AN ANALYSIS OF THE AGGREGATION STAGE IN THE DEVELOPMENT OF THE SLIME MOLDS, DICTYOSTELIACEAE. I. THE POPULATIONAL DISTRIBUTION OF THE CAPACITY TO INITIATE AGGREGATION [1]

M. SUSSMAN AND ELIZABETH NOËL

Dept. of Biological Sciences, Northwestern University, Evanston, Illinois

The results of a previous investigation (Sussman, 1951), indicate that the complex sequence of development displayed by the Dictyosteliaceae can occur in a population derived from a single parent cell. Moreover, the progeny of single cells, taken from widely divergent stages of growth and differentiation, retain this capacity. This would imply that, within a clonally isolated population of myxamoebae, the required cellular varieties can arise and become apparent in the necessary proportions and at the correct times so as to insure normal development. One of the interesting aspects of this implication involves the nature of the mechanisms which provide for the origin of the cellular heterogeneity observed.

The aggregation stage of the development was chosen as the subject of an initial approach to this problem. As a result of past investigation, some features of this phenomenon are well understood. A brief summary of the available information follows.

After vegetative proliferation has ceased, the myxamoebae, heretofore randomly distributed, elongate and become radially oriented. They move toward the center of orientation, at first as individuals and later in streams which become progressively larger and more ramified. In this manner, a rounded mass of cells is built up at the aggregative center. Ultimately the aggregate assumes the typical appearance of a pseudoplasmodium.

Studies by Raper (1941) indicate a complete dissociation between vegetative growth and the onset of aggregation. The results of Bonner (1947) suggest that the process occurs as the result of a response of the outlying cells to the production, by individuals at the aggregative center, of a diffusible substance or substances which Bonner has named "acrasin." Other work by Raper (1940a) indicates that the attracting substances may be specific since cell mixtures of different species will, in many cases, form entirely separate aggregation figures.

Consideration of this phenomenon suggests several pertinent questions which are capable of experimental elucidation.

(a) Is the number of aggregative centers formed a function of the number of cells in the population?

(b) If such a relationship does indeed exist, does it remain constant during the course of growth and differentiation of the population?

[1] The latter portion of the investigation was supported by a grant from the National Institutes of Health, U. S. P. H. Service.

(c) Is the capacity to initiate aggregation possessed by all the myxamoebae or by only a part of the population?

(d) Can a single cell initiate the formation of a center?

The present communication summarizes the salient results achieved during an attempt to answer these questions. The results indicate that the number of aggregative centers is both a function of the number of cells and of the population density. The number of centers/cell at the optimal population densities was determined for two species and was found to be constant for cells at the developmental stages immediately prior and subsequent to aggregations. Under the conditions employed the capacity to initiate center formation is not omnipresent but is possessed by only a small portion of the population. Finally, the results offer some support to the contention that aggregation centers are initiated by single cells.

Methods

A. The strains employed and their preparation for experiment

Single clone isolates of two species of Dictyosteliaceae, *D. discoideum,* strain NC-4 and *D. purpureum,* strain V-1, were employed in this investigation. Stocks were carried by plating with *Aerobacter aerogenes* on a previously specified medium (Sussman, 1951). Three to five spore masses were picked from a mature culture and mixed with a few drops of a 24–48 hour culture of *A. aerogenes.* The mixture was spread and the plates were incubated at 22° C. This inoculum requires between 44 and 50 hours to grow to maximal number and dispose of almost all of the bacteria. Subsequently, the myxamoebae aggregate and form fruiting structures.

To prepare the cells for experiment, cultures were washed off the plates with cold distilled water and the resulting suspension centrifuged five minutes at 1000–1200 rpm. in an International refrigerated centrifuge. This speed and time are sufficient to free the myxamoebae from most of the occluded bacteria. After three more washes in distilled water, the cells were suspended in either water or a salt solution devised by Bonner (1947).

B. Direct cell counts and surface density determinations

Direct counts were made with the aid of a Levy counting chamber. At least six replicate samples of each suspension were surveyed. The errors involved were found to be less than the fluctuations due to random sampling.

