Evidence for differential cellular adhesion as the mechanism of sorting-out of various cellular slime mold species

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SUMMARY

Various authors have shown previously that if the amoebae of two species of slime molds are mixed they have the ability to sort themselves out. In the work reported here, the sorting-out of cells of several slime mold species was examined in clumps of cells in suspension.

Cells of four species, Dictyostelium discoideum (Dd), D. mucoroides (Dm), D. purpureum (Dp), and Polysphondylium violaceum (Pv), were mixed in pairs in suspension and clumps of cells formed. Dd and Pv cells sorted out completely and formed separate clumps, each of single species. Both Dd and Dm, when mixed separately with Dp, formed clumps containing both species. Sorting-out took place in these clumps such that the cells of Dd and Dm partially enveloped the Dp cells. Finally, in the Dd–Dm mixtures, the Dm cells always sorted out such that they surrounded the Dd cells. When mixed in a 1:2 ratio (Dd:Dm) the Dm cells formed a complete shell around a sphere of Dd cells.

Sorting-out of cells in clumps in suspension can occur by either of two possible mechanisms: response of cells to a chemotactic gradient or differences in cell surface strengths of adhesion (Steinberg, 1964). Mixing of two species in a clump of cells and observing the process of sorting-out permits one to distinguish between these two mechanisms (Steinberg, 1964). By such an analysis it was found that the sorting-out observed in mixtures of Dd and Dm is consistent with the mechanism of differential cellular adhesion. The major reasons for this are (1) when the adhesive properties of the cells are known to change the Dd cells began to move inside the clumps, (2) the Dd cells coalesced into islands rather than streaming inward independently, and (3) the Dd cells and cell masses did not lie at the center of the clumps but rather lay randomly within the clumps. The partial envelopment observed in the Dd–Dp and Dm–Dp mixtures and the separate clumps formed by the Dd–Pv mixtures are also consistent with differential cellular adhesion. They represent cases in which the interspecific strengths of adhesion are low (Dd–Dp and Dm–Dp) and near zero (Dd–Pv).

INTRODUCTION

Vegetative cells of cellular slime molds aggregate to form the multicellular stage of the life-cycle when the food supply has been exhausted. Olive (1902) discovered that when two species of slime molds were mixed together during the feeding stage they were able to sort themselves out and form separate fruiting

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bodies. Raper & Thom (1941) and later Bonner & Adams (1958) extended this work. They found that combinations of different species remained together through various stages of development before sorting-out took place. Interest in this phenomenon stems from several points of view. Sorting-out can be viewed as a mechanism which maintains species identity. It may be considered as a model for the sorting-out of prestalk and prespore cells normally observed in the life-cycle of slime molds (Bonner, 1959; Takeuchi, 1969; Bonner, Seija & Hall, 1971). Finally, the mechanism causing slime mold species to sort-out is of interest as it may be a common one for morphogenetic events in many biological systems.

In the work presented here, the process of sorting-out was examined using several species of cellular slime molds. Two alternatives were considered as possible mechanisms of sorting-out: response of cells to a chemotactic gradient and differential cellular adhesion.

The process of aggregation in cellular slime molds is controlled by a chemotactic substance; cAMP in the large *Dictyostelium* species (Konijn, van de Meene, Bonner, & Barkley, 1967; Bonner et al. 1972) and a small peptide in *Polyspondylium* (Wurster, Pan, Tyan & Bonner, 1976). It is possible that a sorting-out mechanism might also involve a chemotactic gradient. Such a gradient, established in the developing pseudoplasmodium or slug, could be used to guide sorting-out and create a segregation of species or cell types. In the *D. discoideum* slug, a gradient of cAMP has, in fact, been shown to exist (Bonner, 1949; Garrod, 1974; Brenner, 1978).

Another possible means of sorting-out is suggested by the work of Steinberg (1964) on cells of embryonic chick tissues. Steinberg has shown that if cells of two tissues are dissociated and then mixed in suspension they will form balls of cells. The cells in these aggregates or clumps then sort out such that like cells are grouped together. Steinberg (1970) and his co-workers have provided much evidence showing that these cells sort out because they have different relative strengths of cell surface adhesions. Furthermore, his work describes critical observations and experiments which can be used to distinguish whether the mechanism of sorting-out is due to (1) cells responding chemotactically to a gradient or (2) differential cellular adhesion.

