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INHIBITION OF AGGREGATION OF THE SLIME MOULD *DICTYOSTELIUM DISCOIDEUM* BY A FACTOR DIFFUSING FROM *ESCHERICHIA COLI*

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SUMMARY

A factor diffuses from *Escherichia coli* that temporarily or permanently prevents separate pre-aggregation cells of *Dictyostelium discoideum* from forming aggregation centres or streams. Centres are inhibited at a greater distance from the bacteria than are streams. The inhibitor does not support the slime-mould's growth, and it has different properties from previously discovered aggregation-centre inhibitors released by the amoebae themselves. The factor, as produced, does not inhibit *Polysphondylium violaceum* cells from forming centres or streams. Yet although *E. coli* itself attracts pre-aggregation *P. violaceum* cells, it does not mimic a *P. violaceum* aggregate in inducing them to form streams.

INTRODUCTION

The collective amoebae, or cellular slime moulds, have a solitary phase in which they feed and multiply, and a social phase, initiated some time after the food is exhausted, in which they aggregate and co-operate to build fruiting bodies. Aggregation involves positive chemotaxis in a gradient of a factor they secrete, which has been called acrasin (Bonner, 1947). This social chemotaxis has been investigated extensively (see Shaffer, 1962).

Separate slime-mould amoebae are also attracted towards their food. Arndt (1937) thought this was evident from his time-lapse film of these organisms, and Samuel (1961) plotted the paths of the individual amoebae and found that they were strongly oriented towards bacterial colonies. I had independently discovered (Shaffer, 1962) that the cells would collect on top of bacterial colonies when separated from them by a thin sheet of agar. The first clear evidence that chemotaxis was involved was provided by Wright and by Sussman, who each found that an extract of the food bacterium could orient slime-mould amoebae in the agar-block test, which had been designed to detect acrasin (Shaffer, 1956); this was reported in Shaffer (1962). The present work began as an investigation of the relationship between these two chemotaxes, and it has led to the discovery of an aggregation inhibitor. The recent observations of Bonner, Kelso & Gillmor (1966) will be considered in the discussion.

RESULTS

Dictyostelium discoideum

Escherichia coli, taken from plates on which it was growing, was deposited in shallow mounds about $\frac{1}{2}$ mm in diameter among pre-aggregation cells of *Dictyostelium discoideum* on non-nutrient agar plates. The result was the same whether or not there were any *discoideum* cells directly under the bacteria: cells within about $\frac{1}{2}$ mm of the bacterial heaps crawled towards them, so that in 15–30 min the periphery of each heap was occupied by a ring of cells.

If *E. coli* heaps were gently deposited close to continuous aggregation streams without mechanically disturbing them, they commonly had no effect on the aggregating cells. However, if there was a break in the stream, the upstream end might turn towards the bacteria, and the rest of the stream follow it. If the stream was disturbed, or part of it disintegrated into separate cells, more cells independently oriented towards the bacteria. Stream cells gave the greatest response to bacteria if a stream was first gently stirred to destroy its organization and its cells deposited within a few hundred micra of a bacterial heap and much further from any remaining aggregation centre or stream. Whether the original stream organization had broken down spontaneously or been broken down, if the resultant cells were at a sufficiently high density, they re-formed streams as they crawled towards the bacteria. Bonner *et al.* (1966) have obtained a similar stream-cell reaction to *E. coli* after removing the original aggregation centre. Streams and stream cells thus gave apparently the same response to bacteria as to slime-mould aggregates secreting acrasin (Shaffer, 1957). When they reached the edge of the bacterial heap, streams and short cell chains tended to crawl on into its interior, though they took an irregular course, presumably because their leading cells were not then receiving any further guidance. Separate cells or short chains of cells that had previously been attracted into a bacterial heap could emerge again, oriented towards an aggregation centre or slug deposited near it or towards the streams of a normal aggregation that happened spontaneously to extend into its vicinity.

