were

analyzed by projecting them on paper and tracing the amoeba tracks with a pencil.

Bacterial extracts. (i) For the crude extract, *E. coli* B/r was grown in a liter of nutrient broth on a rotary shaker at 21 C for 48 hr. The bacteria were concentrated into pellets by centrifugation, resuspended in a liter of distilled water, and shaken for 3 hr (21 C). The bacteria were removed by centrifugation, and the supernatant fluid was boiled down to a volume of 50 ml. This concentrate was centrifuged at 20,000 × *g* to remove the debris, and the supernatant fluid was vacuum-evaporated to dryness in six tubes.

(ii) For the osmotic shock method, bacterial extracts were prepared by the method of Neu and Heppel (12) for *E. coli* B/r.

RESULTS

Evidence that crude bacterial extract contains a chemotactic agent in addition to cyclic AMP. When the cellophane square test was originally devised, there was some question as to whether it measured orientation of the amoebae or their rate of movement, or a combination of the two. It is shown here that it is primarily a test for orientation, and that rate of movement of the cells alters only at very high concentrations of bacterial extract. However, before the details of these experiments can be given, it is important to understand how the rate of outward movement of cells from the square can be an index of chemotactic orientation (*see* reference 2).

It has been known for some time that there is an acrasinase, an enzyme which inactivates acrasin (14, 16), and Chang (4) has recently shown that this enzyme is a specific phosphodiesterase for *D. discoideum*, converting cyclic-3′,5′-AMP to 5′-AMP. Since the amoebae give off acrasinase in their immediate vicinity, any cyclic AMP in that region would be eliminated. If there originally were a uniform concentration of cyclic AMP in the test agar, there would now be little or none surrounding the cellophane square, with the result that there would be an outward gradient of increasing cyclic AMP concentration as one proceeds away from the squares. It is presumed that this gradient is responsible for the orientation of the amoebae away from the square. The success of the test, then, is thought to be dependent on the cells possessing an enzyme which destroys the attractant, a property that may be important for chemotaxis during the normal development of the slime molds.

As a first check on the time-lapse film method, duplicate cellophane square tests were run both on film and by the standard laboratory procedure, by using an ocular micrometer. The control plates, the plates containing 1.5×10^{-5} M cyclic AMP, and the plates containing bacterial extract showed no statistical difference between the values obtained by the two methods. In other words, the filming operation did not in any way affect the results obtained with the cellophane square test.

The films were then analyzed for the rate of movement of the cells under the three conditions mentioned above. Forty amoebae were chosen at random. The mean rates of movement of the individual cells for the control, the cyclic AMP, and crude bacterial extract 1 are similar (Table 1); using the Student *t* test, there is no significant difference between them. However, a second crude bacterial extract was prepared (extract 2) which had a higher mean rate of cell movement significantly different from the other three mean rates at the 95% confidence level.

To measure chemotactic orientation, we used the McCutcheon (11) chemotactic ratio. This is the net distance away or towards a source over the total distance traveled by the cell. If the cells move directly away from the cellophane square in a straight line, they will be highly orientated and the chemotactic ratio will be near 1.0. If, on the other hand, they take a tortuous path and show very little net progress, then the ratio will be nearer 0.

Again the chemotactic ratios were calculated for 40 vegetative amoebae under each of the same conditions mentioned above, and it is clear from Table 1 that 1.5×10^{-5} M cyclic AMP shows significantly more orientation than the controls,

TABLE 1. *Effects of different experimental conditions on the rate of movement and the orientation of vegetative amoebae in the cellophane square test*[a]

Experimental conditions	Mean rate of cell movement ± SD (n = 40)	Mean chemotactic ratio ± SD (n = 40)	Mean score for cellophane square test ± SD (n = 6)
	mm/hr		*mm/hr*
Control	0.646 ± 0.119	0.135 ± 0.099	0.225 ± 0.014
Cyclic adenosine monophosphate (1.5×10^{-5} M)	0.664 ± 0.067	0.491 ± 0.104	0.433 ± 0.033
Crude bacterial extract 1	0.640 ± 0.080	0.838 ± 0.086	0.530 ± 0.035
Crude bacterial extract 2	0.804 ± 0.079	0.778 ± 0.085	0.818 ± 0.069

