J. Cell Sci. **50**, 9–17 (1981)
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OXYGEN GRADIENTS CAUSE PATTERN ORIENTATION IN *DICTYOSTELIUM* CELL CLUMPS

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SUMMARY

We have investigated the formation of the prestalk–prespore pattern in *Dictyostelium discoideum*. Pattern formation occurs in clumps of *Dictyostelium* cells embedded in agar under a 100% oxygen atmosphere. Agar embedding allows us to control spatially the environment surrounding the cell clumps. Our results suggest that the ambient oxygen concentration plays a role in controlling the size of the multicellular mass. Further, oxygen gradients established across clumps embedded in agar or held in holes in a plastic barrier cause orientation of the prestalk–prespore pattern such that the anterior prestalk region forms at the highest end of the gradient. The results also indicate that developing cells have the ability to migrate up a gradient of oxygen.

INTRODUCTION

Slugs of the cellular slime mould, *Dictyostelium*, exhibit a characteristic pattern in which prestalk cells occupy approximately the anterior $\frac{1}{4}$ of the slug and prespore cells occupy the posterior $\frac{3}{4}$ of the slug. These cell types become the stalk cells and spores of the fruiting body. Formation of the pattern has been studied by several laboratories and possible pattern-formation mechanisms involving morphogenetic gradients (Durston, 1976), plasma membrane components (West & McMahon, 1979), and sorting-out (Bonner, 1959; Takeuchi, 1969; Matsukuma & Durston, 1979; Tasaka & Takeuchi, 1979) have been proposed. However, experimental evidence has not yet singled out any particular mechanism (MacWilliams & Bonner, 1979). A liquid culture system, which has great potential for furthering the investigation of pattern formation in *Dictyostelium*, has recently been devised (Sternfeld & Bonner, 1977; Sternfeld & David, 1979; Takeuchi, Hayashi & Tasaka, 1977; Forman & Garrod, 1977). It allows better control of the environment surrounding developing clumps of cells than is possible with slugs developing at an air–water interface. We have now modified the liquid culture system by embedding cell clumps in agar. In addition to controlling the composition of the environment, this technique permits us to manipulate clumps easily and to apply environmental stimuli locally or as gradients. Here we show that developing cells have the ability to sense a gradient of oxygen. The cells respond by moving up the gradient and the prestalk–prespore pattern can be oriented by the gradient.

MATERIALS AND METHODS

Cells of *Dictyostelium discoideum* (strain NC-4) were grown in liquid culture on live *Klebsiella pneumonia* (strain 29). Cells were harvested by low-speed centrifugation in 17 mM-sodium/potassium phosphate buffer during log phase or just as they cleared the bacterial suspension. The cells were stained with the vital dye neutral red after the first wash by adding 4 to 6 drops of a stock solution (0·2 mg/ml) of neutral red to 2 ml of the cell suspension. The suspension was quickly mixed and then immediately diluted with an additional 10 ml of buffer. The cells were washed twice more before being used. They were either used directly or placed on non-nutrient, unbuffered 2% agar to develop. The developing cells were re-harvested as they formed aggregation centres, or after they had formed migrating slugs, and mechanically dissociated before being used.

The basic technique for embedding *Dictyostelium* cells in agar involved putting cells into a well made in the agar and overlaying with more warm agar. One millilitre of 2% agar in Bonner's salt solution (BSS) (Bonner, 1947) was spread in a 60-mm Petri dish. After cooling, a second layer was spread on top of the first. Small wells about 0·5 mm in diameter, were made in the top layer by removing plugs of agar with a thin-walled capillary pipette. Growth-phase or developing cells were packed tightly by an additional 1 min, 2500 rev./min spin of the cell pellet from the last wash. Cells from the pellet were transferred to agar wells with a mouth pipette attached to a drawn-out Pasteur pipette. About 1×10^6 cells were put into each well. Then 0·8 ml of 1% agar in BSS was spread in the Petri dish to seal the cells in the well and the dish was exposed to a 100% oxygen atmosphere.

