Evidence for a Substance Responsible for the Spacing Pattern of Aggregation and Fruiting in the Cellular Slime Molds

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WITH TWO PLATES

INTRODUCTION

In the cellular slime molds, after the individual amoebae have finished their growth and depleted their food supply, they aggregate into centers. Each center ultimately produces a small fruiting body (a spore mass supported by a slender stalk) that rises into the air. In a previous study (Bonner & Dodd, 1962a) it was shown that the size of the aggregation territories remained constant for any one species under a given set of environmental conditions even though the density of the amoebae in the culture dish might vary considerably. From this it was suggested that one of the possible hypotheses might be that a center-inhibiting substance is diffusing outward from the first formed centers and its effectiveness is independent of the number of cells within a territory. In another study (Bonner & Dodd, 1962b) evidence was brought forth to support the notion that as the fruiting body rises into the air it orients with respect to the environment by producing a gas to which it is sensitive and it orients away from regions of high concentration. In the discussion of this second paper we made the point that the adaptive significance of these two phenomena are one and the same; they both tend to space the fruiting bodies, first by keeping the centers separate during aggregation, and then by keeping the sorocarps separate as they rise into the air. Together they produce optimum conditions for effective spore dispersal.

We would now like to present the evidence that spacing in aggregation and spacing in culmination are governed by a common factor which we have tentatively called the spacing substance. (This term is used in a general sense to include the possibility of one or more chemicals.) The two previous studies which at first appeared quite unrelated except for their adaptive significance now seem to be separate aspects of a single process.

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There are at least three basic mechanisms which control the social existence, the development of the slime mold amoebae. In the first place there is the mechanism by which acrasin orients the amoebae by positive chemotaxis and is responsible for the aggregation pattern. Many details of this process are obscure, but the broad outlines are well established (for recent reviews of this subject, see Shaffer, 1962: Bonner, 1963). It is also known that acrasin is produced in later stages of development within the cell mass (Bonner, 1949) but we remain ignorant of its function during the migration and culmination stages.

The second component of development is center formation. The appearance of centers precedes the acrasin mechanism and, as Shaffer (1961a) has shown, the first sign of center formation is the appearance of a cloud: a dense mass of amoebae which Samuel (1961) found are slow moving compared to the ones outside of the cloud. Certain cells within the cloud become the actual focal point of the center. In Polysphondylium violaceum Shaffer (1961b) gave irrefutable evidence that it is a single cell which he termed the ‘founder cell’. This has also recently been confirmed for P. pallidum by Francis (personal communication).

The third component is the spacing substance. Arndt (1937), as well as numerous workers since, showed that early centers may reverse their progress and disintegrate, to use Shaffer’s term. Recently Shaffer (1961a, b) demonstrated that founders may be inhibited and revert to ordinary cells. Furthermore he has made the interesting observation (Shaffer, 1962, 1963), which fits in perfectly with the argument to be presented here, that if a sandwich is made of pre-aggregating cells of P. violaceum over some aggregating ones, the new centers form in the regions of low cell density. From this he argues that there must be a diffusible center inhibitor which exists in a gradient highest near the old centers. In the present study we should like to show that a diffusible inhibitor exists for all the species tested, and that it is a major control mechanism in the spatial distribution and density of the centers which ultimately develop into fruiting bodies.

With these three mechanisms one has a method of producing centers of attraction, a method of seeing that the number and distribution of these is controlled, a mechanism of gathering the amoebae to the established centers, and finally a mechanism for preserving this spatial arrangement during the remaining period of development, right to the end of culmination.

**METHODS**

In a few of the experiments the slime molds were grown in Petri dishes on non-nutrient agar upon which a generous loopful of *Escherichia coli* was evenly spread over the surface. The spores were inoculated at one point in the
center and allowed to grow and develop radially (Shaffer's modification of Singh's (1946) technique).

In the majority of the experiments the amoebae were grown first and then washed free of the remaining bacteria by centrifugation in standard salt solution (Bonner, 1947). Two methods were used in growing the amoebae; in some cases they were grown in Petri dishes containing nutrient agar (Peptone, 10 g., dextrose, 10 g., Na₂HPO₄·12H₂O, 0.96 g., K₂HPO₄, 1.45 g., agar 20 g., distilled H₂O, 1000 ml.). At the time of the plates were inoculated with the spores and the E. coli, a few ml. of sterile distilled water was added. These were ready for harvesting after 2 days at 23°C. The other method used was that of Gerisch (1959, 1960, slightly modified by Hohl & Raper, 1962). Basically it consists of growing the bacteria first in liquid culture, washing them free of nutrient, and re-suspending the bacteria in Sorensen's buffer. The spores of the slime mold were added to this liquid culture and put on a shaking machine. One advantage of this method, as Gerisch (1962) has shown, is that it is possible to age amoebae in this way for all aggregation will be inhibited during the shaking process.