Measurements of surface density of cells on aggregation plates were made under low power using a Sedgewick ocular grid which had previously been calibrated with a stage micrometer. Except where later specified, the myxamoebae did not clump appreciably and the densities could be adequately controlled.

C. Medium for aggregation experiments

All studies of aggregation were carried out on plates containing a 2% washed agar-distilled water medium, hereafter designated as minimal agar. The agar was washed 5 to 10 times and dried in a vacuum desiccator before use.

Results

A. The ability of washed myxamoebae to aggregate and form fruiting structures

In his studies of the nature of the aggregative stimulus, Bonner (1947) had used myxamoebae washed free of bacteria and suspended in a salt solution. Aliquots of the suspension were dispensed in Syracuse dishes. The cells settled at the glass-water interface and, after about 16 hours, began to aggregate. Eventually, typical pseudoplasmodia were formed and at this point the morphogenetic process stopped.

For the purpose of the present investigation, a system was required which would permit accurate counts of the number of aggregative centers and precise control over population number and density. Preliminary experiments using the previously described procedure suggested that it could not completely satisfy these criteria. In devising an alternative method of study, use was made of an observation by Raper (1940b) that aggregating myxamoebae could be transferred to fresh nutrient agar plates whereupon the aggregation continued. A washed agar-distilled water substratum was substituted for the complete medium in order to suppress the growth of occluded bacteria and thus to prevent growth of the myxamoebae. Under these conditions, washed cells remained constant in number, aggregated, formed pseudoplasmodia and eventually complete and typical fruiting structures. Accurate counts of the numbers of aggregation centers were possible and adequate control over population size and density was comparatively easy to achieve.

B. The dependence of the number of aggregation centers formed upon the population size and density

Preliminary experiments were performed in which different numbers of cells were homogeneously distributed over identical areas of agar surface and, subsequent to aggregation, the number of centers counted. The results indicated a complex relationship between the number of centers and the number of cells. For very large populations, the number of centers was largely independent of the population size. For very small populations, the number of centers appeared to vary exponentially with the population size.

Clearly, a change in the total population number, distributed over a constant area must also involve a change in the population density. The possibility was raised that the number of aggregation centers produced by a group of cells is a function both of the total quantity of cells and their surface density. It therefore became necessary to elucidate the effect of the latter condition before one might hope to understand the effect of the former.

The relations between the number of centers and the population density was determined in the following way. A series of dilutions was prepared from a suspension of washed myxamoebae in Bonner's salt solution. An aliquot of each dilution was chosen such that all the aliquots contained the same number of cells. A typical protocol is given below:

No. cells/cc. of dilution	Vol. of aliquot chosen	Total no. of cells per aliquot
5×10^6	0.02 cc.	1×10^5
2×10^6	0.05 cc.	1×10^5
1×10^6	0.10 cc.	1×10^5
5×10^5	0.20 cc.	1×10^5

These aliquots were then dispensed in replicate on minimal agar plates. Since the surface area covered by an aliquot is a function of the volume delivered, the number of cells per unit area could be varied while the total population remained constant.

Trial experiments were run in order to assay the reproducibility and reliability of the procedure. It was found that the surface area covered by an aliquot is directly proportional to the volume delivered within the range employed. The number of cells/mm.^{2} of surface was determined, (a) by direct count under low power using a Sedgewick ocular grid and (b) by calculation using the known numbers