Oxygen gradients were established across clumps embedded in agar and also across clumps held in holes in a lucite barrier. We exposed clumps embedded in agar to an oxygen gradient by placing a glass coverslip on the top layer of agar before putting the Petri dish into an oxygen atmosphere. The coverslip was positioned such that the clumps were under the coverslip and about 0·5–1·0 mm from the edge. A gradient was established as the oxygen diffused under the glass. To establish an oxygen gradient across clumps in a lucite barrier, slug cells, from the tightly packed centrifuge pellet, were placed in the holes (0·3 mm in diameter) in a sheet of lucite (1·0 mm thick). Only the centre $\frac{1}{3}$ of the holes was filled. The barrier was then immersed in BSS, trapped air bubbles were removed and the barrier was mounted between 2 chambers each containing BSS. Oxygen was bubbled into one chamber and air into the second. After 2 h the barrier was removed, the clumps marked at one end with carbon particles, and popped out with a stream of water. The patterns were then observed.

Prestalk and prespore tissues were identified by neutral red staining. Neutral red has been recently shown to be a stable cell marker (Bulychev, Trouet & Tulkens, 1978; Sternfeld & David, unpublished data), which is specific for cells with large autophagic vacuoles such as prestalk cells (Sternfeld & David, unpublished data; Quivger, Benichou & Ryter, 1980). Estimates of the proportions of prestalk and prespore tissue were made by visual inspection. This procedure proved sufficiently accurate to document the very large differences in the prestalk–prespore proportions reported here.

RESULTS AND DISCUSSION

Development of clumps embedded in agar

The time course of development of cells embedded in agar is the same as for cells at an air–water interface. If the growth-phase cells are put into agar wells, the cells aggregate into several separate clumps after about 12 h. These separate clumps then fuse into one large clump of cells and a single prestalk–prespore pattern forms after about 15–16 h of culture. The pattern is visualized by neutral red, which stains the prestalk region darkly and the prespore region lightly (Bonner, 1952). This time course is shortened if cells from later stages are put into the wells. A staining pattern appears after 7–8 h if starved cells are used and after 1·5–2 h if dissociated slug cells are put

into the wells. Differentiation of mature stalk cells and spores occurs after about 24 h in clumps embedded in agar. However, for the experiments described here, we delayed terminal differentiation, which disrupts the prestalk–prespore pattern, by including 2·7 mM-calcium in the medium (Sternfeld & David, 1979). Under these conditions, the prestalk–prespore pattern persists for more than 24 h (Fig. 1).

