

# A way of following individual cells in the migrating slugs of *Dictyostelium discoideum*

(cellular slime mold/prestalk/prespore/cAMP/ammonia)

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**ABSTRACT** In the development of the cellular slime mold *Dictyostelium discoideum* there is a stage in which the aggregated amoebae form a migrating slug that moves forward in a polar fashion, showing sensitive orientation to environmental cues, as well as early signs of differentiation into anterior prestalk and posterior prespore cells. Heretofore it has been difficult to follow the movement of the individual cells within the slug, but a new method is described in which small, flat (one cell thick) slugs are produced in a glass-mineral oil interface where one can follow the movement of all the cells. Observations of time-lapse videos reveal the following facts about slug migration: (i) While the posterior cells move straight forward, the anterior cells swirl about rapidly in a chaotic fashion. (ii) Turning involves shifting the high point of these hyperactive cells. (iii) Both the anterior and the posterior cells move forward on their own power as the slug moves forward. (iv) There are no visible regular oscillations within the slug. (v) The number of prestalk and prespore cells is proportional for a range of sizes of these mini-slugs. All of these observations on thin slugs are consistent with what one finds in normal, three-dimensional slugs.

In the cellular slime molds the separate amoebae feed on bacteria in the soil, and once the food supply is finished they come together by aggregation to form a multicellular organism that crawls about for a period, finally producing a small fruiting body in which the anterior cells die during the process of forming the stalk, and the posterior cells become encapsulated in hard cellulose cases. In *Dictyostelium discoideum*, the species used in this study, it is well known that during the crawling, or migration stage, the naked slug has a polarity, and as it moves it is extraordinarily sensitive to environmental stimuli; for instance it will go toward light, responding to very small differences in intensity, to minute heat gradients, and to small gradients of ammonia gas that is produced by the cell mass. All these attributes presumably serve to place the final fruiting body in the optimal place for spore dispersal.

Although we have accumulated much information on the nature of this mass movement of cells over the last few decades, as well as a better understanding of the factors that lead to the anterior cells beginning their path of differentiation into stalk cells, and the posterior cells into spores (more on this as I proceed), we have had difficulty in seeing inside the crawling slug to describe the details of the internal movement, simply because the slug is thick and made up of thousands of cells. However, by using various clever devices such as marking some individual cells and by using special microscopic techniques, for example, the confocal microscope, it has been possible to gain some insight of what goes on inside the cell mass.

The first attempt to see the cells directly was only partially successful (1). If the cells from a slug are drawn up into a small glass capillary (with an inside diameter of anywhere between

20 and 170  $\mu\text{m}$ ), and the capillary is placed under mineral oil under a coverslip, with an air bubble abutting against the cells at one end of the capillary, and oil at the other, the optical properties of such preparations make it possible to see the cells clearly and follow and record their motions by using video microscopy. What happens is that the cells near the air bubble are, within a minute, marked off by a sharp division line, and those cells undergo rapid churning movements, after irregular circular and helical courses. Using alkaline phosphatase expression and green fluorescent protein fused to the promoter of a gene that is known to produce a protein found in the anterior prestalk region, we were able to show that these anterior cells behave, at least in some respects, like normal prestalk cells. However, in subsequent work we found that the posterior cells did not have prespore characteristics; rather they seemed to simply be inactive. Since then we have shown (2) that this rapid appearance of prestalk cells in the capillaries is oxygen dependent, and we have evidence that the division line is due to a threshold in oxygen concentration [see also Sawada *et al.* (3) for complementary observations]. The other drawback of these capillary experiments for insights into normal slug behavior, is that the cell mass in the capillary does not move forward, but remains stationary.

Quite by accident I found a way of producing very small two-dimensional (2-D) slugs that are pressed flat between a glass slide and a drop of mineral oil. They are only one cell layer thick, so that it is possible to see all the cells and record them on time-lapse video. Unfortunately they only occur occasionally, but I have recorded enough cases for this initial study.