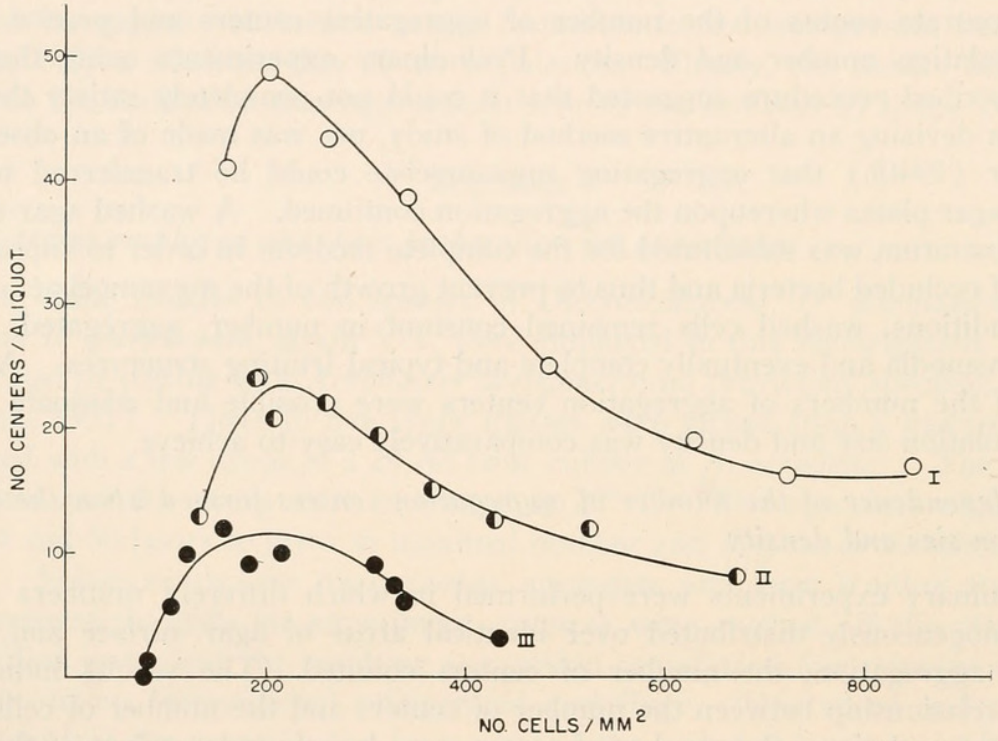

FIGURE 1. Aggregative center formation by *D. discoideum* as a function of population size and density. Aliquots of myxamoebae suspensions were placed on minimal agar plates and were designed to contain a constant number of cells dispersed at different population densities. After incubation, the numbers of centers per aliquot were determined. Curve I represents the data obtained for a population size of 1.0×10^5 cells per aliquot, curve II for 5.0×10^4 cells/aliquot, and curve III for 2.5×10^4 cells/aliquot.

of cells and measurements of the areas covered. The two procedures provided calibration data which are in excellent agreement.

After plates had been seeded in this manner, they were incubated at 22° C. with the covers ajar. Within a short time the fluid was absorbed by the agar and the covers were replaced. Determinations of population density at this time revealed no significant changes due to drying. After about 12 hours, aggregation began. Subsequently pseudoplasmodia were formed, one from each aggregation center, and these later gave rise to complete sorocarps. Counts of the numbers of centers were usually made at 16, 24 and again at 36 hours. Each aliquot was replicated between four and eight times and the means were calculated. The variance was low enough to assure the significance of the values obtained.

Figure 1 summarizes the results of a number of experiments with 44-hour cul-

tures of *D. discoideum*. The cells were harvested immediately prior to the normal occurrence of aggregation. Each point on the curves represents the mean of at least 12 replicate determinations. Three different total population levels were used.

The curves indicate that at very high population densities, the center-forming cells appear to interact competitively resulting in the suppression of a portion of them; the remaining centers are proportionately larger than those at lower population densities. At very low densities, the limit of activity of the aggregation stimulus seems to be reached so that the number of centers falls to zero. At a density of about 200 cells/mm.^{2}, no limitation is apparent and the number of centers is maximal.