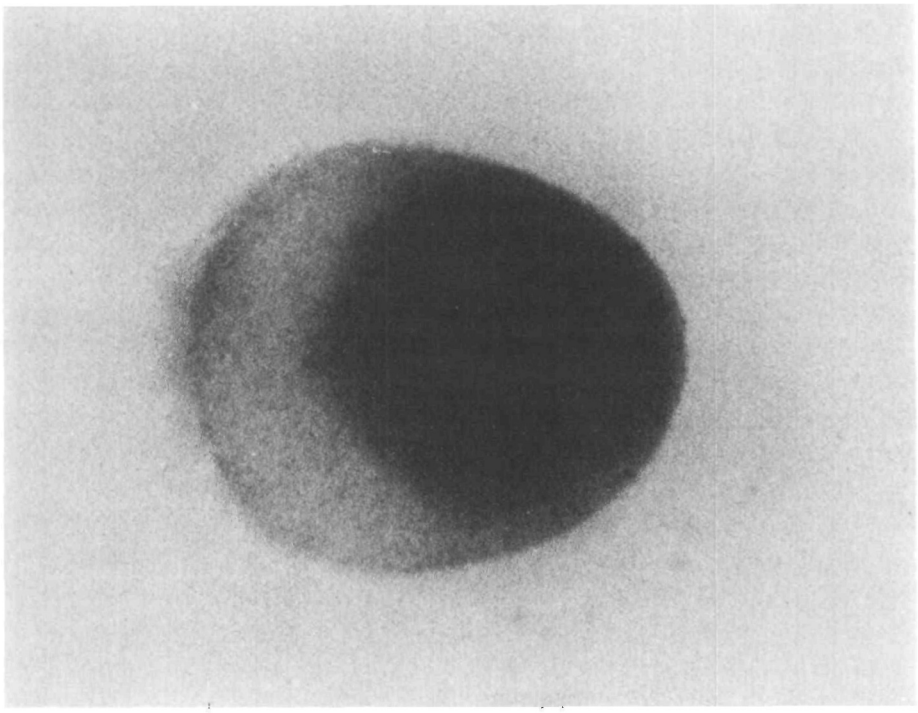

Fig. 1. A clump of developing *Dictyostelium* cells embedded in agar. Cells were harvested after 6 h of starvation, stained with neutral red, washed, and embedded in agar as described in the text. The cells were maintained in a 100 % oxygen atmosphere for 16 h. The prestalk cells stain darkly and the prespore cells lightly. The clump is about 0·4 mm in diameter and contains about 100000 cells. The prestalk/prespore ratio is about 60 %/40 %.

By making drawings of the clumps at intervals with a *camera lucida* the pattern is seen to move around inside the clumps. That this is due to actual movement of the cells and not to a continual repatterning is demonstrated by injecting into the clumps a small number of cells stained with a second vital dye (Bismarck Brown). The injected cells move with the pattern. Movement of the cells is also observed when cell debris and slime sheath material is extruded and left to collect at one point on the surface of the clump. In effect, the embedded clumps appear to form slugs which crawl around over themselves leaving slime sheath and cell debris behind.

Although the timing of development in embedded clumps under 100 % oxygen is normal, the proportion of prestalk tissue is markedly increased in clumps compared to that in slugs. Submerged clumps have a 60 % prestalk/40 % prespore ratio (Fig. 1)

compared to a 25% prestalk/75% prespore ratio in slugs. Furthermore, when slug tissue is dissociated and the cells used to form submerged clumps the prestalk proportion increases from 25% to 60% over a period of several hours. This change in proportion, documented here by neutral red staining, has also been observed in submerged clumps by Tasaka & Takeuchi (1979) using antibody to prespore cells to distinguish prestalk and prespore cells.

Two observations suggest that the altered proportions are not due to differences in oxygen concentration between slugs on agar and submerged clumps. (1) The 25%–75% prestalk–prespore pattern in slugs is unaffected by oxygen concentrations of 21% (air), 50% or 100%; and (2) the 60%–40% pattern in embedded clumps is unaffected by all oxygen concentrations (40–100%) that allow formation of a pattern (see below). The observation of an enlarged prestalk region can be explained, however, if the proportion of prestalk to prespore tissue is under the control of an endogeneous diffusible factor. For example, if the prestalk cells secrete a diffusible inhibitor of prestalk differentiation, loss of the factor would be greater in clumps completely surrounded by medium than in slugs lying on a surface. Greater loss of the inhibitor would allow more prestalk cells to differentiate.

Oxygen concentration affects clump size and morphology

While the prestalk/prespore ratio is not affected by oxygen concentration, the size and morphology of clumps formed by cells embedded in agar is strongly oxygen-dependent. An atmosphere of 60% oxygen or more above the agar produces a single large clump per well (100000 cells, 0·4 mm in diameter). Under a 50% oxygen atmosphere some clumps break up into smaller clumps and under a 40% oxygen atmosphere all of the clumps separate into smaller units each of which may have two or more prestalk regions. Despite being smaller, these clumps still exhibit the altered 60%/40% prestalk/prespore ratio. At even lower oxygen concentrations, clumps break up and many of the cells leave the wells by migrating out between the agar layers. These results suggest that, when the oxygen concentration in the atmosphere above the agar is less than 50%, oxygen becomes limiting for the maintenance of a single large clump per well. Presumably, under these conditions, the cells at the surface of the clump are consuming the oxygen as rapidly as it diffuses through the medium. This leaves the cells at the centre of the clump anoxic and incapable of movement. As the oxygenated cells continue to move around inside the well they leave behind the cells in the centre just like slime sheath material. Clearly, as this occurs the immobilized cells become oxygenated themselves and are able to form a separate unit. Under extreme oxygen deprivation multicellular units cannot form at all and only as single cells can migration continue.