Recently, Nicol & Garrod (1978) have done experiments similar to those presented here. They, however, could not make a judgement as to a possible sorting-out mechanism. In more detailed experiments reported here, various slime mold species are shown to sort out in a ball of cells in suspension. Evidence is presented which shows that the mechanism of differential cellular adhesion is sufficient, alone, to cause the sorting-out of two *Dictyostelium* species.
Mechanism of sorting-out of various slime mold species

MATERIALS AND METHODS

Four slime mold species were used for this work. Dictyostelium discoideum (strain NC-4 haploid), D. mucoroides (strain 2), D. purpureum (strain 2), and Polysphondylium violaceum (strain 1). These will be abbreviated as Dd, Dm, Dp, and Pv, respectively.

Growth conditions. Amoebae were grown both on agar and in liquid culture. Slime mold spores and Escherichia coli B/r were spread on peptone-dextrose buffered agar (Bonner, 1967). After 36-40 h at 21 °C, when the amoebae began to clear the bacteria and become aggregative, they were harvested by differential centrifugation in standard salt solution (Bonner, 1947). For liquid culture, spores were added to 50 ml of a suspension of autoclaved E. coli (O.D. of 4·0 at 550 nm) in phosphate buffer (17 mM, pH 6·0) in a 250 ml flask. The flask was put on a gyrotory shaker table at about 200 rev./min. The suspension cleared in approximately 4 days. If radioactively labeled cells were required, 5 μCi per ml of [3H]thymidine (Amersham/Searle Co., specific activity about 26 mCi per mmol) was added to culture flasks at the same time as the spores.

Experimental procedures. After harvesting and washing, three volumes of standard salt solution were added to the volume of the packed pellet. The resulting stock suspension was used in the mixing experiments. Pairs of species were mixed by adding drops of the stock suspension (or dilutions of this suspension) to roller tubes containing 2 ml of standard salt solution. The proportions of the species were varied by adding different numbers of drops. The total number of cells in the roller tubes ranged from 2-8 × 10⁶ cells per ml. Clumps formed in the roller tubes shortly after they were placed on a roller drum.

Clumps were fixed, embedded, sectioned and stained for observation. 2 % glutaraldehyde followed by 1 % OsO₄ (both in standard salt solution and both ‘EM’ grade from Polysciences, Inc.) were used for fixing. Clumps were embedded in methacrylate (Hartung Associates) and 1–5 μm sections were cut on a Porter-Blum microtome. The periodic acid–Schiff reaction (PAS) was used to stain the sections, after a 10 min digestion in amylase at 40 °C (Gomori, 1952). Kodak emulsion NTB-2 was used for autoradiography. The slides were allowed to expose for a minimum of 2 weeks at 4 °C.

Requirements of two sorting-out mechanisms. A chemotactic response by cells to a gradient is one possible means by which two cell types can sort out. The requirements for this mechanism are simply that one cell type has the ability to sense a gradient and move in response to it. Because of the small size of clumps and the fact that they are agitated in suspension, a gradient of a freely diffusible substance would be highest at the center of the clumps. The cell type which could respond to this gradient would move toward the center. The final configuration achieved would be one in which a sphere of one cell type would be surrounded by a sphere of the second type.
A second possible means of sorting out is by differential cellular adhesion. The essential requirement of this mechanism is that the two cell types have quantitative differences in their cell surface strengths of adhesion (Steinberg, 1964). Then, from purely thermodynamic reasons, the most stable configuration would exist when the sum of the strengths of adhesion is maximized. For example, say that the intercellular strengths of adhesion of cell types a and b are in the order, from greatest to least, $a-a$, $a-b$, and $b-b$. If, further, the strength of the $a-b$ adhesion is less than the average of the sum of the strengths of $a-a$ and $b-b$ cohesions, then the two cell types will sort out such that the more adhesive $a$ cells will form a sphere within a sphere of $b$ cells.