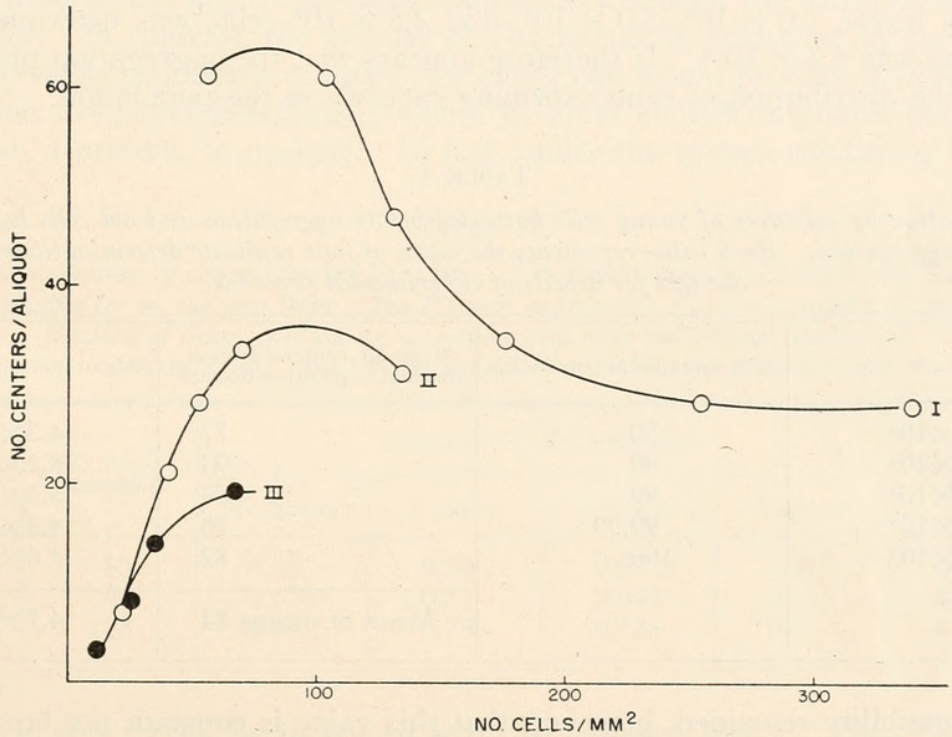

FIGURE 2. Aggregative center formation by *D. purpureum* as a function of population size and density. Aliquots of myxamoebae suspensions were placed on minimal agar plates and were designed to contain a constant total number of cells dispersed at different population densities. After incubation, the numbers of centers/aliquot were determined. Curve I represents the data obtained for a population size of 2.5×10^4 cells/aliquot, curve II for 1.0×10^4 cells/aliquot, and curve III for 5.0×10^3 cells/aliquot.

In the region of this optimal density, the number of centers was found to be directly proportional to the total number of cells present. Calculation of the number of centers/cell within this region revealed the following: for 1×10^5 cells, the number of centers/cell was 4.85×10^{-4}; for 5×10^4 cells this figure was 4.70×10^{-4}; and for 2.5×10^4 cells a value of 4.65×10^{-4} was obtained. The mean value was $4.73 \times 10^{-4} \pm 0.15 \times 10^{-4}$ (standard deviation), equivalent to one center for about 2100 cells.

A similar set of experiments was performed with washed cells from 50-hour cultures of *D. purpureum*, harvested immediately before aggregation. Figure 2 summarizes the results obtained. The number of centers/cell was found to be much higher in this species than in *D. discoideum*, the mean value amounting to

$3.3 \times 10^{-3} \pm 0.5 \times 10^{-3}$. This corresponds to a distribution of one aggregation center for about 300 cells.

C. The number of aggregation centers among cells which already have aggregated

In order to determine if the number of centers/cell was different in a population which had already aggregated, 55–60-hour cultures of *D. discoideum* were harvested, washed, and used for the experiment previously described. These cells had already aggregated or were in the process of doing so. Such cells have great tendency to clump, a condition which could be partially alleviated by the use of distilled water as the suspending medium. Even so, appreciable clumping remained and the results were somewhat erratic. The number of centers/cell for three population levels, 1.0×10^5, 5.0×10^4, and 2.5×10^4 cells, was determined. The mean value was 4.3×10^{-4}. It therefore appears that the aggregation process does not alter the distribution of center-forming capacity in the population.