In the foregoing experiments, cells crawling towards bacteria were never induced to form streams unless they had already been stream cells. It thus seemed unlikely that bacteria could stimulate the onset of true social aggregation in previously unaggregated cells. But to examine this possibility, some 200 heaps of *E. coli* were deposited among *discoideum* cells at various times ranging from 0 to 6 h before aggregation could be detected. Although cells accumulated around them, in no case did one of the heaps become the centre of an aggregation with streams leading towards it. It might have been, however, that the bacteria did provide a diffusible stimulus to the cells to enter the social phase, but that, before they had had time to respond to it, the cells made contact with the bacteria and were then induced to start feeding again. To test this, it was necessary to prevent the cells from reaching the bacteria.

Hot non-nutrient agar was poured into an optically flat Petri dish until the bottom was covered; almost all of it was immediately poured away; and the dish was left on a level bench until the residual agar set. This produced an agar sheet about 100 μ

thick. Pieces about 1 cm square cut from the sheet were then laid on top of pre-aggregation cells on non-nutrient agar. In sandwiches of this kind, *discoideum* cells aggregated much as they did on an ordinary culture plate, except that the streams tended to be more extensive and to persist longer. *E. coli* taken in the logarithmic growth phase from a nutrient agar plate was deposited on top of the agar rectangles in heaps 100–200 μ in diameter and 1–2 mm apart. Traces of nutrient in the agar were sufficient for the heaps to grow appreciably during the ensuing 24 h. Cells were attracted towards each heap; and although the densest accumulation was in fact directly underneath it, the most conspicuous increase in density was in the zone immediately surrounding each heap, because the cells were more clearly visible where they were not overlain by bacteria. When aggregation began, in the 'stippled' pattern typical of this species—in which the cells oriented before making much contact with one another—it was obvious that not only were there no aggregation centres in areas near the bacterial heaps, but that the rest of the aggregations also avoided these areas, the stippled streams sweeping around them. The compact, continuous streams that formed over the next few hours also invariably skirted the bacterial heaps and sometimes nearly encircled them. Thus the cells in the immediate vicinity of the bacteria were inhibited from forming streams and from relaying the centre's influence (Shaffer, 1958) to the cells beyond each heap on the side farther from the centre.

Very similar patterns are seen in an ordinary culture plate when the pre-aggregation cells are not homogeneously distributed and stippled aggregations develop in areas where small patches of the agar are almost devoid of cells. Despite their apparent dissimilarity, these bare patches and the cell accumulations produced by bacteria occupy equivalent positions in the resultant patterns, because they are both regions lacking cells able to form streams.

In the bacterial preparations, amoebae from the margins of the inhibited areas were slowly recruited over a period of hours into nearby streams, if there were any, and thus the accumulations induced by the bacteria gradually disappeared. In no case were true aggregations seen to form under the bacteria. Without plotting the movements of individual cells it was impossible to be certain whether the cells in the centre of the accumulations were directly attracted towards the existing streams, or whether they only wandered to the periphery of the accumulations and were attracted from there, but the observations made suggested that the cells were attracted at least from some distance inside the accumulations. This is not surprising, for many mutants, although unable to form true aggregations, are nevertheless attracted by acrasin (Sussman & Lee, 1955).

In an experiment in which observations were temporarily discontinued 9 h after aggregation had begun in the culture, very small compact aggregates were found under 7 of 68 bacterial heaps 18 h later. If these aggregates had in fact formed at these sites, at least some cells must have slowly overcome the inhibition, or the ageing bacteria must have become less inhibitory. However, it was quite possible that these aggregates had formed outside the bacterial areas and then been attracted into them. Certainly there were still at that time many lengths of stream crawling about within the sandwich layer.

It was thought desirable to find out how cells at an agar/air interface (as in an ordinary culture plate) would respond to bacteria, because, even in the absence of bacteria, cells form fewer aggregation centres if they are sandwiched between agar. However, it was not possible simply to reverse the positions of the slime mould and the bacteria in the foregoing experiment, because if the bacterial heaps were covered with a thin block of agar they dispersed in the sandwich layer. To prevent dispersal, the bacteria had to be grown within the agar, and they had to produce widely separated colonies about 100 μ from the surface.