Predicted results of the two sorting-out mechanisms. By mixing two cell types in a ball of cells a situation is established by which the two sorting-out mechanisms can be distinguished (Steinberg, 1964). Both mechanisms predict the same final configuration of cell distribution: a sphere of one cell type surrounded by a sphere of the second. The actual means of distinguishing between a chemotactic response to a gradient and differential cellular adhesion, then, is based on observations of the movements of the cells toward this final configuration. If differential adhesion is the sorting-out mechanism, then the earliest event seen should be the disappearance of the more adhesive $a$ cells from the surface of the clump. The $a$ cells should coalesce in larger and larger islands. If cells were chemotactically responding to a gradient, on the other hand, all of the $a$ cells (the chemotactically responding cells) would stream toward the center without coalescing in islands. The final configuration of cells responding to a gradient would be a concentric sphere within a sphere, while if differential adhesion was operating the $a$ cell sphere could lie anywhere within the $b$ sphere. A critical experiment that can distinguish between these mechanisms can be done by reducing the $a/b$ ratio. If the cells are responding to a gradient by chemotaxis then all of the $a$ cells should come to lie at the center of the clump. If differential adhesion is involved, the $a$ cells will lie randomly within the clump except at the surface, which should be completely devoid of $a$ cells.

RESULTS

General morphology of clumps. Within a few minutes after being put on the rotator, the cells in the roller tubes began to clump. At first the clumps were very small and the cells only loosely adherent. By 2–4 h, the cells formed tighter clumps. Complete segregation of the species had occurred by 48 h. The slightly elongate clumps ranged from less than 100 μm to about 500 μm in diameter. The larger clumps tended to have a very irregular shape and often a lumen was present in their center. Small clumps, less than 200 μm in diameter, usually had the clearest segregation of species.

$Dd$–$Dm$ mixtures. Both the PAS reaction, which stains Dm cells more darkly than Dd, and autoradiography was used to distinguish the two species. The
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Table 1. *Dd–Dm* mixtures

<table>
<thead>
<tr>
<th>Proportions of Dd:Dm</th>
<th>Total number of clumps</th>
<th>Percentage of clumps in which Dm surrounds Dd</th>
<th>Percentage of unclear results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>12</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>1:4</td>
<td>10</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>1:2</td>
<td>87</td>
<td>94</td>
<td>6</td>
</tr>
<tr>
<td>1:1</td>
<td>59</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>2:1</td>
<td>51</td>
<td>61</td>
<td>39</td>
</tr>
<tr>
<td>4:1</td>
<td>9</td>
<td>56</td>
<td>44</td>
</tr>
<tr>
<td>10:1</td>
<td>7</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

*Dd* cells were grown in liquid culture and *Dm* cells on agar. The cells were mixed in different proportions. After 48–72 h the clumps which formed were fixed. The results are from three separate experiments and a total of 235 clumps were scored. *Dm* cells surrounded *Dd* cells in all clumps in which the results were clear. In 7% of all of the clumps scored *Dm* cells only partially surrounded the *Dd* cell mass. Other configurations, such as *Dd* cells surrounding *Dm* cells, were not found.

Fig. 1. *Dd–Dm* mixtures. Stained sections of clumps formed from a cell suspension of *Dd* cells and *Dm* cells mixed in a 1:2 proportion. The clumps were fixed after 48 h of culture. *Dd* cells stain lightly and *Dm* cells stain darkly. One clump shows the coalescence of two *Dd* cell masses. The clumps are about 90 µm in diameter.
Table 2. *Dd–Dm* mixtures: reversed growth conditions

<table>
<thead>
<tr>
<th>Proportions of Dd:Dm</th>
<th>Total number of clumps</th>
<th>Percentage of clumps in which Dm surround Dd</th>
<th>Percentage of unclear results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1:4</td>
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<td>40</td>
</tr>
<tr>
<td>1:2</td>
<td>4</td>
<td>100</td>
<td>0</td>
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<tr>
<td>2:1</td>
<td>14</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>4:1</td>
<td>12</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>10:1</td>
<td>11</td>
<td>36</td>
<td>64</td>
</tr>
</tbody>
</table>

Dd cells were grown on agar and Dm cells in liquid culture. The cells were mixed in different proportions. After 72 h the clumps which had formed were fixed. Fifty-one clumps were scored. Dm cells surrounded the Dd in all clumps in which the results were clear. There were no cases in which Dd cells surrounded Dm cells. The growth conditions did not affect the inside–outside configuration.