They will be described in some detail below, but let me say briefly, that (i) they have an antero-posterior polarity, (ii) they appear to exude a slime sheath, (iii) they move forward, and as they do it is possible to observe the movement of the individual cells in all parts of the 2-D slug in detail, (iv) they can split in two, or twin, (v) they show two clear zones that would appear to reflect the early differentiation into prestalk and prespore cells, (vi) they will migrate from about 2 to 6 hr (and in one instance for 24 hr), and (vii) they occasionally form flat fruiting bodies with mature stalk cells and spores, but in forming the stalk they get thicker near the tip and it is impossible to see the individual cells.

**How 2-D Slugs Are Formed.** Cells from a migrating slug or late aggregate of *D. discoideum* (strain NC-4) are sucked up (using a mouth pipette) into a hand drawn capillary whose internal diameter may vary from about 30–120  $\mu\text{m}$ . The tip of the capillary is immediately placed in a drop of mineral oil on a glass slide, and with a small spear broken up into two to four segments, and then covered with a coverslip. For best results it has been found helpful to place the slide at 12°C overnight before observing it at room temperature. A 2-D slug begins as a small group of active cells with prestalk characteristics that emerges from a broken capillary fragment. They sometimes initially move directly up into the oil as minute three dimen-

sional (3-D) slugs. Right from the beginning of their formation they show two zones: a small, very active anterior zone, and a passive posterior zone. In these minute 3-D slugs it is easy to see a definite slime sheath that collapses behind the slug as it moves forward, much the same as one sees in normal 3-D slugs on an air-agar surface.

At some point, either right from the beginning, or after some of the 3-D migration just described, the cell mass will touch the oil-glass interface and as it progresses it spreads into a flat sheet of cells (Fig. 1). This 2-D slug moves forward and continues to show the two zones. A sheath appears to surround the entire slug, although it is impossible to know if this is a genuine slime sheath or just a band of water between the glass and the oil. It is formed by the active cells wandering out from the leading edge of the slug. (In one case the slug circled around and ran into its previously deposited sheath, and the two sheaths immediately dissolved at the juncture. Still, this does not tell us the composition of the line that is so clearly visible.)

In what follows I will present the new insights gained from these 2-D slugs in the form of a series of questions. Each of these questions will center around an important unresolved issue from past work concerning slug movement and differentiation.

**Does the Slug Tip Consist of a Group of Special Cells?** It has been recognized since the early work of Raper (4) that the tip of the slug has special properties in that it determines the direction of movement of the slug and it has the power of entraining cells to follow it. Because of these features it has often been referred to as an "organizer" region (5) in that it bears some similarities to dorsal lip of the blastopore in amphibian embryos (6). Heretofore it has been impossible to know the cellular composition of this tip region—is there a keystone cell at the very tip, or a special group of cells that are a group of "directors"? I have always assumed one of these, but the actual state of affairs turns out to be quite different.

All the cells of the anterior zone are highly active in their locomotion, and the cells at the very tip are constantly replacing one another; they seem to take turns being at the front end (Fig. 2). It would appear that the tip is a region, an area, with a constantly changing cell population; the region itself must have special properties that no doubt are produced by the itinerant cells. Later in the discussion I will consider what those special properties might be.

Siegert and Weijer and their colleagues (7–9) have made the interesting discovery of the helical movement of cells in the prestalk region of normal slugs, while the cells in the posterior, prespore region move directly forward. These anterior movements can occasionally be seen beautifully in small 3-D slugs

in oil examined in this study. The anterior cells will move rapidly in a helical fashion for some periods of time. They will sometimes reverse their direction, and sometimes the movement in the tips of these mini-3-D slugs will appear quite chaotic, similar to what one invariably finds in 2-D slugs. The fact that, for simple mechanical reasons, one never sees helical movement in 2-D slugs would indicate that such movement is not required to guide a slug's motion.

Because in these minute 3-D slugs one cannot follow the individual cells clearly, one could reasonably assume that the cells at the very tip are not a fixed group, but constantly changing. This postulate is reinforced by the observation that the motion sometimes becomes periodically chaotic.