To gain a better understanding of this oxygen dependence we calculated the oxygen concentration gradient within and around clumps embedded in agar under varying oxygen concentrations (Fig. 2). The details of the calculations are given in the legend to Fig. 2. Because the calculations assume an idealized geometry, which does not precisely match our experimental conditions, the curves should be taken only as estimates of the actual oxygen profiles. Nevertheless, they clearly suggest that a clump

under a 50% oxygen atmosphere is oxygenated whereas a large portion of the clump under 40% oxygen is oxygen-depleted. The calculations are thus consistent with the observations that, when oxygen becomes depleted, clumps break up and the cells migrate out of the wells. Further, the curves in Fig. 2 indicate that the concentration

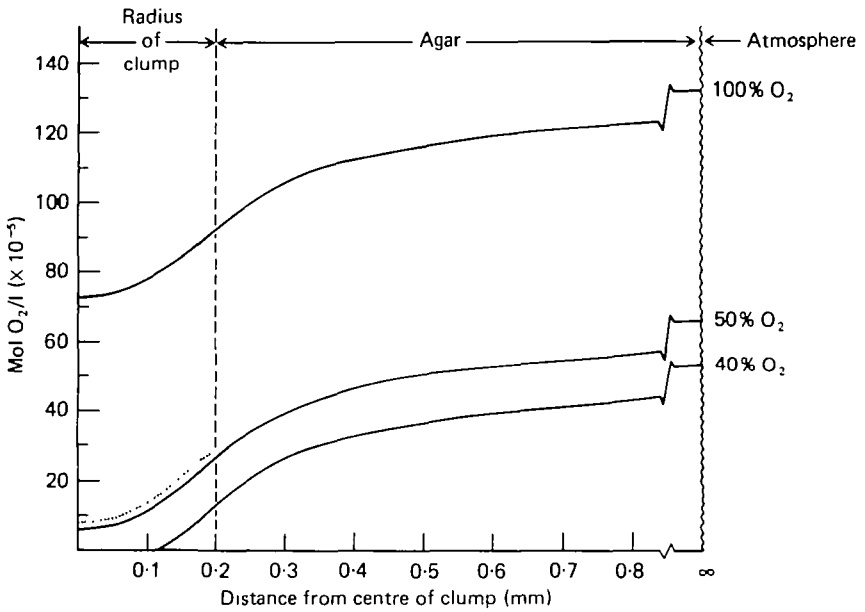

Fig. 2. Oxygen concentration gradients within and around a clump embedded in agar. The curves shown are oxygen profiles through the agar and along the radius of a clump under atmospheres of 40, 50 and 100% oxygen. The broken line represents the oxygen profile within a spherically shaped aggregate at an air–water interface under an air atmosphere (21% oxygen). The oxygen profiles were calculated using equations provided by Boag (1969). The calculations are dependent on the number of cells in the clump, the size of the clump, the rate of consumption of oxygen, and the oxygen concentration at an infinite distance. Typical clumps contain 1×10^5 cells and have a radius of 0·2 mm. (These dimensions were also used for calculation of the oxygen profile of the aggregate at an air–water interface.) *Dictyostelium* cells consume oxygen (as measured with an O_2 electrode in well-stirred suspensions) at a constant rate of 2×10^{-17} mol/s per cell down to 4×10^{-5} M. The curves shown should be taken as lower estimates of the actual oxygen profiles because the equations assume an infinite agar reservoir while the agar around the clumps in our experiments is only about 0·8 mm deep.