Table 3. Delayed addition of Dd cells

<table>
<thead>
<tr>
<th>Amount of delay</th>
<th>Proportions of Dd:Dm</th>
<th>Total number of clumps</th>
<th>Percentage of clumps in which Dm surrounds Dd</th>
<th>Percentage of unclear results</th>
<th>Percentage of pure clumps</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>1:2</td>
<td>64</td>
<td>100</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>52</td>
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<td>0</td>
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<td>2:1</td>
<td>37</td>
<td>78</td>
<td>22</td>
<td>0</td>
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<tr>
<td>24 h</td>
<td>1:2</td>
<td>16</td>
<td>44</td>
<td>38</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>40</td>
<td>38</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>19</td>
<td>53</td>
<td>0</td>
<td>47</td>
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<tr>
<td>48 h</td>
<td>1:2</td>
<td>17</td>
<td>41</td>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>14</td>
<td>21</td>
<td>14</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>16</td>
<td>50</td>
<td>0</td>
<td>50</td>
</tr>
</tbody>
</table>

Dm cells were added to three sets of roller tubes at 0 h. Dd cells were added to one set at 0 h, one at 24 h and one at 48 h. All clumps were fixed 48 h after the addition of the Dd cells. Dm cells surrounded Dd cells in 91% of the 153 clumps formed from the set in which Dd and Dm cells were both added at 0 h. 52% of the 122 clumps from the delayed addition sets (24 and 48 h) were either pure Dd or pure Dm clumps. Dd cells were surrounded by Dm cells in all of the remaining clumps in which the results were clear. There was only one clump (grouped in the 'unclear results' column) in which a patch of Dd cells were attached to the outside of an otherwise pure Dm clump.

two methods provided useful confirmation of one another. Dd was grown in liquid culture with [3H]thymidine and Dm was grown on agar.

The two species were mixed in different proportions in the various experi-
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The results of three experiments are compiled in Table 1. Sorting-out clearly takes place and a definite inside-outside pattern is seen. Dd is surrounded by Dm in all cases in which the clumps yield clear results (Fig. 1). There are no cases of Dd surrounding Dm. The best results were achieved when twice as many Dm cells were used as Dd. In this case 94% of the 87 clumps scored showed Dm surrounding Dd. Dm cells only partially covered the Dd cells in some clumps. These, however, represent only about 9% of all the clumps in which Dd was surrounded by Dm. Clumps which gave ‘unclear results’ were ones in which the inside-outside configuration could not be determined. This was due to a technical difficulty, such as poor fixation or sectioning, or to the absence of enough cells of one species. If too few cells of one species were used they could not be satisfactorily distinguished among the cells of the second species.

Control experiments. As the two species were grown under different conditions in the above experiments, a control experiment was done in which the growth conditions were reversed. Dm was grown in liquid culture with [3H]-thymidine and Dd was grown on agar. This is an important control because it has been found that growth conditions can affect sorting-out in the slug (Leach, Ashworth & Garrod, 1973). The sections were stained with the PAS reaction. The results were essentially the same as in the experiments described above (Table 2).

A second control experiment was done to examine the possibility that the Dd cells clump more quickly than the Dm cells, resulting in Dm cells adhering to the outside of already formed Dd clumps. Accordingly, Dm cells were allowed to form clumps alone for 24 and 48 h before the addition of any Dd cells (Table 3). All clumps were fixed 48 h after the addition of the Dd cells. The only new effect of delaying the addition of the Dd cells to the suspension was the generation of a large number of clumps containing only a single species. Fifty-one clumps, however, contained both species and in 50 of these the Dd cells lay inside the Dm cells, as in the above experiments. The Dd cells had moved through the Dm cell mass. Only in one anomalous clump was a patch of Dd cells seen on the surface of an otherwise pure Dm clump.