Occasionally the anterior zone of a 2-D slug is transformed into a whirlpool pattern: one great flat spiral—a headless mass of amoebae chasing their tails. When this occurs all anterior forward motion of the entire slug ceases and the front end remains fixed in one spot, while the posterior cells continue to follow the cells in front of them. If one places a group of amoebae from a slug or an aggregation mound directly on the glass slide under the mineral oil, the cell mass never forms a slug but often one of these whirlpools. This is similar to the formation of rings of cells that occur on the surface of agar where the cells just follow one another for long periods of time as Shaffer (10) and others have shown. In all these instances there is an absence of a tip, a dominant organizing center.

**Does Turning Involve Differences in Speed of the Amoebae on the Two Sides of a Slug, or Does the Tip Itself Shift Its Position?** These two possibilities can be found in the literature: in phototaxis or thigmotaxis, for instance, is there a difference in the speed of movement of the cells on one side of the slug compared with the other, as I had initially assumed (11), or does turning consist of a displacement of the organizing center as first suggested by Fisher and his coworkers (refs. 12 and 13; see also ref. 14).

This second mechanism is consistent with what one sees in 2-D slugs: there is not a faster movement of the amoebae on one side of the slug than the other as the slug turns. At the same time it is equally clear that the tip shifts at the beginning of a turn. The active cells move as a group at the edge of what appears to be a slime sheath to push the tip in a new direction. So again it is an area that has a special property, and that while the cells no doubt are responsible for that property, many different and changing cells are involved in its production.

In two instances I was able to record the bifurcation of a tip, similar to the natural occurring twinning so often observed in ordinary 3-D slugs crawling on agar. In both cases two closely associated regions show separate expanding protuberances, and once they have been initiated, they rapidly move away from one another, each tip bringing with it many follower cells (Fig. 3).

**Do the Anterior Cells Pull the Posterior Ones, or Do the Posterior Cells Push the Anterior Cells?** The most striking phenomenon in the videos is the difference between the movement of the amoebae in the anterior and the posterior zones. The anterior cells zoom about at a rate 3 or 4 times faster than the posterior cells (see below), and they appear to be moving in all different directions (Fig. 2). The anterior amoebae are constantly changing their position; often a cell will suddenly move forward for quite some distance, and then later move off in a different direction. Not all the cells are moving at the same speed, in fact any one cell will move in spurts; they appear to have excess energy and dart into and through any empty space they can find. By contrast the posterior cells seem to all move forward in unison in a comparatively sedate fashion, similar to the movement of amoebae in an aggregation stream. This is consistent with unpublished observations in scanning electron micrographs where the posterior prespore cells were all neatly aligned in the antero-posterior direction (15).

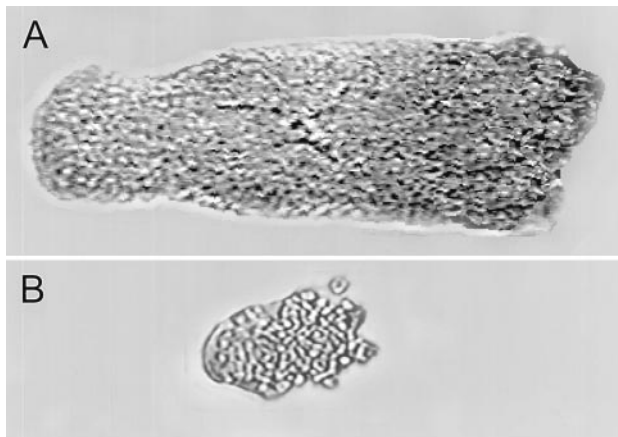


FIG. 1. A large (A) and a small (B) 2-D slug photographed from a TV monitor showing a videotape. The large slug is 470  $\mu\text{m}$  long and contains  $\approx 2,020$  cells; the small one is 80  $\mu\text{m}$  long and has  $\approx 110$  cells.