[a] Abbreviations: n, number of cases and SD, standard deviation.

and the crude bacterial extract shows especially strong orientation. Because the rate of cell movement is the same for the first three conditions (control, cyclic AMP, and bacterial extract 1), the values for the cellophane square test only reflect the degree of orientation or chemotaxis. Obviously, in some of the crude bacterial extracts (e.g., extract 2), there are also substances which affect the rate of movement, a matter that needs further investigation.

A whole range of different concentrations (10^{-4} to 10^{-10} M) of cyclic AMP have been tried on the cellophane square test, and there is a broad peak of maximal activity which extends from 10^{-5} to 10^{-8} M; i.e., the concentration used in Table 1 is within the optimal range. Since the bacterial extract gives very much higher values, it must contain some additional chemotactic agent other than cyclic AMP.

It is also possible to demonstrate the new chemotactic agent by a modification of the Konijn test (6–9; Konijn, Ph.D. Thesis, University of Wisconsin, 1961). This substance has the peculiarity of not being able to diffuse readily through agar; therefore, when dialyzed bacterial extract (i.e., extract presumably devoid of cyclic AMP) is put near small populations of vegetative amoebae on the Konijn test, there is no evidence of attraction. However, when the dialyzed bacterial extract is directly mixed with the hydrophobic agar, the positive orientation of the vegetative amoebae away from the original populations is striking.

Stage specificity in the response of the amoebae to the two different chemotactic agents. The Konijn assay is designed to test substances with amoebae which are ready to aggregate. One of the essential elements of the assay is a method of ensuring that the amoebae are at this stage: the vegetative amoebae on agar are kept in the dark for 3 to 4 hr at 21 C, overnight at 5 C, and then returned to room temperature the next morning after a 60-min transition period at 16 C. Konijn has shown that this routine insures that all the amoebae are at the right stage at the same time.

The cellophane square test was originally tried on amoebae of different ages, with no apparent difference in result, but from what we know now, we never before tried sensitive amoebae on the threshold of aggregation. To do this, the cellophane squares (on 2% agar), well covered with amoebae, were subjected to the Konijn temperature routine; the next morning, they were placed on test plates containing different concentrations of cyclic AMP. At the same time, cellophane squares containing fresh vegetative amoebae were placed on similar plates. The difference between the two was striking. At certain concentrations (especially 10^{-7} M cyclic AMP), the aggregating amoebae formed large waves of cells that moved outward at a rapid rate, often in a solid front, whereas this movement was never so marked with the vegetative amoebae (Fig. 1). The formation of a front of fused or adhesive cells appears to be a phenomenon especially associated with cyclic AMP and cells at the aggregating stage.

It is possible to measure the extent of the chemotactic response in the cellophane square test for a range of concentrations of cyclic AMP with vegetative and aggregating amoebae (Fig. 2). As previously mentioned, the vegetative cells show a small increase for a wide range of concentrations (approximately 10^{-8} to 10^{-5} M). In contrast, the aggregating cells show a much higher and sharper peak, which reaches its zenith at about 10^{-7} M cyclic AMP.

The same experiment was run with dialyzed bacterial extract, which was prepared by the osmotic shock method. This extract (which is free of cyclic AMP) was highly active with the vegetative amoebae, giving cellophane square test values varying from 0.56 to 0.96 mm/hr, depending on the character of the particular extract. When the dialyzed extract was used with aggregating amoebae, there was no effect at all; the amoebae did not leave the square, but simply proceeded to aggregate on the square.

Clearly then, the vegetative amoebae are strongly attracted to the second chemotactic agent in bacteria, but respond only weakly to cyclic AMP. In contrast, the aggregating amoebae respond strongly to cyclic AMP, but have no response to the second chemotactic factor (Table 2).