All experiments were run at 23° ± 1°C. in the light (constant ceiling fluorescent lights).

THE ORGANISMS AND THEIR AGGREGATION CHARACTERISTICS

One of the most interesting and often the most disheartening, aspects of development of the cellular slime molds is their extraordinary variability, not only among species, but within any one clone. Even under ideal controlled conditions aggregation patterns may take on variable characteristics. In the following brief description of the five species used, particular phenomena will be emphasized, but probably all of these species can show all the phenomena to varying degrees, and it is merely a matter of emphasis. Except for one phenomenon, none of these observations are original but have been noted by one or more previous workers.

Dictyostelium discoideum (Strain No. 1) (Plate 1). Usually this species has very long streams on which subsequently there may appear secondary centers. It is as though all the centers do not initiate at the same instant, but slowly over a period of time. It is even possible to see in a that some centers have already formed before general aggregation has occurred. Also note that some of the secondary centers which form on streams are subsequently attracted inward to the main center.

Dictyostelium purpureum (Strain No. 2) (Plate 1). Besides providing a good demonstration of clouds, this species shows a new and interesting phenomenon. If two centers arise close together, and they both retain their power as centers, then they move away from one another to a small but definite extent. This occurs even before any signs of migration or stalk formation.
Dictyostelium mucoroides (Strain No. 11) (Plate 2). In this series one may see the well known phenomenon of center suppression or dominance. A number of small centers appear which ultimately disband and join the larger centres.

Polysphondylium violaceum (Strain No. 6, Shaffer's original 'founder' strain) (Plate 2). This species consistently shows the most perfect and ideal spacing pattern. Note that one of the centers divides into two. For this species and others, this phenomenon becomes more common as the amoeba density is increased.

Polysphondylium pallidum (Strain No. 4). This species is not illustrated for it shows no additional characters that have not already been illustrated. Suppression of secondary centers is often very marked, and the streams of the aggregating amoebae tend to be symmetrical, like the spokes of a wheel.

Evidence for a substance that inhibits aggregation

It has been noted on previous occasions that *D. mucoroides* frequently did not develop at all in confined conditions. If, for instance the amoebae were dispensed (after centrifugation) on non-nutrient agar in small plastic culture tubes stoppered tight with a cork (Falcon No. F25, 25 ml.) they frequently did not show any signs of aggregation, or if they did the aggregates were few and unhealthy in appearance. We have always assumed that this was caused by a lack of oxygen, for we know from the work of Gregg (1950) that aggregation is an aerobic process.

To test the oxygen deficiency hypothesis a set of stoppered culture dishes in which total inhibition of aggregation occurred (for about 12 hr. after the control with cotton plugs) were unstoppered and in some a few grams of activated charcoal were added, while nothing was added to the others. Each was again stoppered and placed at 23°C. The agar was on the upper surface of the tissue culture dishes so that the charcoal at the bottom of the dish lay about a centimeter away. In the controls without charcoal no aggregation occurred at all (they were observed for about 48 hr.) while in the dishes with the charcoal, after an hour, beautiful, normal aggregation occurred and vigorous, healthy fruiting was soon completed. Presumably, then, the inhibition is not caused by the deficiency of a gas (oxygen) but by the accumulation of an inhibitory gas which is effectively removed by the charcoal.

It was also found that aggregation and culmination would proceed in stoppered vessels if mineral oil (Parke-Davis, Heavy) was added. This could either be added, as with the charcoal, in the bottom of the dish, not touching the agar, or by filling the entire air space with oil. Therefore mineral oil is also capable of removing the inhibitory gas.

It is assumed that the amount of the gas produced is proportionate to the number of amoebae present in a tissue culture dish. To test this hypothesis stoppered dishes without charcoal were supplied with different numbers of
EXPLANATION OF PLATES

Each of the photographs on the two plates represent an actual area on the Petri dish of 6.3 x 4.2 mm.

PLATE 1

Dd, a–d. A series of photographs of aggregation of centrifuged *D. discoideum* amoebae. The original amoeba density was 1820 amoebae/mm². The final density of the centers was 36 centers/cm². Each photograph is taken exactly 1 hr. apart. Note the attraction of centers that form in some streams to the large center (left of the middle) and the appearance of centers on streams (e.g. right of the middle). Also note that the centers do not arise at the same time.