of oxygen in an aggregate at an air–water interface is similar to the oxygen concentration within an embedded clump under a 50% oxygen atmosphere. The experimental observations above and these calculations provide an explanation for previous observations that high levels of oxygen are required for development in submerged clumps of cells (Sternfeld & Bonner, 1977). In addition they imply that oxygen concentration can control the size of the multicellular mass that forms a prestalk–prespore pattern. Thus it is possible that oxygen plays a role in size regulation.

Oxygen gradients can polarize the prestalk–prespore pattern

The calculations above suggest the presence of oxygen gradients within submerged clumps and aggregates at an air–water interface. To test whether such gradients play a role in establishing the polarity of the prestalk–prespore pattern, clumps were exposed to an exogeneous oxygen gradient. This is accomplished by placing a coverslip over

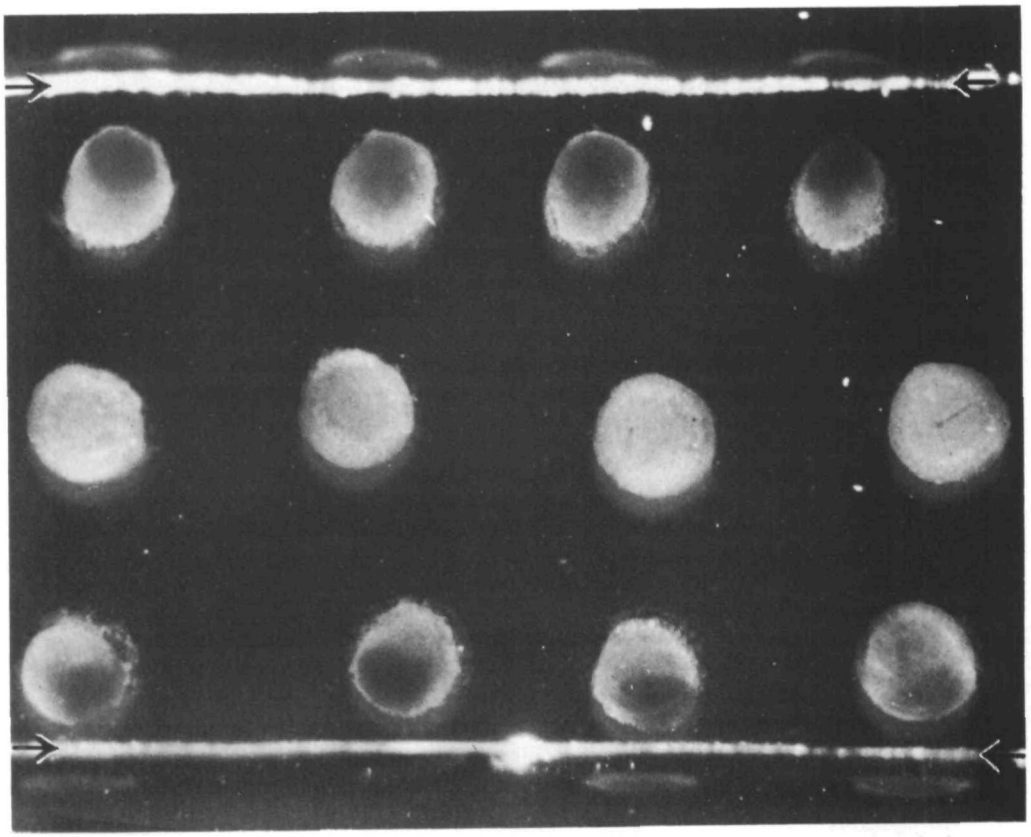

Fig. 3. Orientation of prestalk-prespore patterns. Three rows of wells were made in a thin layer of agar. Cells dissociated from neutral red stained slugs were put into each well. After sealing the wells with warm agar a narrow piece of coverslip was placed over the wells such that the edges (arrows) of the coverslip were near the outside rows of wells. This photograph was taken after 2 h when oriented patterns began to appear in the wells near the coverslip edges. Clumps in the centre row of wells did not form patterns because of insufficient oxygen (see text). The clumps are about 0·3 mm in diameter.