To achieve this, 2% non-nutrient agar was poured into optically flat Petri dishes, 7 cm in diameter, to produce a layer about 100 μ thick, as previously described. The agar was flooded with a dilute suspension of *E. coli*, and this was immediately poured off again. The density of the bacterial suspension was adjusted until this procedure left about 1000 bacteria on each plate. The plates were left with their covers ajar until the residual water had evaporated. Two per cent agar containing 0·1% glucose and 0·1% peptone, buffered with 0·002 M phosphate at pH 6·5, was then poured on top of the non-nutrient agar and bacteria to produce plates of normal thickness. When this agar was set, the agar combination was turned out upside-down into 9 cm Petri dishes, using a mounted razor blade as a flexible spatula. The bacteria were now about 100 μ from the surface in contact with the air. After incubation at 37 °C for 24 h, during which time the bacteria produced colonies within the agar, the plates were returned to room temperature and the agar covered with a suspension of *discoideum* cells taken from 42-h growth plates (1% glucose, 1% peptone, 0·02 M phosphate buffer, 2% agar) freed from residual bacteria by centrifugation (Bonner, 1947), and resuspended at such densities that there were 250–1500 cells/mm^2 after they had settled on the agar. The water was drained off after 10 min, and the plates again left with their covers ajar until they were dry.

The cells soon formed dense accumulations on top of the bacterial colonies, and each colony was also surrounded by a halo of about twice its diameter in which cell density was considerably greater than in the rest of the culture. Stippled aggregations began to develop about 10–12 h after the cells were deposited. In these preparations, in which more centres developed than when the cells were covered with agar, it was more obvious that the centres were invariably sited well away from the bacteria (Figs. 1–4). Indeed, they were so often approximately midway between the nearest colonies on opposite sides of them as to suggest that the inhibitor acted at distances of more than a millimetre from its sources. The streams were shorter and often ended at the edge of an accumulation, but those that continued always skirted it (Figs. 1–4). The results were the same, except for the size of the aggregations, whether the cells were left to aggregate in light or darkness.

Fig. 1 shows moderately early aggregations in which many of the streams are still discontinuous or 'stippled'. The bacterial colonies are so dark that in most cases it is not easy to see that there are still large numbers of cells sitting on top of them. The streams extend nearly up to most of the colonies, but not above any of them. Haloes of separate unoriented cells are especially clear around the colonies on the left. The left half of Fig. 2 is the same field as the right half of Fig. 1 rotated anticlockwise

a quarter turn. The aggregations are a few hours older. With all the streams now continuous, it is perhaps more evident that they never lie on top of the bacteria. There are still quite a few separate amoebae above almost all the colonies, and especially those on the right. In the largest aggregation in Fig. 3 a stream almost encircles a colony; the stream directly above it in the photograph shows much the same relationship at an earlier stage. Another such stream is seen in Fig. 4. In Fig. 3 marked cell accumulations still exist above some colonies, and in Fig. 4 aggregation is inhibited completely in areas of heavy bacterial growth. Fig. 4 also clearly shows how centres tend to form at maximal distances from the surrounding colonies.

Cells in the accumulations were slowly recruited into neighbouring streams, as they were when in the sandwich layer; but in the present preparations, because the streams withdrew into the centres more quickly, a rather greater proportion of the cells was left behind when this had happened. Sometimes there were enough scattered cells between the colonies for a few small aggregates to develop there later; these too could recruit some of the residual cells from on top of the bacteria. But the cells that remained above the bacteria never aggregated, and after about a week, starvation had reduced many of them to dwarfs.

Aggregates could, however, develop directly above the bacteria (as well as elsewhere in the culture) in two circumstances: first, if the centrifuged cells were allowed while still in suspension to form rather large clumps that did not disperse when they settled on the agar, but eventually transformed themselves into true aggregates (which attracted other cells and developed into fruiting bodies); secondly, if older cells were used that had been aggregating when brought into suspension and that reaggregated almost immediately they were deposited on the agar.