Dp, a–d. A series of photographs of aggregation of centrifuged *D. purpureum* amoebae. The original amoeba density was 2020 amoebae/mm². The final density of the centers was 37 centers/cm². The time between a and b is 1 hr., while 30 min. have elapsed between b–c and c–d. Note the appearance of clouds and the fact that the centers which lie close together move apart slightly.

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*(Facing page 574)*
PLATE 2

Dm, a–d. A series of photographs of aggregation of centrifuged *D. mucoroides* amoebae. The original amoeba density was 1630 amoebae/mm$^2$. The final density of the centers was 41 centers/cm$^2$. The photographs are taken 30 min. apart. Note the disintegration of a number of the small centers which appear in a, and also note in comparing c and d (left of the middle) that a small center has joined a larger one.

Pv, a–d. A series of photographs of aggregation of centrifuged *P. violaceum* amoebae. The original amoeba density was 1120 amoebae/mm$^2$. The final density of the centers was 36 centers/cm$^2$. a–b is a 30-min. interval, while b–c, c–d are 1 hr. each. Note that the appearance of the centers is particularly well synchronized and that in one case (d, middle) a center divides into two.

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In a series of experiments the range of cell densities was from 21,500 to 1200 amoebae/mm$^2$. Since the agar surface area is known (1653 mm$^2$) as well as the total air space within the dish (24 cm$^3$) it is possible to calculate the number of amoebae per mm$^3$ of air space. Aggregation and fruiting occurred at the following cell densities 86, 126, 146, 250 amoebae/mm$^3$ and total inhibition occurred at 236, 415, 546, 638 and 1479 amoebae/mm$^3$. Even though these ranges overlap they show that if the number of amoebae is greater than roughly 250 amoebae per cubic mm of air space, the inhibitor they produce rises to a concentration where it is totally effective. This is the threshold of self inhibition.

It should be added parenthetically that under threshold conditions or conditions which in general favor the accumulation of the inhibiting substance, many familiar abnormalities appear, such as large aggregation swirls, that often have no centers but whirlpool-like central areas (Arndt, 1937; Raper, 1941); rounded humps after aggregation that stop completely in their development; all sorts of odd forms during migration and culmination including total disintegration (Shaffer, 1957). All of these well known deformities can be dramatically and quickly cured by a spatula full of fresh activated charcoal.

*Evidence that the gas inhibiting aggregation is a spacing substance*

Continuing with experiments on *D. mucoroides* it is possible to choose conditions in which the air space and the ventilation is sufficient so that development will occur without the help of activated charcoal and then compare these to similar preparations in which charcoal has been added. Both Petri dishes and plastic tissue culture dishes with cotton or plastic plugs (which allow some air passage) were used and the results have been summed, for there is apparently no significant difference between the two. In the case of the dishes with charcoal it was again placed in the bottom of the dish, some distance away from the agar on the upper surface. In nineteen controls the mean number of centers per cm$^2$ was 58 ± a standard deviation of 77. In ten dishes with charcoal the mean was 246 centers/cm$^2$ ± 234. Clearly if the gas is removed by the charcoal the territories are smaller and therefore we may assume that the greater the amount of inhibitor, the larger the territory.

If the agar is submerged in mineral oil then the mean fruiting body density is 1352 centers/cm$^2$ ± 1690. Again one might assume that the oil, which is in contact with the amoebae, is even more effective in removing the inhibitor. But then the oil may have other effects as well since the gas phase has been totally removed. It should be added parenthetically that one of these effects might be dessication, for, as Raper (1940) showed, a lowering of the humidity produces smaller and more numerous fruiting bodies. In all experiments reported here great care was taken to avoid drying conditions.

Another brief but revealing experiment further confirmed that the inhibitory
gas is a spacing substance. Open Petri dishes containing the amoebae of *D. mucoroides* were placed in a desiccator jar lined with wet toweling. Moist air (passed through a sintered glass bubbler in a water trap) was circulated through the jar by means of a small aquarium pump. The control Petri dishes were in a similar jar in still moist air with their covers off. In two experiments the controls were 63 and 63 centers/cm.\(^2\) while in the circulated air the values were 210 and 186 centers/cm.\(^2\). Presumably the circulating fresh air has blown away enough of the spacing substance to significantly reduce the territory size.