Equilibrium configuration. It is important to know if the inside-outside configuration is a final configuration. One reason for this is that both sorting-out mechanisms stipulate that the cells move toward a final configuration in which one cell type is surrounded by the second. Furthermore, one of the requirements of the differential adhesion hypothesis is that this final, equilibrium configuration can be reached from different starting conditions (Steinberg, 1964). One starting condition, described in the first experiments, is a mixture of two cell types in suspension. A second is achieved by fusing a Dd clump to a Dm clump. Experiments of this kind were done. Dd clumps and Dm clumps were allowed to form for 24 h. Then a glass wool fiber was run through one clump of each species so that they were in contact. Within 30 min they had
Time course of Dd–Dm sorting-out. Stained sections of Dd–Dm clumps showing different stages of sorting-out of lightly staining Dd cells and darkly staining Dm cells. (A) 4 h of culture. Dd and Dm appear to be randomly intermixed in patches except on the surface where Dd cells are predominant. The clump is 230 μm long. (B) 8 h. Dd cells are still predominant on the surface. Dm cells tend to surround single Dd cells (arrows). The clump is 350 μm in diameter. (C) 15 h. This clump shows the Dm cells surrounding the Dd cells which had been on the surface. The clump is 330 μm long. (D) 36 h. These clumps show Dm cells almost entirely surrounding the Dd cell mass. The largest clump is 230 μm in diameter. Dd cells appear to be coalescing in (C) and (D) (arrows).
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Fig. 3. Asymmetrical placement of Dd cell masses. A stained section of a clump in which Dd cells and Dm cells were mixed in a 1:4 proportion. This clump was fixed after 82 h of culture. It shows the asymmetrical placement of lightly staining Dd cell masses (arrows) within the darkly staining Dm cells and also the partial envelopment of one Dd mass by Dm cells. Why some of the Dd cells have persisted on the surface of this clump is not clear. The clump is 185 μm long.

fused well enough to remain attached when the fibers were withdrawn. Twenty fused clumps were put back into roller tubes for an additional 48 h, after which they were fixed and sectioned. It was clear that, although the clumps did not develop perfect spheres within spheres, the Dm cells, again, surrounded the Dd cells. Thus, the same final configuration was achieved from two different starting conditions.

Time-course experiments. Careful time-course studies were made on the process of sorting-out of dissociated cells in Dd–Dm mixtures. Many of the distinguishing features of the two sorting-out mechanisms are apparent in these observations. The cells of the two species were mixed in a 1:2 ratio (Dd:Dm). Stages of this sorting-out process are shown in Fig. 2. After 4 h of culture,
sorting-out had clearly begun. The clumps consisted of small patches of Dd cells and Dm cells. At this time no inside–outside configuration existed, but the peripheries of the clumps were in large part made up of Dd cells. By 8 h the clumps had become larger and the cells more tightly packed, except for a very loosely packed region that existed in the center of many of the clumps. Although the peripheries of the clumps were still predominantly made up of Dd cells, the Dm cells did not appear to play the role of an internally segregating cell type. Rather than being surrounded by Dd cells, the Dm cells showed a tendency to surround individual spherical Dd cells not attached to the surface. By about 12 h the Dm cells had clearly become the externally segregating species. They had begun to surround the Dd cells which at first lay on the surface. The Dd cells appeared to coalesce as they came to lie internally. Although a few Dd cells remained on the surface, by about 36 h the Dd cells were almost completely surrounded by Dm cells. As shown in Fig. 1, sorting-out was complete by 48 h. A sphere of Dd cells was surrounded by a sphere of Dm cells. This configuration was maintained until the clumps began to disintegrate at about 120 h.

Experiments with a low Dd to Dm ratio. Dd and Dm were mixed in proportions of approximately 1:4 and 1:5. In the clumps that formed it was seen that only a few small islands of Dd lay within the Dm cell mass. These islands were randomly and asymmetrically distributed (Fig. 3).

In two experiments the Dd:Dm proportion was further reduced to 1:100. [3H]thymidine-labeled Dd cells were mixed with unlabeled Dm cells. The clumps that formed were fixed after 70 h of culture. The autoradiographs of the sectioned material showed that the Dd cells were randomly scattered throughout the clumps (Fig. 4).

Mixtures with Dp and Pv. Each of the species studied here had its own distinct morphology. In particular, Dp clumps were quite irregularly shaped with several large protrusions. When either Dd or Dm were mixed with Dp, the clumps that formed were much more irregular than the Dd–Dm clumps. Rising out from the center of the clumps were projections of Dp cells. These irregularities made the recording of the results more difficult. While sorting-out definitely occurred, a clear inside–outside configuration was not observed. Therefore, each area of contact between the species was examined. The results were recorded in terms of which species partially enveloped the other at each of these points of contact. As can be seen in Table 4, Dp cells tended to be surrounded by both Dd and Dm cells.