TABLE I

Center formation by mixtures of young cells harvested before aggregation and old cells harvested after aggregation. Each value represents the mean of four replicate determinations. See text for details of experimental procedure

No. cells per drop	% young cells in population	Mean no. centers/drop at optimal population density	Mean no. centers per cell
1.8×10^5	50	77	4.3×10^{-4}
1.8×10^5	90	93	5.2×10^{-4}
1.8×10^5	99	94	5.2×10^{-4}
1.8×10^5	99.99	76	4.3×10^{-4}
1.8×10^5	100	82	4.6×10^{-4}
		Mean of means 84	4.7×10^{-4}

The possibility remained, however, that this value is constant not because more cells had not attained the ability to initiate center formation but rather because the remainder of the population could not respond to the stimulus imposed. In order to rule out this possibility determinations were made of the number of centers/cell of a series of mixtures of old cells (55–60 hours) and young cells (44 hours). Table I shows the results obtained. Regardless of the proportion of young and old cells, the number of centers formed did not differ beyond experimental error. The similarity was evident for each of the population densities employed.

D. The populational distribution of center-initiating capacity

Under conditions where the degree of cellular dispersion does not limit the initiation of aggregative centers, the number of centers which are formed was found to be directly proportional to the number of cells present. At least two interpretations may be raised to explain this fact.

(a) All individuals in the population are capable of initiating aggregation, acting either singly or in association. However, the realization of this potency does not occur simultaneously in all individuals but rather is distributed in time. Thus, the small proportion of cells which attained this capacity first would be the only

ones to initiate centers. The rest of the population, attaining this ability at a later time, would already have been attracted to previously established centers and so could not inform the observer of their acquisition.

(b) The formation of a center depends upon the presence in the population of a single cell or group of cells which is uniquely constituted so as to be able to initiate the formation of the center.

A distinction between the alternatives was made by examining the distribution of center-forming capacity within very small population samples at population densities previously shown to be sufficient to permit the expression of aggregating ability. Were alternative (a) valid, one might anticipate that at least one center of aggregation would appear in every sample even though the times of appearance might differ. In contrast, the validity of (b) would be evidenced by the fact that, for small enough population samples, not every one would produce a center despite the fact that the population density would be great enough to enable the initiating mechanism, if present, to operate. In fact, under the proper conditions the distri-

TABLE II

Population distribution of center-forming capacity. The mean number of centers/cell was calculated by solving for m, the first term of the Poisson expression, $P_o = e^{-m}$, where P_o is the fraction of drops containing no centers and m is the average number of centers/drop. See text for details of experimental procedure

No. cells per drop	No. drops examined	Fraction of drops containing:				Mean no. centers/cell
		No centers	1 center	2 centers	3 centers	
2100	85	0.42	0.50	0.07	0.012	4.1×10^{-4}
1025	91	0.626	0.32	0.044	0.011	4.6×10^{-4}
900	123	0.69	0.285	0.025	0.0	4.1×10^{-4}

bution of centers should be in accord with the Poisson series. Thus, the proportion of samples containing 0, 1, 2 and 3 aggregative centers would be given, respectively, by the expression, e^{-m}, me^{-m}, $m^2e^{-m}/2!$, and $m^3e^{-m}/3!$, where m is the mean number of centers per sample.

Suspensions of washed 44-hour *D. discoideum* were prepared and replicate, direct cell counts were made in the Levy chamber. A Cenco machined loop of 1.0 mm. inside diameter was used to deposit drops of the suspension on minimal agar. The fluid was quickly absorbed leaving the cells homogeneously distributed. The average volume of the drops delivered by the loop was found in trial experiments [2] to be 1.1×10^{-4} cc. $\pm 0.1 \times 10^{-4}$. Surface density measurements were made as before with a Sedgewick ocular grid and demonstrated the duplicability of the method. The variances of the determinations were in all cases approximately equal to the means, indicating that the only significant error is that incurred by random sampling.

[2] Very dilute suspensions of myxamoebae were dispensed with the loop. The mean numbers of cells deposited were determined by direct count under low power. The original suspensions were counted with a Levy chamber. From the two sets of data, the volume delivered by the loop could be calculated.