To test the possibility that this might be due to desiccation, a gas insensitive species (to be explained presently) was used. *D. purpureum* gave the following values: 41 and 25 centers/cm.\(^2\) in control conditions and 33 and 43 centers/cm.\(^2\) in circulating air. Since drying affects territory size for this species (as well as all others), and since the controls and the experimentals are clearly comparable, the air circulation does not produce any drying effect on territory size. In one final experiment another gas sensitive species was also tested, and *P. pallidum* showed 14 centers/cm.\(^2\) in the controls and 128 centers/cm.\(^2\) in circulated air.

It should be added that in all the experiments where there is a reduction of the spacing substance, either by charcoal or air circulation, the aggregation starts somewhat sooner (usually \(\frac{1}{2}\) to 1 hr. sooner). Therefore the spacing substance would appear to delay aggregation, the extreme case being total inhibition in closed vessels.

At the suggestion and with the help of Dr David Francis tests were run to see if the distribution of centers in space was random or non-random, the latter being expected if the spacing substance is actively suppressing center formation.

Using the test of Clark & Evans (1954) it is possible to show that the distribution of centers is in all cases non-random. Also a series of dots were plotted on a graph by using random numbers obtained from the tables of Fisher & Yates (1943). If the distance between any dot and its nearest neighbor is plotted in a frequency distribution the resulting curve is approximately normal, while if the same is done for the distribution of aggregation centers, the curve is definitely skewed in such a way as to indicate that very short distances between centers are less frequent in the slime mold patterns than one would expect on a random basis. This is consistent with the notion that the centers are producing the spacing substance that inhibits new centers from forming in their immediate vicinity.

Also some experiments were performed in which two agar surfaces containing amoebae were separated from one another by a 5 mm. air space. It was found that the range of distances between centers and their nearest neighbor were the same for those cases in which the nearest neighbor was on the same agar surface as those where it was on the opposite agar surface (i.e. the diagonals across the air gap between centers). In other words for *D. mucoroides* the spacing substance can act as effectively across the gap as it can through or along the agar surface.
The chemical nature of the spacing substance

The first and the most crude experiment was one which indicated that there is no evidence for species specificity in the gaseous spacing substance. The experiment consisted merely of placing agar both on the bottom and on the top of a Petri dish. In the experiments two species confronted one another and in the control one species was confronted with cell-free agar. The result was that in seven experiments for *D. mucoroides* the controls showed a mean of 146 centers/cm.² while the summed experimentalss (*D. mucoroides* confronted with *D. purpureum* in four cases and *D. discoideum* in three cases) showed an average of 99 centers/cm.². Perhaps it would be more helpful to state that in four of the cases there was total inhibition with the partner and in the remaining three the number of centers/cm.² was reduced. Brief tests were also run with the two other gas sensitive species and *P. pallidum* (two cases) produced 211 centers/cm.² in the controls and 62 centers/cm.² when confronted with *D. purpureum*. Total inhibition was produced when it was confronted with itself (two cases). *P. violaceum* (two cases) produced 267 centers/cm.² in the controls and 130 centers/cm.² when confronted with *D. purpureum*. In the case of *D. discoideum* (three cases) and *D. purpureum* (eight cases) their territory size was clearly not increased by the addition of a partner producing additional gaseous spacing substance on the opposite side of a Petri dish. Thus as far as could be determined from these limited experiments all species produce the gaseous spacing substance, but only three of the five species are sensitive to it.

Partly inspired by the interesting observations of Loomis (1959) on the effect of CO₂ on sexual differentiation in hydra, we attempted some experiments in which specific CO₂ absorbants (KOH and diethanolamine) were placed in the bottoms of plastic tissue culture dishes (as in the charcoal experiments). To rule out the possibility of desiccation effects, in controls H₂SO₄ was added at a concentration to give a relative humidity of approximately 95 per cent., which was slightly drier than that produced by 5 per cent. KOH (97 per cent. RH), but the number of centers/cm.² was comparable with the control. Also some of the tissue culture dishes (unstoppered or with cotton plugs) were placed in a desiccator jar containing moist air. Through a side opening alveolar air was blown in and then the jar sealed off (for the method of obtaining alveolar air see Best & Taylor, 1961, p. 486). This produced a CO₂ concentration inside the jar of over 5 per cent.

The results of these experiments are shown in Table 1 and also included are the previously mentioned experiments with charcoal and oil. It is obvious that conditions which allow a high concentration of CO₂ either increase territory size or result in total inhibition of aggregation, while the reverse is true when CO₂ is specifically adsorbed. The situation is not quite so clear cut in the case of some experiments on *P. pallidum*; although the added alveolar CO₂ did reduce fruiting body density from 96 centers/cm.² (corked control) to 26 centers/cm.²
(five cases), the CO\textsubscript{2} absorbants gave results comparable in magnitude to the H\textsubscript{2}SO\textsubscript{4} controls, indicating that the desiccating effects are obscuring any possible CO\textsubscript{2} deficiency effects. The territory sizes of \textit{D. discoideum} and \textit{D. purpureum} are unaffected by either the addition or the absorption of CO\textsubscript{2}.