The question then arose whether the amoebae were prevented from aggregating simply because they received sufficient nutrient to be kept in the growth phase. Certainly the nutrients in the agar had no appreciable effect, since centrifuged cells deposited on agars containing buffered 1% glucose and 1% peptone, buffered 0·1% glucose and 0·1% peptone, and solely Bonner's saline (Bonner, 1947), aggregated simultaneously. But did sufficient nutrient leak from the bacteria?

It was not possible to examine this question with the previous preparations because of the extensive changes in cell distribution resulting from both kinds of chemotaxis: to make meaningful population counts it was necessary to use preparations in which the bacteria were uniformly distributed and all the cells were inhibited. To achieve this, compound agar plates were prepared in the same way as before, except that the first layer of agar, 100 μ thick, was completely covered with a very dense bacterial suspension, which was not drained off, but left until the liquid had evaporated; and the second layer of agar, poured on top of the bacteria, contained ten times as much nutrient as before (1% glucose, 1% peptone, phosphate-buffered), so as to encourage vigorous bacterial growth within the agar. After 24-h incubation at 37 °C the plates were returned to room temperature, and centrifuged pre-aggregation cells deposited on them. Only those plates were used in which the myriad of separate bacterial colonies that had developed initially had fused to form a continuous sheet, for otherwise it was difficult to count the amoebae.

Whereas in the absence of bacteria, cells on this agar aggregated within about 12 h, in the presence of a bacterial layer most of the areas of the culture plates remained devoid of aggregations for as long as observations were continued, namely a week. The populations were measured at marked sites in the plates, using an ocular micrometer. The original densities ranged from 270 to 1050 cells/mm^2. After 96 h there had been in fact a slight decline: $12{\cdot}8 \pm 19{\cdot}6$ (S.D.)% (26 sites in 5 plates). Thus the bacteria did not inhibit aggregation by stimulating cell growth.

In some plates after several days, cells managed to gain access to the bacterial layer, usually entering at a point on the perimeter; these cells aggregated after consuming the bacteria locally available, and this allowed the previously inhibited cells directly above them to aggregate too. In addition, careful scanning of the plates revealed occasional small fruiting bodies even in areas where the underlying bacterial layer was intact; thus a small percentage of cells must have been able to escape from the inhibition.

Polysphondylium violaceum

On an ordinary agar culture plate, the pattern of aggregation in *Polysphondylium violaceum* is different from that in *D. discoideum*: instead of cells throughout the area occupied by an aggregation responding at about the same time, continuous streams gradually grow outwards from a point centre by recruiting cells that have previously been separate, and it takes several hours for the streams to reach their greatest extent (Fig. 5). Transfer experiments show that once aggregation has begun, amoebae from all parts of a homogeneous culture can be transformed into stream cells; which of them are induced to do so in any given interval depends on the proximity of centres or streams (Shaffer, 1958).

Small heaps of *E. coli* were placed in cultures of *P. violaceum* on non-nutrient agar at various distances beyond the outer ends of streams. In no case did they mimic the action of the streams in inducing the separate cells near them to form streams. Nor were cells from interrupted or dispersed streams found to re-form streams leading towards the bacteria, and in this they differed from *discoideum* stream cells. In preparations in which pre-aggregation *violaceum* cells were over-laid by a 100-μ thick agar sheet bearing small *E. coli* heaps, the bacteria had no detectable effect on the pattern of outgrowth of the very extensive stream systems that eventually developed in the sandwich layer.

In combined-agar preparations, in which the cells were on the surface of the agar and the bacterial colonies 100 μ below the surface, the pre-aggregation cells gathered above the bacteria, though to a smaller extent than did those of *D. discoideum*. Again stream outgrowth was unrelated to the position of the bacteria (Fig. 5). In these preparations the streams were shorter and the centres more numerous than when the cells were in the sandwich layer, and so it was easier to see whether the bacteria strongly inhibited centres from forming. In fact, centres appeared in all possible positions, and they could readily be found directly on top of a bacterial colony, or slightly to one side of it, but still within the cell accumulation it had induced (Fig. 5). Again there was no evidence that the colonies ever directly induced separate cells to

form streams leading towards them. In those cases where streams did converge on colonies, they always fed into ordinary centres, and these were formed by single cells in the normal way (Shaffer, 1961).