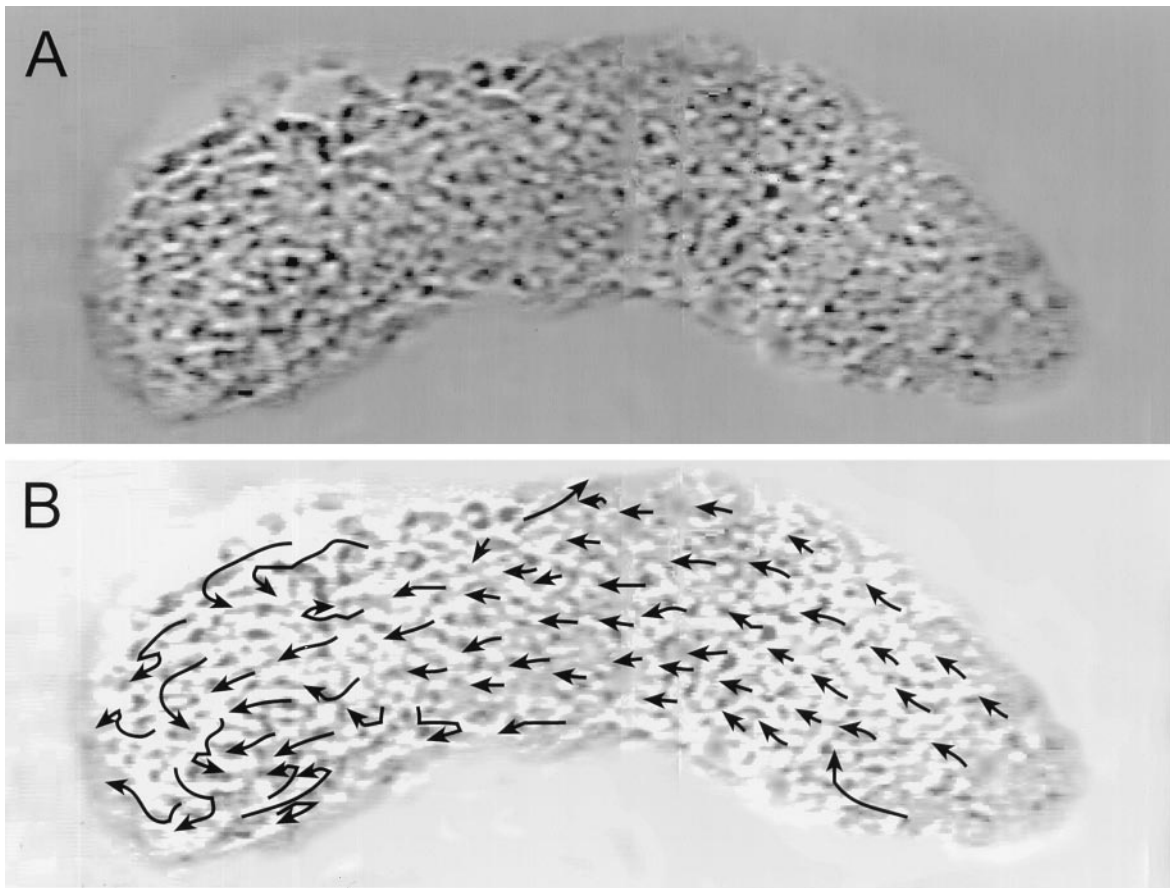


FIG. 2. The movement of individual cells in a migrating 2-D slug. (A) A photograph of an individual frame (the slug is  $175\ \mu\text{m}$  long and is made up of  $\approx 226$  cells). (B) The tracings of selected cells. 50 frames from a video of the slug, spanning 6 min in real time, were caught on a computer and played repeatedly so a few individual cells could be followed by tracing them on the screen. Note the difference in the speed and the direction between the anterior and posterior amoebae. [This was done on a Silicon Graphics (Mountain View, CA) O2 workstation and a Silicon Graphics media recorder utility to capture a sequence of the individual video frames.]