**Table 1**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Centers/cm\textsuperscript{2}</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alveolar CO\textsubscript{2} added</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Corked control</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>Open control</td>
<td>58</td>
<td>19</td>
</tr>
<tr>
<td>5 per cent KOH</td>
<td>134</td>
<td>6</td>
</tr>
<tr>
<td>60 per cent Diethanolamine</td>
<td>136</td>
<td>3</td>
</tr>
<tr>
<td>Activated Charcoal</td>
<td>246</td>
<td>10</td>
</tr>
<tr>
<td>Mineral oil</td>
<td>1352</td>
<td>10</td>
</tr>
</tbody>
</table>

A few runs were made on a Beckman gas chromatograph (Model CG-2) with the help of Mr R. Hyde. Gas samples from tissue culture dishes containing large concentrations of \textit{D. mucoroides} amoebae were analyzed using a 6 ft. silicon column and these samples differed from air only in that the one large peak (which includes CO\textsubscript{2}) is slightly broader in the slime mold samples.

These preliminary experiments are consistent with the idea that CO\textsubscript{2} could be the gaseous spacing substance. But it is also possible that besides CO\textsubscript{2} there are other significant gaseous substances. Furthermore it is possible that CO\textsubscript{2} has effects which are merely similar to the normal gaseous spacing substance. These possibilities have not been rigorously ruled out and must be before we can reach any final conclusion.

There is also the puzzling fact that some species are completely insensitive to the gas yet they clearly do have spacing and a non-random distribution of aggregation centers. In all the species it is possible to produce aggregation and demonstrate territory formation under a layer of agar, or in the bottom of a glass dish under a layer of standard salt solution (Bonner, 1947). The territory size under these conditions is roughly the same as on the surface of agar. Therefore in all species the spacing substance can diffuse through an aqueous medium and a gas phase is not required. In the gas sensitive species this could still be CO\textsubscript{2}, but this would not account for territory formation in the gas insensitive species. It was thought that perhaps in these latter species they were sensitive to bicarbonate and therefore a series of tests were run both underwater and on agar surfaces at different pH's. Unfortunately the results are thus far confusing and contradictory and require an extensive and systematic study which we hope to undertake. All that can be said at the moment is that changes in pH do have a very marked if inconsistent effect on aggregation territory size. It is for all these reasons that it still seems advisable to use the term \textit{spacing substance} in the general sense of being possibly one or more chemicals.
Variations in the aggregation patterns

As already emphasized, the variations in aggregation patterns are considerable and now our problem is to attempt to understand these variations. First a series of observations will be described with the hope that they will provide some basis for interpreting the underlying causes.

In a previous study on aggregation territories (Bonner & Dodd, 1962a) we were very fortunate in doing the majority of these studies with growth plates, for such preparations give remarkably constant territory size over a great range of amoeba densities. In the last year and a half we have run many hundreds of tests with all five species using centrifuged amoebae, and we now find that the only species that holds this amoeba density relation completely with centrifuged amoebae is *D. purpureum* (see the solid lines in Text-fig. 1). In *D. discoideum* there is a definite decrease in territory size as amoeba density increases. In *D. mucoroides*, and particularly *P. violaceum* and *P. pallidum*, there is so much variation that a definite relation requires too much imagination to perceive with any confidence. The question is how to explain these differences.

One way in which the species differ is in their susceptibility to the spacing substance, as has already been discussed. This is shown in summary in Table 2 and it can be seen that as one proceeds down the list the species are progressively more affected by the conditions which remove the spacing substance (oil and charcoal). In other words *D. purpureum* which is so especially rigid in producing fixed territories under all conditions is especially insensitive to the volatile spacing substance, and this correlation may be of significance.

The other important feature, which is obscured by the averaging in this table, is the fact that *P. violaceum* apparently can exist in two states. It alternates through periods of relative insensitivity to periods of great sensitivity. *D. purpureum* and *D. discoideum* are at all times relatively insensitive and *D. mucoroides* and *P. pallidum* are at all times sensitive to the spacing substance. *P. violaceum* seems to possess both characters which switch on and off in succession. The causes of the switching are not understood.