DISCUSSION

It has long been known that by continually transferring them to fresh supplies of food, slime-mould amoebae can be kept indefinitely in the solitary phase. If the nutrient is exhausted, aggregation follows after some hours—the precise length of the interval depending on the species and on various environmental conditions—and it has been generally assumed that it is simply the absence of food that initiates the biochemical change resulting in aggregation. In the present work *E. coli* has been found to release a diffusible factor that inhibits *D. discoideum* cells from entering the social phase: it prevents them not only from starting aggregation centres but even from being converted into stream cells and from propagating 'chemotactic pulses' (Shaffer, 1956, 1962). The centres are inhibited at much greater distances, and presumably at a much lower concentration, than are the streams. The inhibitor is not simply a supply of soluble nutrient sufficient to keep the cells in the growth phase: it does not support cell growth, and the amoebae may remain inhibited until they die of starvation. The factor does not inhibit centre or stream formation in *P. violaceum* in the conditions used. This difference agrees with what has previously been found to happen when cells from an aggregation are dispersed among bacteria: *D. discoideum* cells revert to feeding (Raper, 1940); *P. violaceum* reaggregates (Shaffer, 1961).

Slime-mould amoebae themselves are known to produce various inhibitory effects. Aggregates of *P. violaceum* release a diffusible factor that prevents cells from founding aggregation centres, but not from entering streams (Shaffer, 1961, 1963). Calculations based on Sussman and Noel's data (Shaffer, 1962) showed that in *D. discoideum* and another species, the number of aggregation centres was largely independent of cell density, which meant that here too one centre tended to inhibit the development of another one within a certain average distance of itself. Bonner & Dodd (1962) made further measurements on the spacing of centres, and Bonner & Hoffman (1963) discovered that all five species examined produced a gas that reduced the number of centres formed by three of them. In *D. mucoroides* it could prevent any centres from appearing. Although it is not clear whether it directly inhibits the cells from forming streams, it can hardly be the same factor as the bacterial inhibitor, because of the difference in species specificity: *P. violaceum* is sensitive to it, *D. discoideum* insensitive. There must also be a non-gaseous factor responsible for spacing centres, at least in the two species insensitive to the gaseous one; but this must presumably be produced by the centres themselves, and therefore cannot suppress all centres; nor does it appear to affect streaming. Thus it too can hardly be the same as the bacterial factor.

The relationship between the attraction exerted by the bacteria and by the amoebae is more obscure. Bacteria or a bacterial extract were known to attract *D. discoideum* cells, both those in the solitary phase and those from aggregation streams. Bonner *et al.* (1966), using a variant of the method for producing a stable acrasin extract from

D. discoideum aggregates (Shaffer, 1956), recently reported that preparations from non-aggregating as well as from aggregating cells of *D. discoideum*, from *P. violaceum* amoebae and from *E. coli* were indistinguishable in their multiple effects on test amoebae. These authors suggested that the same factor—acrasin—might be released by the amoebae of all species during both the solitary and social phases, and also by their food bacterium, and that if this were the case, the change from the solitary to the social phase would be due only to an increase in the amoebae's sensitivity to the orienting action of acrasin. However, it is not clear how such a scheme could explain the known patterns of aggregation. For example, at what moment during a period of several hours separate *P. violaceum* cells become stream cells depends only on their position in relation to the centre or existing streams. Thus we should apparently have to postulate the existence of a diffusible factor that increases the acrasin sensitivity of the still-separate amoebae, and is released first from the cell that founds the centre and then from the other cells as they join the aggregate. An additional hypothesis would seem necessary to explain why the separate cells, once rendered more sensitive, should not respond to the acrasin secretion of the separate cells around them and thus form clumps rather than a radial aggregation.