The question of where lies the motive force for slug movement is an old and contentious issue; there are those that have argued that the steady posterior cells push the anterior cells (7), and those that have argued that the more active anterior cells pull the posterior cells (14). From the observations on 2-D

slugs, it is evident that the amoebae of both parts of the slug are moving forward of their own accord. The evidence for this comes from videos of slugs in which there develops a narrow isthmus of cells between the anterior and posterior regions. Clearly the posterior cells are moving forward regardless of how narrow the thread of connecting cells, and the same is true of the anterior cells. They each, in their very different ways, are moving forward. In fact, in one instance the isthmus ultimately breaks off and the anterior cells wander away, yet the now isolated group of posterior cells continue to move forward for a short period of time, before forming a whirlpool at their anterior end. Inouye and Takeuchi (16) showed that this was also true for severed posterior portions of 3-D slugs if they were crawling inside an agar tunnel; they continue to crawl forward, while amputated posterior ends on an agar surface do not.

#### Do 2-D Slugs Move at the Same Speed as Normal Slugs?

The answer to this question is that 2-D slugs are slower than normal slugs, even taking their smaller size into account. This might be due to the mineral oil, because in one case a small 3-D slug first migrated in the oil and then flattened out in the oil-glass interface, and the speed of forward movement was approximately the same in the 3-D and the 2-D state. The oil not only provides less oxygen than air, but its increased viscosity will produce a lower Reynold's number; either one could slow the slug down.

This difference in speed between our small slugs and normal ones in air is reflected in the speed of the individual amoebae. By measuring the cells from the videos of 2-D slugs (Fig. 2), the anterior cells move at an average of  $2.8 \pm 0.8\ \mu\text{m}/\text{min}$  ( $n =$

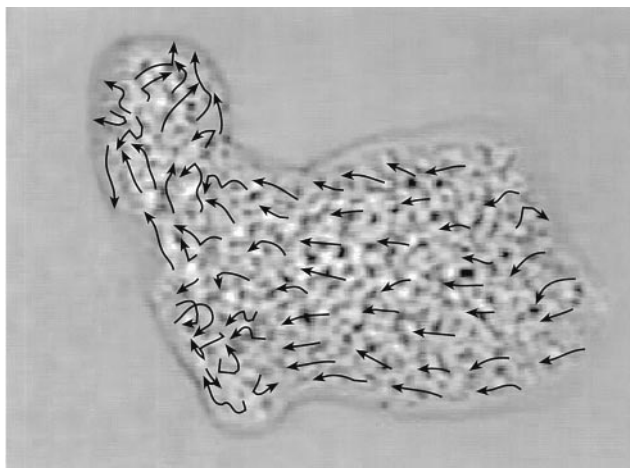


FIG. 3. A computer image of a single video frame of a 2-D slug in the process of splitting in two. The arrows represent 6 min of cell movement, using the same methods described in the caption of Fig. 2. Note the two active regions of cell motion that are moving away from one another. (The cell mass is  $139\ \mu\text{m}$  long and is made up of  $\approx 330$  amoebae.)

12) while the posterior cells average  $0.81 \pm 0.2 \mu\text{m}/\text{min}$  ( $n = 12$ ). This is considerably lower than what Siegert and Weijer (6) find for normal slugs ( $23.7 \pm 6.4 \mu\text{m}/\text{min}$  for prestalk cells,  $n = 118$ ;  $17.3 \pm 5.2 \mu\text{m}/\text{min}$  for prespore cells,  $n = 64$ ).

The important thing to remember in both of these instances is that the slug—be it 2-D or 3-D—keeps its overall shape as it moves, and therefore the faster anterior amoebae must twist and turn in such a fashion that their sum forward movement is the same as that of the slower posterior cells that are moving ahead in a straight line (Fig. 2).