clumps embedded in agar before exposing the Petri dish to oxygen. Then, when the dish is put into an oxygen atmosphere, an oxygen gradient is established as oxygen diffuses under the coverslip. Because dissociated slug cells form a pattern rapidly, observations are made on clumps of these cells near the edge of the coverslip where the gradient is first established. Within 2 h clumps near the coverslip edge begin to form patterns and the patterns are uniformly oriented such that the prestalk cells

appear at the higher oxygen concentration (Fig. 3). Clumps at a greater distance from the coverslip edge do not form patterns.

The above results suggest that an oxygen gradient orients the prestalk–prespore pattern. However, there is an alternative explanation: the prestalk cells might be moving away from a volatile metabolic product. The lowest concentration of such a substance would be at the coverslip edge. To test this possiblity we established an oxygen gradient using an entirely different experimental design. We placed slug cells in holes in a lucite barrier mounted between 2 chambers containing BSS. Any metabolic product would be lost by both ends of the clumps equally. When oxygen is bubbled in one chamber and air (or a lower oxygen concentration) in the other, the clumps form patterns oriented in only one direction; the prestalk cells are located on the oxygen side (Table 1).

Table 1. *Pattern orientation in a lucite barrier*

Position of prestalk cells	No. of clumps
At 100 % O_2 chamber	38
At air chamber	1
At centre	2

The position of the stained prestalk cells relative to the unstained prespore cells was scored in clumps mounted in a lucite barrier. Dissociated slug cells stained with neutral red were placed in a lucite barrier and the barrier was inserted between 2 chambers containing BSS. One chamber was equilibrated with 100 % oxygen (100 % O_2 chamber) and the other with air (air chamber). After 2 h carbon particles were used to mark one end of the clumps and they popped out of the barrier with a stream of water. Clumps were examined to determine the orientation of the prestalk–prespore pattern. Results are shown for clumps that formed a single pattern. Large clumps, which invariably separated into 2 clumps, one at each end with a mass of dead cells in the centre, were not scored. Small clumps occasionally formed with the prestalk cells in the centre of the clump. Presumably this condition is favoured when the clump does not completely plug the hole and the oxygenated salt solution can leak around the clump.

A second effect of oxygen gradients is seen when the concentration of the agar used to embed clumps is reduced from 1 % to 0·5 %. If slug cells are used, more than half of the clumps, after they have formed an oriented pattern, are actually able to move through the agar. The clumps invariably move toward the coverslip edge and, if they reach the air–agar interface (a distance of 0·5–1·0 mm), they form slugs and fruiting bodies. If, under these conditions, aggregation-stage cells are put into the wells, most of the cells do not form clumps, but stream out of the wells toward the coverslip edge (Fig. 4). This response is not due to the cells moving away from a volatile metabolic product since clumps placed in lucite barriers crawl out of the holes on the oxygen side after 4–5 h.

These results clearly demonstrate that developing *Dictyostelium* cells exhibit a chemotactic response to oxygen. This may be another mechanism, in addition to the cells' sensitivity to light and heat (Bonner, Clarke, Neely, & Slifkin 1950; Poff & Loomis, 1973; Whitaker & Poff, 1980), that the slug can use to move out of the soil