What might be another explanation of how acrasin could be present during the feeding and pre-aggregation stages without inducing aggregation (though again not in itself an explanation of the pattern of aggregation) would be the presence of an aggregation inhibitor in the food. At first sight, the *E. coli* factor seems to fill this role. However, as we have seen, this factor does not inhibit *P. violaceum*, at least in the concentration at which it occurs in cultures. Yet the bacteria have never been found to mimic an aggregate in inducing separate *violaceum* amoebae to form streams. Even when these cells are covered by thin agar, they do not join into streams converging on overlying bacteria. This is particularly significant because, in this species, an agar cover itself promotes the growth of streams, while partially, or in darkness completely, inhibiting the formation of ordinary centres (Shaffer, 1961).

What at least is clear is that in continuing the hunt for acrasin, it will be essential to try to obtain it from amoebae that have been grown not on bacteria but on a synthetic medium, just as Francis (1965) has done.

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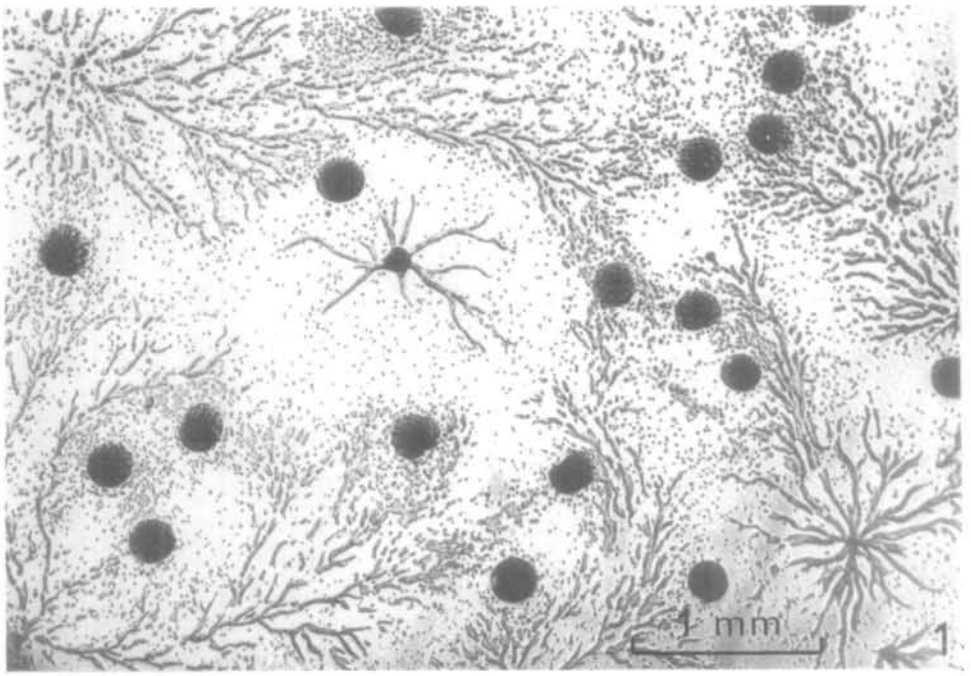
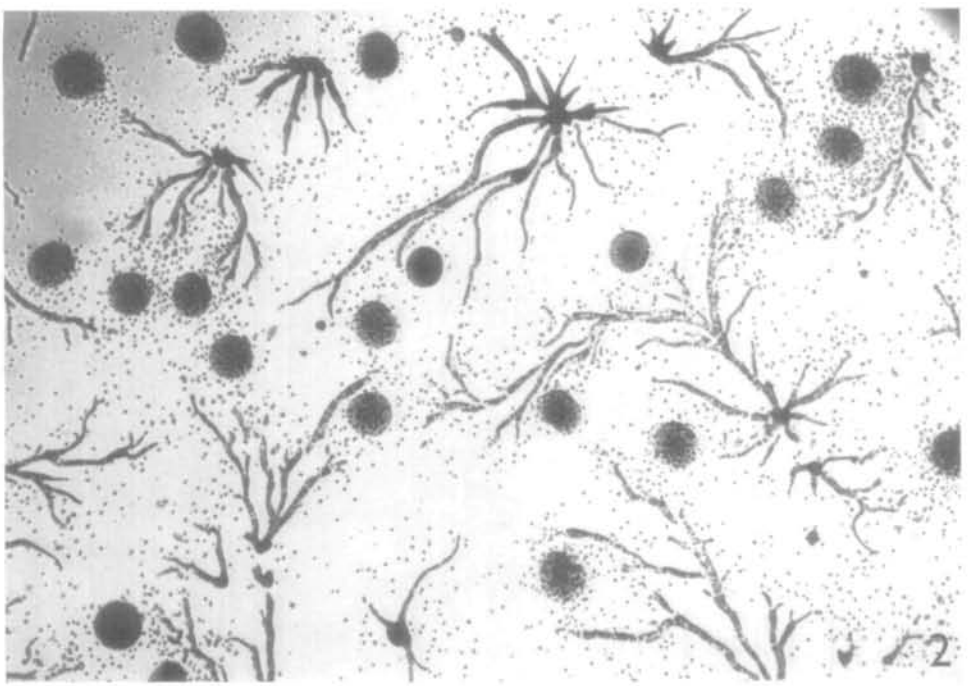
Fig. 1. *Dictyostelium discoideum* aggregating on the surface of a culture plate. The black circles are bacterial colonies about 100 μ below the agar surface. The aggregation centres are sited well away from them. The relatively early and still discontinuous streams extend up to and around the bacteria but not on top of them, where there are still accumulations of separate cells. These accumulations extend as haloes around some of the colonies.