Another feature which has been studied in some detail is the reversibility of center formation. Two extreme cases were chosen; *D. purpureum* and *P. pallidum*, and for each their amoebae were allowed to begin aggregation and then were flooded with standard salt solution, stirred, filtered through cotton, and re-suspended on fresh agar. In both cases (the dotted lines in Text-figs. 1 and 2) the number of centers formed became density dependent, as shown by the fact that the lines have a slope of 1 on a logarithmic scale. In other words, to examine *D. purpureum* first (Text-fig. 1), if the amoeba concentration is halved, the number of centers is halved, which is contrary to what happens when aggregation occurs for the first time and the territory size is fixed at all amoeba densities. Once a center of *D. purpureum* is formed and territories have been blocked out, the process is irreversible, at least under these circum-
stances. The centers can be fragmented and each one produces many new centers upon replating, but they do not lose their tendency to produce centers.

TEXT-FIG. 1. The log of the amoeba density (amoeba/mm.$^2$) plotted against the log of the aggregate density (centers/cm.$^2$) for *D. purpureum*. The solid lines are of amoebae taken directly from shaker flasks (modified Gerisch (1959), technique) while the dotted lines are of amoebae that have aggregated once and have been partially separated and replated on fresh agar. Each line represents one experiment with successive dilutions of the amoebae. If the line is vertical this indicates that center formation is independent of the amoeba density; if the line is at 45° then if the number of amoebae is halved, so is the number of centers (i.e. dependent on the amoeba density).
Table 2

Number of centers/cm.$^2$ under different conditions for five species ± standard deviations. Numbers in brackets are the number of cases

<table>
<thead>
<tr>
<th></th>
<th>Air</th>
<th>Air with charcoal</th>
<th>Under oil</th>
</tr>
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<tbody>
<tr>
<td>D. purpureum</td>
<td>35 ± 14 (22)</td>
<td>50 ± 14 (5)</td>
<td>58 ± 27 (19)</td>
</tr>
<tr>
<td>D. discoideum</td>
<td>75 ± 71 (13)</td>
<td>111 ± 122 (7)</td>
<td>90 ± 82 (9)</td>
</tr>
<tr>
<td>P. violaceum</td>
<td>130 ± 182 (32)</td>
<td>762 ± 695 (9)</td>
<td>1100 ± 2520 (25)</td>
</tr>
<tr>
<td>D. mucoroides</td>
<td>58 ± 77 (19)</td>
<td>246 ± 234 (10)</td>
<td>1352 ± 1690 (10)</td>
</tr>
<tr>
<td>P. pallidum</td>
<td>80 ± 66 (24)</td>
<td>345 ± 94 (3)</td>
<td>3043 ± 5840 (22)</td>
</tr>
</tbody>
</table>

The whole mechanism of territory formation and suppression can no longer operate. The only exception to this rule for *D. purpureum* is in underwater preparations where after a day or so the first centers (which cannot develop further under water) will disintegrate and form new ones with larger territories.
P. pallidum is far more fickle, for in a matter of approximately 8 hr. many of the new centers are suppressed so that the number of centers/cm.² reverts from a density dependent to a density independent condition (Text-fig. 2). Perhaps sensitivity to the spacing substance and center reversibility are correlated phenomena.

Center sensitivity might also account for another phenomenon observed previously (Bonner, 1960). It was found that if very small groups of cells were isolated on a growth plate by scraping away the cells immediately about them, then their success in completing development depended upon the number of cells in the group. In the case of D. mucoroides (Fig. 1–4, Bonner, 1960) if there were slightly over 100 cells in a group its chances of culminating rather than disintegrating were 50 per cent. In the case of D. purpureum (Figs. 1–5) dispersal was almost impossible to achieve unless the centers were very small (ca. 25 cells) but culmination was abnormal unless the number of cells in the center was fairly large. On the other hand Konijn & Raper (1961) showed that in isolated drops small centers never disintegrated nor formed abnormal fruiting bodies. We would now propose the hypothesis that in the growth plates (as compared to Konijn and Raper's preparations) there was an accumulation of the spacing substance from the surrounding amoebae and pseudoplasmodia and small groups of cells are particularly sensitive to it. In fact, the curves in Figs. 1–4 and 1–5 of the 1960 paper show the number of cells necessary to resist disintegration in the presence of a given but unknown concentration of spacing substance. This hypothesis is further supported by the fact that the types of abnormalities observed in D. purpureum culmination are now known to be cured by the addition of activated charcoal in the culture dish. It is also worthy of note that again D. purpureum shows greater resistance than D. mucoroides to the spacing substance.