#### Are Regular Oscillations Necessary for Slug Movement?

One of the surprising things about 2-D slugs is that there are no signs of oscillations or pulses, which, as is well known, are prevalent in aggregating amoebae and evidence for them in later stages goes back to early work (17, 18) and more recently by Schaap and coworkers (19, 20), and Siegert and Weijer (7). The answer may be very simple. In the first place on purely theoretical grounds Goldbeter (21) has shown that depending upon the relative concentrations of the chemoattractant cAMP and the phosphodiesterase that enzymatically degrades it, there are conditions that will lead to oscillations, and others that will not. Furthermore, it is well known from a number of old time-lapse films, as originally pointed out by Shaffer (22), that even during aggregation regular oscillations come and go. Therefore it would be easy to imagine that the conditions in which 2-D slugs find themselves, sandwiched between glass and mineral oil, might be ones that would suppress pulses, keeping in mind that pulses are due to a synchronized cAMP relay system, and there is no reason why the cell-to-cell relay could not be asynchronous.

**Are the Prestalk and Prespore Zones in 2-D Slugs Proportional?** The two zones can be seen in the time-lapse videos because the anterior (presumably prestalk) cells are tremendously active, whereas the posterior (presumably prespore) cells move in a more passive, deliberate fashion and the division line between can be drawn. It is possible to make an estimate of the cells in the two zones simply by tracing an outline of the slug and the two zones on the video screen and use a value for the number of cells per unit area determined by making a careful count of a sample area. (The average cell diameter is  $4.6 \mu\text{m}$ ; the area or the volume that a cell occupies—not the area or volume of the cell itself—is approximated by taking the square or the cube of this diameter.) By this method one can calculate the number of cells in the two zones and plot them against each other on a log-log graph (Fig. 4). On the same graph they can be compared with normal slugs using old data on anterior-posterior slug volumes calculated

from slugs stained with a vital dye (23). For these the volumes were divided by  $97.3 \mu\text{m}^3$  (the volume occupied by a cell, not the cell volume) to estimate the number of cells. It is clear from Fig. 4, that although the 2-D slugs in the present study are much smaller, they approximate the same prestalk-prespore proportions of normal slugs.

## DISCUSSION

The 2-D slugs provide some new insights into early differentiation and movement in migrating slugs of *D. discoideum*, ones that no doubt apply directly to normal 3-D slugs. From the 2-D slugs it is clear that the prestalk and prespore zones, which are normally proportioned, are characterized by each having totally different internal cellular movements: the prestalk cells move very rapidly in all directions, while the prespore cells move more slowly and go straight forward. Like normal slugs they move as a whole; their movement is directed, or polar. Furthermore they can split in two, or twin as do normal slugs. Both the anterior and the posterior cells actively move forward in their different ways as the whole slug migrates. There are no signs of regular oscillations or scrolls, indicating that they are not a requirement for migration. The anterior tip does not have a fixed set of cells, but a constantly changing one. Some of these facts are known to be true for normal 3-D slugs, while others are new—they are most likely telling us what is happening inside the standard large slugs where it is difficult to see the interior cells.

There are three particular aspects of these observations that invite an explanation.

(i) The first is how are we to understand the polar movement of these 2-D slugs in the light of the chaotic behavior of the anterior cells? It is well known that there is a gradient of secreted cAMP produced by the slug (24) and that the anterior prestalk cells possess a greater number of cAMP receptors on their surface than the prespore cells (25). This high tip concentration is not produced by particular cells, but by any cell that enters this peak region, and once there it is stimulated to release more cAMP—it is an autocatalytic phenomenon that involves whole cells that are moving about inside the slug. Because it is achieved by separate, moving entities it closely resembles the kind of autocatalysis described by Deneubourg and Goss (26) to explain the patterns found in foraging ant colonies, and schools of fishes and birds. And at the same time it can be perfectly described in detail by using a Turing type reaction-diffusion model (27, 28).