Fig. 2. The left half is the same field as the right half of Fig. 1 rotated a quarter turn anticlockwise. A later stage of aggregation, showing that the continuous streams never pass over the bacteria. Magnification as in Fig. 1.



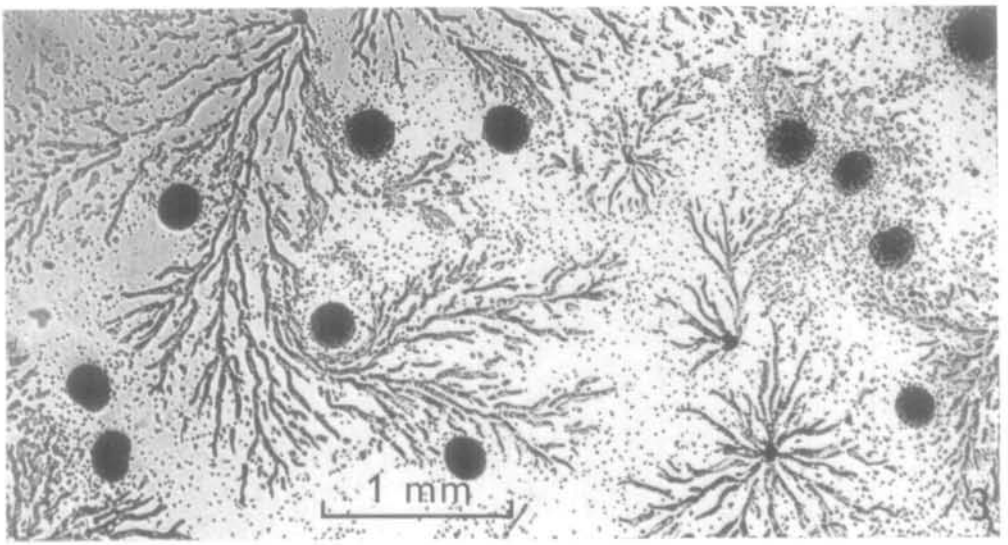


(*Facing p.* 400)

Fig. 3. *Dictyostelium discoideum*. One of the streams of the largest aggregation almost encircles one of the bacterial colonies. Dense cell accumulations are still associated with some of the colonies.