There is another source of variations that can be demonstrated by using the Gerisch (1959, 1960) technique, as Gerisch (1962) himself has shown. It is possible to prevent the amoebae from aggregation by keeping them continuously on the shaker. In the case of D. purpureum it makes no difference when the amoebae are taken: they all give fixed territories, and as can be seen from Table 2 the territory size in air is roughly comparable to that under mineral oil. However, at the other extreme, 72-hr.-old amoebae of P. pallidum under mineral oil show an extraordinary increase in the number of centers/cm.² (Text-fig. 3). This is not true in air so we must assume that the spacing substance suppressed all these new centers, but that the mineral oil prevented the spacing substance from acting and therefore the new centers could express themselves without inhibition. There is no explanation for the increase in territory size with amoeba age in the air experiment, except possibly that with age the amount of spacing substance increases or the susceptibility to it increases.

The importance of timing is also shown in the normal development of D. discoideum. As can be seen from Plate 1, the centers in this species do not form
at one time (as they do, for instance, in *P. violaceum*, Plate 2); rather they appear slowly over a period of time. If the time at which a center is produced can vary as well as the timing of susceptibility which is illustrated (as already mentioned) in *P. violaceum*, then the timing alone would provide many possibilities in variation.

Another cause of variation can only be mentioned in passing for we have no specific experiments to report. This is the important effect of environmental changes. As already mentioned Raper (1940, 1962) showed that lowering the humidity decreased territory size. Potts (1902), Harper (1932) and Raper (1940) have also shown that an increase in light causes a reduction in territory size. More recently Shaffer (1958) has shown that a sudden onset of light will induce aggregation in some species, and specifically produce a spate of new founder
cells in *P. violaceum* (Shaffer, 1961b). In this study every attempt was made to reduce these variables.

In conclusion it may be stated that the variation in aggregation patterns have in general as their basis (1) variation in center formation, (2) variation in spacing substance production, and (3) variation in susceptibility to the spacing substance. On top of these one must add the activities of acrasin and how they follow through with aggregation. Each one of these aspects is in turn affected independently by the genetic constitution of the species or strain, the environment, and finally by the timing of the events. Considering all these possibilities it is perhaps hardly surprising that the possible permutations of pattern are considerable.

*The spacing substance and the later stages of development*

From the fact that the distribution of centers in all species is non-random we assume that all species produce and respond to a spacing substance and from the experiments in which one species confronted another there was evidence that during aggregation all species produced the gas form of the spacing substance, even those species that are insensitive to its effects during aggregation. Now arises the question of whether or not the substance continues to be produced by the migrating and culminating pseudoplasmodia.

The fact that it is continually produced during the later stages was demonstrated by a simple experiment. If a growth plate (i.e. non-nutrient agar covered with a thin layer of *E. coli*) is inoculated one at spot with a few spores of *D. mucoroides*, as the amoebae grow and eat their way outward radially there are continuously expanding concentric circles of successive stages of aggregation and fruiting. If, on the other hand, a huge mass of amoebae is placed at the inoculum spot of a comparable culture plate, then simultaneously the amoebae will spread and large fruiting bodies will arise from the inoculum spot over the vegetative and pre-aggregation amoebae. When this occurs, as long as the large aerial pseudoplasmodia are actively migrating or culminating, none of the amoebae on the surface of the Petri dish will begin aggregation even though their density and age be suited for aggregation. This suppression of aggregation will occur even when the rising pseudoplasmodia are not touching the agar surface (except for the bases of their stalks at the point of inoculation).

The adaptive function of such inhibition would appear to be the same as during aggregation, namely the prevention of cell masses from forming near others: the fact that the original group of cells is at a very much later stage of development does not alter the spacing action of the inhibitory substance. But inhibiting aggregation is not the only activity of the spacing substance produced during later stages of development.

Another is the curtailing of migration. This is especially obvious in *D. discoideum* where the migration stage is sharply defined. If two sets of stoppered plastic tissue culture dishes are inoculated with the same quantity of *D. discoideum*
amoebae, but in one group activated charcoal is added, then on the average migration lasts from 12 to 24 hr. longer in the presence of the charcoal than in the controls. In conditions which favor the accumulation of the spacing substance there is reduction of the duration of migration. (It should be noted parenthetically that during migration lowering the humidity produces the same effect as the spacing substance while they have opposite effects on aggregation, i.e. dryness causes aggregation to occur sooner (Raper, 1940). This is one more bit of evidence to support the fact that the spacing substance effects are quite distinct from the effects of desiccation.)