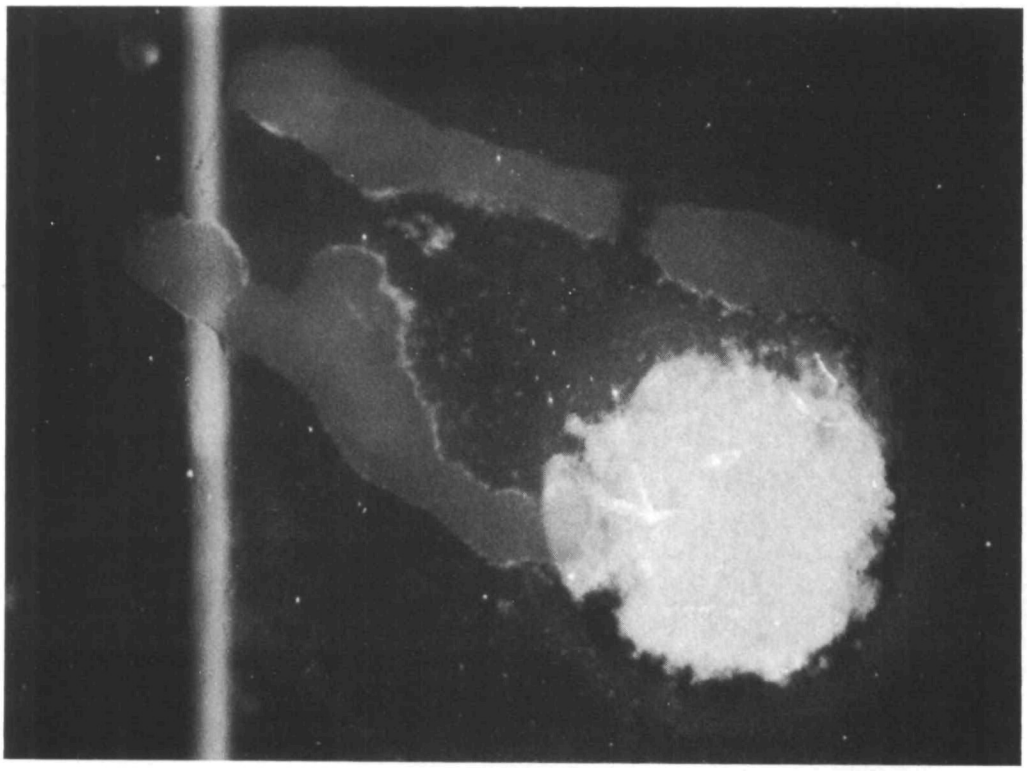

Fig. 4. Movement of aggregation-stage cells up an oxygen gradient. Cells harvested during aggregation on non-nutrient agar were placed in agar wells, sealed with 0·5 % agar and covered with a glass coverslip. The edge of the coverslip is to the left of the photograph. Cells can be seen streaming out of the well (which appears as a white disk) in 2 fingers toward the edge of the coverslip. The well is 0·5 mm in diameter. The photograph was taken after about 4 h. Depending on their position on the surface of clumps some stained regions appear larger than others.

so that fruiting can take place on the surface. Further, since (1) our results indicate that oxygen gradients can orient the prestalk–prespore pattern in clumps (Fig. 3); and (2) diffusion calculations suggest the presence of endogeneous oxygen gradients in aggregates at an air–water interface (Fig. 2), it appears likely that oxygen controls the polarity of the prestalk–prespore pattern during normal development. This suggestion is in agreement with early observations of Gerisch (1968) that an air–water interface can polarize the prestalk–prespore pattern in aggregates. In summary, it is interesting to note that oxygen has also been shown to polarize hydranth regeneration in *Tubularia* (Miller, 1937) and that physiological gradients generally have been hypothesized to play a role in morphogenesis (Child, 1941).

We thank J. T. Bonner, S. C. Kayman, H. K. MacWilliams, P. M. Silverman and C. D. Town for helpful discussions and critical reading of the manuscript. The research was supported by grants from the NIH (HD07154-02) and the NSF (77-25426). C.N.D. is a recipient of a Career Development Award (FRA-132) from the American Cancer Society.

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(*Received* 3 *November* 1980)