Fig. 4. *Dictyostelium discoideum*. The centres are sited at maximal distances from the nearest colonies, a tendency that can be seen also, though less obviously, in the preceding figures. Where there is heavy bacterial growth, there are many separate cells and aggregation is totally inhibited. One of the colonies is half encircled by a stream. Magnification as in Fig. 3.

Fig. 5. A similar culture of *Polysphondylium violaceum*. Aggregations are developing over the whole field, both where there are and where there are not bacteria. The centre of the aggregation on the right is sited directly above the edge of a colony. The streams of the aggregations at the top of the field have grown out from their centres unaffected by the position of the bacteria. Magnification as in Fig. 3.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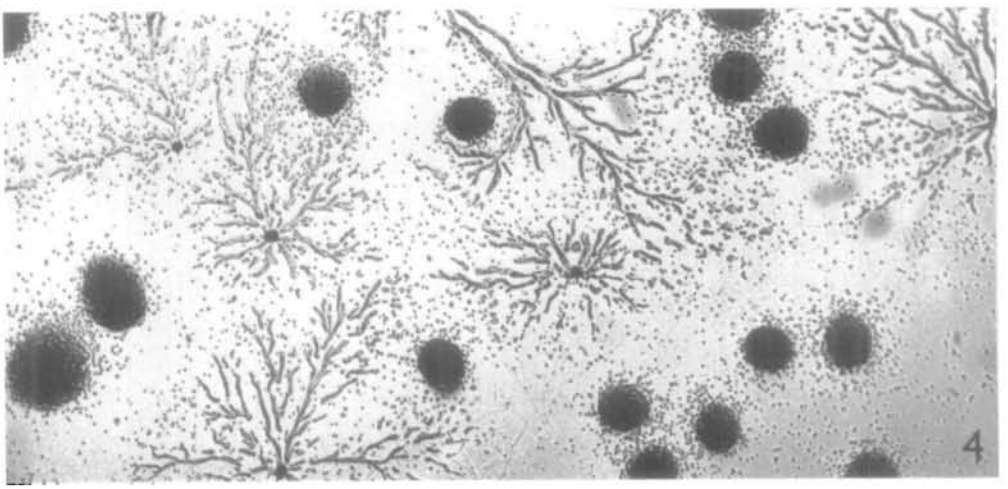
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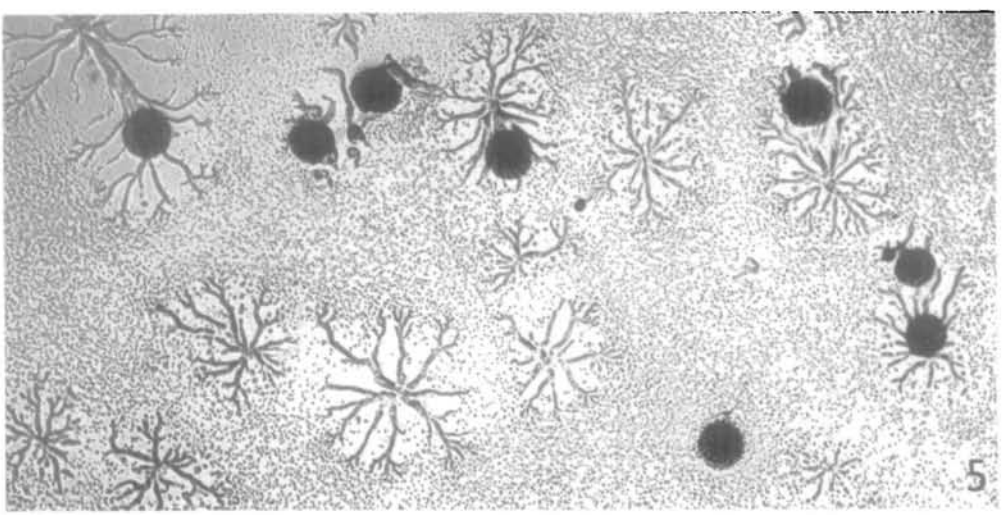
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