(ii) This leads us directly to the problem of turning—what can be its biochemical basis? From earlier work we know that slugs and rising fruiting bodies will move away from ammonia (29–31). Although it is true that ammonia increases the speed of amoebae and slugs at critical concentrations (32), as discussed above we now know that differential speed is not the basis of turning, but rather it involves shifting the tip that we have just assumed is the point of highest cAMP concentration. It is also known from earlier work that ammonia inhibits adenylyl cyclase and therefore cAMP production (33, 34) that means that an increase in ammonia on one side of the tip would shift the position of the tip by reducing the cAMP production on that side. Note that this makes ammonia a possible candidate for the inhibitor in a reaction-diffusion model, and cAMP for the autocatalytic activator.

(iii) The third point that needs explanation has to do with the proportionality between the number of prestalk and prespore cells. This proportionality is also true of normal slugs and it has been argued that it too could be explained by a simple reaction-diffusion model (27, 28).

What is more difficult to understand is our recent observation that the very active state of the anterior amoebae is directly dependent on the oxygen tension (2, 3, 35). The presence of oxygen not only makes the amoebae highly active

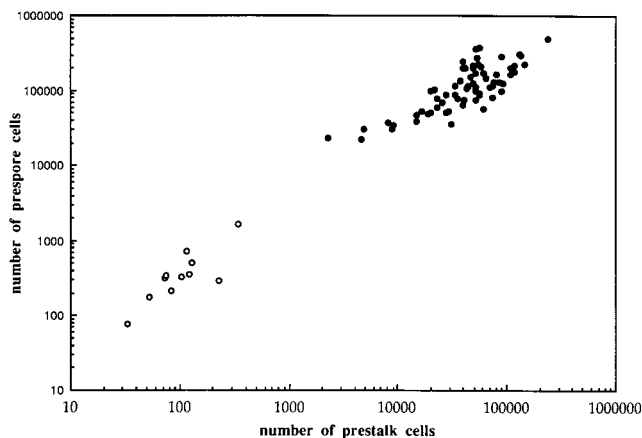


FIG. 4. A log-log graph showing that the number of prestalk cells is proportional to the number of spore cells in slugs of different sizes. ●, Normal slugs taken from an early study (23). They were stained with a vital dye to distinguish the two zones and their respective volumes estimated and converted to cell numbers. ○, 2-D slugs of this study.

and pseudopodial, but does so within a minute or two, and contrary to an earlier claim (1), it does not appear to produce the normal prestalk-prespore proportions.

How can we reconcile this fast effect of oxygen that produces such a sharp division line between the two groups of cells (but no proportions), with normal slugs and 2-D slugs where proportions exist? This is especially puzzling because in 2-D slugs clearly both ends get the same amount of oxygen from the oxygen rich mineral oil. We do not know the answer to this question, although we must assume that other morphogens, such as cAMP, ammonia, differentiation-inducing factor, adenosine, calcium, and no doubt others, might play a role in controlling proportions (for reviews see refs. 36 and 37). Somehow those morphogens work in conjunction with the initial stimulus provided by oxygen.

Perhaps the key is that there are two separate phenomena: polarity and proportional differentiation. Establishing the antero-posterior polarity by an oxygen gradient comes first, and this leads directly to the mechanisms responsible for the proportional pattern of early differentiation. In the case of these 2-D slugs, when they emerge from their prison in the broken piece of capillary, they have more oxygen on their oil side than on their opposite side flush against the glass; they are born in an oxygen gradient (as indeed are mounds at the end of normal aggregation). These two phenomena, polarity and proportionality, occur both in two, as well as in the normal three dimensions.

There are many reasons that support the idea that what we have learned from these 2-D slugs directly applies to normal 3-D slugs: the polarity, the proportions, the fact that fruiting with final differentiation into mature stalk cells and spores can occasionally occur between oil and glass, and other reasons discussed earlier. For the future there are some important matters to be solved in studies that I have already started. The first is to develop a method that ensures 2-D slugs can be produced on a regular and reliable basis. Then it will be vital to use marking methods with reporter labels attached to the promoters of stalk and spore specific genes. Another key matter to be examined is a better understanding of the extent of the slime sheath, and particularly knowledge of its chemical composition.

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