A few experiments were performed in which various concentrations of cyclic AMP were combined with the dialyzed extracts and placed with aggregating amoebae (Table 3). In the case of weak concentrations of cyclic AMP (10^{-9} and 10^{-8} M), there appears to be a definite synergistic effect for the combination of the small amount of cyclic AMP and the dialyzed bacterial extract, having a significantly greater effect than either one alone.

Evidence for an enzyme which inactivates the second bacterial chemotactic factor. As was pointed out earlier, the cellophane square test depends on enzymatic destruction of the chemotactic agent in the region of the square, so as to produce an outward gradient of the attractant.

To determine whether there was an enzyme for this new substance, amoebae were grown on autoclaved *E. coli* B/r by using Sussman's (15)

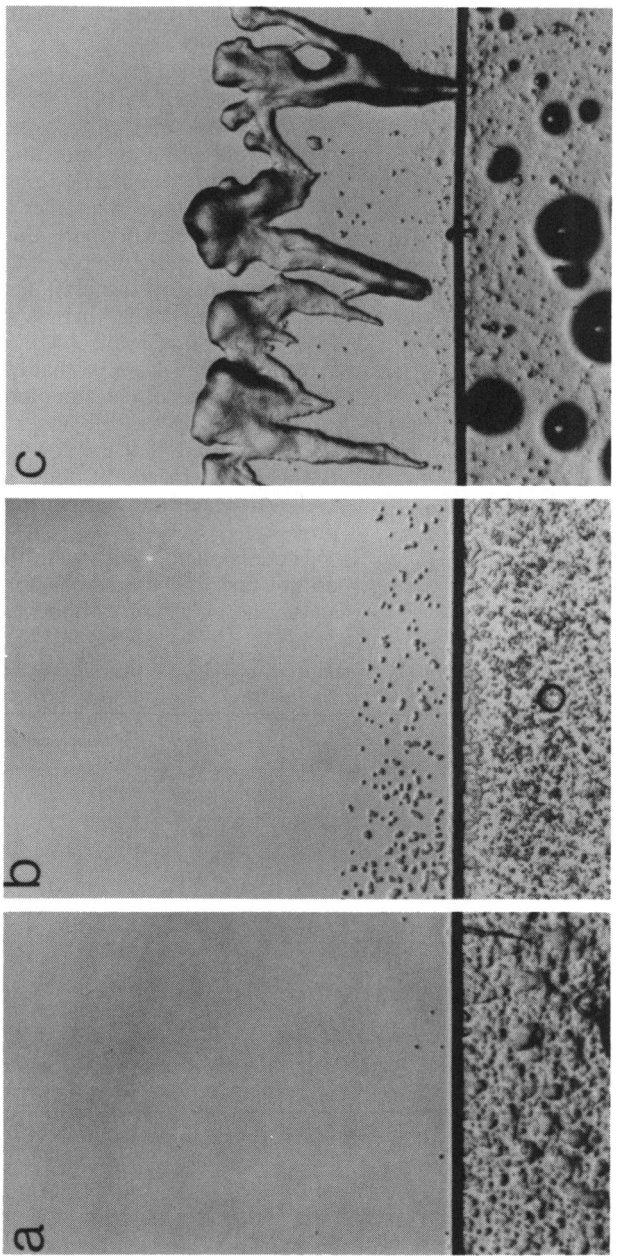

FIG. 1. *Effect of 10^{-6} M cyclic AMP on amoebae at different stages in the cellophane square test. The edge of the squares were photographed 2.25 hr after being placed on the agar. (a) Control with 1% salt solution alone, (b) vegetative amoebae, and (c) amoebae at the aggregation stage.*

liquid culture method. After growth, the amoebae were removed by centrifugation; the supernatant fluid was precipitated with ammonium sulfate, and the precipitate was resuspended and dialyzed.

When the dialyzed bacterial extract (osmotic shock method) was incubated at 30 C with this crude enzyme preparation (secreted by the amoebae in liquid culture), it lost all activity within 30 min (as measured by using vegetative amoebae in the cellophane square test). There is clearly an enzyme that inactivates the second chemotactic agent found in bacteria, a matter which is being pursued further.