Preliminary experiments with Dd–Pv mixtures yielded mostly pure Dd clumps and pure Pv clumps. There was a small percentage of clumps that contained both species and in these Dd cells lay in patches on the surface of the almost pure Pv clumps.
FIGURE 4
Random placement of Dd cells in a clump. An unstained section of a clump in which radioactively labeled Dd cells were mixed with unlabeled Dm cells in a 1:100 proportion. It was fixed after 70 h of culture. (A) A phase contrast micrograph showing the cells. (B) A bright field micrograph showing the random distribution of the silver grains of the autoradiograph. The clump is about 370 μm long.
Table 4. Dd-Dp and Dm-Dp mixtures

<table>
<thead>
<tr>
<th>Proportions</th>
<th>Total number of clumps</th>
<th>Total number of contact areas</th>
<th>% of areas in which Dd or Dm tend to surround Dp</th>
<th>% of areas in which there is no tendency to surround</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dd:Dp</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Dm:Dp</td>
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<td>10:1</td>
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<td>3</td>
<td>67</td>
<td>33</td>
</tr>
</tbody>
</table>

Dd and Dp cells and Dm and Dp cells were mixed in different proportions. The clumps which formed were fixed after 72 h. Because there was only partial envelopment of one species by the other, each area of contact between the species was examined to determine which species tended to surround the other. Dd cells tended to surround Dp cells in all of the 65 contact areas except one. This one area, where Dp cells surrounded Dd cells, is included in the 'no tendency to surround' column. Dm cells tended to surround Dp cells in all of the 49 contact areas which gave clear results. There were no areas in which Dp cells tended to surround Dm cells.

**DISCUSSION**

This work has shown that cells of several slime mold species sort out from one another and come to lie in particular positions in submerged clumps. Further, the sorting-out of the two species, Dd and Dm, appears to be due to differential cellular adhesion. The evidence for this is based on the observations of the sorting-out events which are consistent with the predictions of the differential adhesion hypothesis (Steinberg, 1964). These observations are outlined below.

1. The Dd cells in the Dd-Dm clumps were seen in the time-course experiments to coalesce into larger islands as they moved inward. This fact implies that chemotaxis cannot be the sole mechanism of sorting-out. If chemotaxis were acting alone the Dd cells would stream inward, independently, rather than coalescing. Coalescence is the expected means of sorting-out if differential adhesion is operating.

2. Once the final, equilibrium configuration has been reached, the Dd cells often do not lie at the center of the clumps. This fact suggests that chemotaxis is not acting in slime mold sorting-out. If it were a factor, then the Dd cells...
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would be expected to lie in the exact center of the clumps.\(^1\) Asymmetrical positioning of the internally segregating species is, however, the expected result if the differential adhesion hypothesis applies.

3. When Dd cells were mixed with Dm cells in a ratio of 1:100 the Dd cells were found to lie randomly within the clumps. Again, this is the expected result if differential adhesion were operating. If chemotaxis were the sorting-out mechanism, then the Dd cells would have been located at the center of the clumps.

4. The same configuration, Dd inside Dm, is reached from different starting conditions (a mixed suspension of cells and the fusion of pure Dd and pure Dm clumps). This is predicted by, and is a necessary condition of, the differential adhesion hypothesis.

5. The differential adhesion hypothesis further predicts that the internally segregating cell type should rapidly disappear from the surface of the clump. At first this does not appear to be the case with the slime mold species Dd and Dm. During the early period of incubation, Dd cells lie predominantly at the surface of the clump. Later, by 12 h, these cells have begun to take up the internal positions which they then maintain. The adhesive characteristics of slime mold cells are known to change after about 8 h of culture (Gerisch, 1968; Beug et al. 1970). These new adhesive properties may cause a reversal in the distribution of the cells in the clumps. Such a reversal is not without precedent. Similar results have been described for certain amphibian embryonic tissues (Townes & Holtfreter, 1955). In addition, Wiseman, Steinberg & Phillips (1972) showed that a similar reversal of position of two embryonic chick tissues was due to a change in their relative strengths of cohesion. If one considers that the disappearance of Dd cells from the surface starts after about 8 h of culture then this prediction of the differential adhesion hypothesis is fulfilled also.