In a recent study Wescott (1960) was able to show that 5 and 10 per cent. concentrations of CO₂ did not inhibit fruiting in *D. discoideum* but that 5 per cent. CO₂ caused some reduction of migration, while 10 per cent. CO₂ almost completely eliminated the migration stage. We have confirmed these results using alveolar air. This again is a case where CO₂ has the same effect as the spacing substance.

The most important activity of the spacing substance during the last stages of development is orientation (Bonner & Dodd, 1962b). As the pseudoplasmodium rises into the air it apparently gives off a gas to which it is characteristically sensitive and it moves away from regions of high concentrations. This results in the repulsion of pseudoplasmodia rising less than 0.8 mm. from one another. Also solitary pseudoplasmodia orient with respect to the physical structures of their immediate surrounding space: normally the substratum is a flat surface and the pseudoplasmodium therefore rises perpendicularly, since this is the only position in which the gases will be of equal concentration on all sides.

The assumption that the gas responsible for the orientation of the cell mass is the same as that which inhibits aggregation is based upon the fact that neither are species specific and they are both adsorbed by charcoal and by mineral oil. The latter, in the case of orientation, was first observed by Shaffer (personal communication). If a small drop of oil is placed on the surface of agar both migrating and culminating pseudoplasmodia will curve right into it, as they do with the charcoal. It should be added that the repulsion shown between close aggregation centers (*D. purpureum* in Plate 1) might be another argument that the same spacing substance is present throughout development. If the spacing substance is CO₂ this, of course, would be expected.

**DISCUSSION**

A question of prime importance is how the spacing substance acts. One hypothesis might be that it speeds the rate of movement of the amoebae. Center formation seems to be associated with the cessation of movement and therefore inhibition of center formation could be produced by stimulating the movement of the cells so that they break up from the groups. This would also explain the negative chemotaxis during culmination for by having faster move-
ment on the side of high concentration of the spacing substance, the cell mass would tend to curve away.

There are of course other possible explanations and furthermore it would not be surprising if the spacing substance did its center inhibiting by a different process than its orientating of the pseudoplasmodia. There is also the interesting question whether or not the mutual repulsion of the vegetative and pre-aggregating amoebae demonstrated by Samuel (1961) is also an action of the same spacing substance and what its mechanism of action might be in this case.

Finally it is also interesting to speculate as to the possible evolutionary origin of the cellular slime molds in the light of these observations on the spacing substance. We might assume that the spacing substance existed first, producing a mutual repulsion of the amoebae, a property which is apparently possessed by solitary soil amoebae. Center formation might have been the next evolutionary step; certain regions became ones of immobilization and there resulted a struggle between these centers and the spacing substance. The aggregation of cysts in the soil amoeba Hartmanella might be an example of this (Ray & Hays, 1954). If this congregation by immobilization has selective advantages, then if a mechanism were to arise which actively guided the amoebae inward, such as the acrasin mechanism, we would have the situation found in all the higher cellular slime molds. Acrasin has the further advantage of aligning the cells and from this polarized group of cells we find all sorts of new properties arising, especially oriented movement and differentiation.

**CONCLUSIONS AND SUMMARY**

The development of cellular slime molds is dependent upon three major factors; (1) the acrasin mechanism which gathers the cells together by positive chemotaxis, (2) the process of center formation which determines the locus of the gathering, and (3) the spacing substance that determines; (a) regions in which centers will be prevented from arising, and (b) inhibition of some centers that are already formed (i.e. the spacing of the aggregation centers): (c) the orientation or spacing of the fruiting cell masses.

Certain of the facts fit in with the hypothesis that the spacing substance is CO₂. However two species (*D. discoideum* and *D. purpureum*) always, and another (*P. violaceum*) sometimes, are insensitive during aggregation to both the naturally produced gaseous form of the spacing substance and added CO₂. Yet they do have non-random spacing during aggregation and therefore must have some form of substance diffusible in water.

There are great variations in the pattern of aggregation and these may be accounted for on the basis of variation in (1) center production, (2) spacing substance production, and (3) susceptibility to the spacing substances, and the changes with time of any of these three components for any one species under a particular set of environmental conditions.
During the later stages of development the pseudoplasmodium continues to produce the spacing substance. It has the effect of: (1) inhibiting a possible wave of secondary aggregation until the first wave of fruiting is completed; (2) inhibiting migration; (3) orienting the rising sorocarps of all species, for the pseudoplasmodia orient by negative chemotaxis in a gradient of the gaseous form of the spacing substance (Bonner & Dodd, 1962b).

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