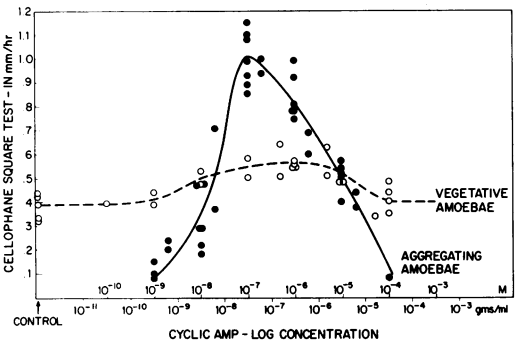

FIG. 2. *Values for the cellophane square test (ordinate) for different concentrations of cyclic AMP (abscissa) by using vegetative amoebae (○) and aggregation amoebae (●). In the control tests of the aggregating amoebae, no cells left the cellophane square; they all aggregated on the square.*

DISCUSSION

Perhaps the most important aspect of these experiments is the demonstration that there can be a switch in chemotactic response of the amoebae from one stage to another in the life cycle of *D. discoideum*. Furthermore, during the vegetative stage when bacteria are present, there is a normal abundance of the bacterial factor, and, during the aggregation stage, there is a great increase in the production of cyclic AMP, as was previously shown (2). We suggested that cyclic AMP is used both as a means of detecting food and as a means of bringing the cells together. Now we would rather postulate that the new bacterial substance is the prime means of food seeking, and that cyclic AMP acts only as acrasin in the aggregation process.

There is one difficulty with this hypothesis: we do not find that the new factor diffuses through agar. The only way to understand this problem

TABLE 2. *Comparison of the chemotactic response of vegetative and aggregating amoebae to cyclic adenosine monophosphate and the new bacterial factor (dialyzed bacterial extract)*

Experimental conditions	Cellophane square test	
	Vegetative amoebae	Aggregating amoebae
	mm/hr	*mm/hr*
Control	0.30 (n[a] = 13)	0[b]
Cyclic AMP (10^{-7} M)	0.56 (n = 4)	0.97 (n = 7)
Bacterial factor (dialized bacterial extract)	0.74 (n = 9)	0[b]

[a] Number of cases.
[b] If these figures were positive, they were not considered to be fully developed aggregating cells.

TABLE 3. *Comparison of the chemotactic response of aggregating amoebae in solutions containing both cyclic adenosine monophosphate and the new bacterial factor (dialyzed bacterial extract), and with each of the attractants alone*[a]

Experimental conditions	Cellophane square test with a cyclic AMP concn of				
	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M
Cyclic AMP alone	0.08 (n = 4)[b]	0.24 (n = 6)	0.97 (n = 7)	0.84 (n = 6)	0.47 (n = 5)
Dialyzed extract alone (n = 4)	0	0	0	0	0
Cyclic AMP and dialyzed bacterial extract	0.34 (n = 4)	0.46 (n = 4)	1.13 (n = 4)	0.83 (n = 3)	0.52 (n = 3)

[a] Controls with no attractant gave a value of 0.
[b] Values expressed as millimeter per hour; n = number of cases.

is to purify and hopefully to identify the substance, a program which is now under way. To mention some preliminary results from the chemical study, we know that the substance is heat-stable, nondialyzable, negatively charged, and inactivated by an enzyme given off by the amoebae. It is a substance specifically secreted by bacteria (and not, for instance, by the amoebae), and it can be separated by paper chromatography from cyclic AMP which is also given off by the bacteria. That it does not diffuse through agar may be insignificant in nature, where soil and humus are the normal substrates. The problem of food seeking invites further investigation, for it is even possible that both the bacterial cyclic AMP and this new factor are responsible for food location of the slime mold.

There is also the final intriguing point that suboptimal concentrations of cyclic AMP combined with the second bacterial factor produce a greater response in aggregating amoebae than either one alone. Again, this is a matter which can be effectively examined only when the chemical identity of the factor is known, but there are numerous interesting possibilities.

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