The configurations seen in the mixtures of Dd and Dm with Dp and Dd with Pv, meet another basic set of conditions of Steinberg's differential adhesion hypothesis. These conditions exist when the strength of the \(a-b\) adhesion is less than that of the \(b-b\) adhesion (the weaker of the two homotypic adhesions). Under these conditions the \(b\) cells only partially envelop the \(a\) cells. The mixtures of Dm and Dd with Dp clearly show the partial envelopment configuration. If the strength of the \(a-b\) adhesion were zero then separate clumps would form. The Dd–Pv mixtures appear to represent a case in which the \(a-b\) adhesion is close to zero as these mixtures almost always produce separate clumps.

\(^1\) One model of chemotaxis can account for an equilibrium configuration in which asymmetrically placed islands of the internally segregating species \((a)\) lie within the externally segregating species \((b)\). This configuration could occur if \(a\) cells produce a chemotactic substance which diffuses poorly and to which only the \(a\) cells are attracted. Evidence against this model can be clearly seen in the 1:10 Dd to Dm mixtures. Within very short distances of Dd islands (sometimes within two cell diameters), free Dd cells could be seen. Even if a very localized chemotactic gradient existed these cells would be expected to have joined the Dd island. The differential adhesion hypothesis, however, requires the contact of cells for sorting-out.
Oliver (1902) first performed experiments in which different species were mixed. He found that if Dm and Dp were grown together in culture they would form separate fruiting bodies. Raper & Thom (1941) extended this work by showing that some mixtures of different species on agar remained together longer than others. Combinations of Dd and Pv immediately sorted out while mixtures of Dd and Dp and Dd and Dm remained together for varying lengths of time. The results presented in this paper are consistent with these observations; Dd and Pv tended to form separate clumps while Dd, in combination with Dm or Dp, formed common clumps. Bonner & Adams (1958), in an extensive study of sorting-out of different species on a solid substratum, found that regardless of how Dd and Dm were mixed, Dm always sorted out to the anterior end of the slug. The mechanism controlling this anterior–posterior sorting in the slug may be the same as that controlling inside–outside sorting in clumps in suspension which appears to be one of differential adhesion of cell surfaces.

As mentioned above, cell surface changes take place as slime mold cells enter the aggregation stage (Gregg, 1956; Gerisch, 1968; Beug et al. 1970; Muller & Gerisch, 1978). Species-specific ‘slime mold lectins’ which appear at this time were discovered by Rosen and his co-workers (Rosen, Kafka, Simpson & Barondes, 1973). In a recent paper, Springer & Barondes (1978), using a binding assay for adhesion, present evidence for differences between Dd and Dp strengths of adhesion. While this assay does not permit one to judge which species is more adhesive, the fact that they found differences in adhesiveness is consistent with the work presented here.

Nicol & Garrod (1978), using methods similar to those described here, have also studied sorting-out of different species. These workers found that in the Dd–Pv and Dm–Pv mixtures the Pv cells tended to sort out inside the other species. This tendency, though, appears to be transitory, as at later times more of these clumps are entirely composed of one or the other species. In the experiments presented here, it was found that the Dd–Pv mixture formed clumps mostly of single species which is consistent with their results.

Nicol & Garrod (1978) further state that in the Dd–Dp mixtures neither species has a tendency to surround the other. Their figures, however, depict results which are very similar to those described here and which were interpreted as Dd surrounding Dp. Although the Dd cells do not completely surround all of the Dp cells, clearly, at the areas of contact, the Dd cells do consistently cover the Dp cells. This partial envelopment is, as explained above, an example of one corollary of the differential adhesion hypothesis.

Finally, these authors found that there was no tendency for one species to surround the other in the Dd–Dm mixtures. In the experiments reported here, this mixture was the one that most clearly and consistently sorted-out with Dd inside Dm. An explanation for this difference may be that Nicol and Garrod did not examine any clumps after 1–24 h of culture. The equilibrium configura-
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Pattern formation is a major event in all developing systems and sorting-out is one mechanism of establishing a pattern. It appears that sorting-out of slime mold species is controlled by differential cellular adhesion. Bonner (Bonner, 1971; Bonner et al. 1971) and later Garrod (1974) proposed that this mechanism might well play a role in prestalk–prespore sorting-out. Whatever the mechanism of sorting-out in the slug, it is clear that it is not the only control of pattern formation in slime mold development. Sorting-out may establish the original pattern but later events, such as regulation (Raper, 1940), are more likely controlled by gradients of diffusible factors (MacWilliams & Bonner, 1979). All of the controls on pattern formation, including the mechanism of sorting-out of prestalk and prespore cells require exploration.

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