After 16, 24 and 48 hours incubation, each spot of cells on the agar was examined under a dissecting microscope for the presence of aggregations and fruiting structures. At the end of this time, it was found that not all of the spots contained centers of aggregation. Moreover, all the centers which appeared did so before 24 hours. Table II summarizes the data obtained from three experiments in which samples of 900, 1025 and 2100 cells were used. The last column on the right contains calculations of the number of centers/cell. These were obtained by solving the first term of the Poisson expression, $P_0 = e^{-m}$, where P_0 is the proportion of samples containing no centers and m is the mean number of centers per sample. The average number of centers per cell was found to be 4.3×10^{-4}. This compares well with the figure, 4.73×10^{-4}, obtained by the procedure described in Section B.

One may ask whether the suppression of center formation by high population densities might not increase the proportion of samples displaying no centers. That

TABLE III

Comparison between the observed and expected distributions of aggregative centers. See text for details

No. cells/sample	2100		1025		900	
m	0.86		0.47		0.37	
Total samples	85		91		123	
No. with 0 centers	36		57		85	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
No. with 1 center	42	30.9	29	26.6	35	31.4
No. with 2 centers	6	13.3	4	6.2	3	5.8
No. with 3 centers	1	3.4	1	0.96	0	0.71
χ^2	45.6		1.00		2.44	
p	<0.01		0.6		0.3	

this is not the case can be seen by comparison between the data obtained with 900 cells and with 2100 cells. In the latter samples the population density was more than twice as great as in the former. Yet, despite the greater cell density, the proportion of samples displaying no centers was much smaller. It must be noted in this connection that high cell densities do not inhibit all center formation *per se*. From the results summarized in Figure 1, it would appear that when the population density is high, center initiators are close enough to compete for aggregatee cells and, as a result of this competition, some prospective centers are incorporated by their neighbors. In no case has the existence of a high population density led to the suppression of all centers but merely has acted to reduce the total number. Thus one might expect that in the small population samples at high cell densities, the presence of one initiator would lead to the production of a center but where two or more initiators were present in a single sample, the number of centers formed might be less than the number of initiators. Were this condition to exist, the P_0 values would provide accurate reflections of the distribution of initiators but the

P_1, P_2, etc., values would be biased. For this reason, only the P_0 values have been utilized in the calculation of the number of centers/cell.

It must be emphasized that the crucial point of the experiment has to do with whether or not every sample can produce a center. The question of agreement with the Poisson series is of secondary importance, for its purpose is to enable us to calculate the number of centers/cell and thus permit a comparison with the figure derived previously.

A rough estimate of the agreement of the observed data with the Poisson distribution was made in the following way. The values of m, obtained from the P_0 figures, were employed in order to calculate the corresponding P_1, P_2 and P_3 values assuming random distribution. Table III compares these figures with those obtained experimentally. The accompanying chi-square estimates indicate that, for the experiments using 900 and 1025 cells/sample, the differences between observed and expected results should be exceeded by chance 30 and 60% of the time, respectively. For 2100 cells/sample, the agreement is very poor. Here, the biasing of the P_1, P_2, etc., values, anticipated previously, was encountered. This is not surprising in view of the fact that the population density of these samples was between 500 and 600 cells/mm.^{2}.

It appears that more efficient utilization of the data might be accomplished by making use of the P_1, P_2, etc., values from the experiments where these values were not biased. However, it is felt that the precision required in order to obtain a definitive answer to the question posed does not warrant this refinement.

Discussion

The reported findings indicate that, by the end of the growth period, a clonally derived population of myxamoebae exhibits a condition of heterogeneity with respect to the ability to initiate aggregation. The question of whether this initiation is contrived by a single cell or by groups of cells acting in concert cannot be answered conclusively from the data at hand. However, two considerations point to the likelihood that the former interpretation is correct.

(a) Microscopic observation of the process reveals that at the beginning of aggregation, the cells elongate and become radially oriented. If the population density is small, it generally may be observed that the center of orientation is occupied by very few cells and often merely by one.

(b) The distribution of aggregative centers among small, replicate samples of a population indicates a constant value for the number of centers per cell despite the fact that in these experiments the mean number of cells per sample was varied and therefore the population density was not held constant. Yet, it is clear that, were a group of cells to cooperate in the initiation of a center, an increase in density would encourage center initiation. In the experiments described, however, increases in density did not affect the proportions of samples with no centers but merely acted to suppress the formation of additional centers in samples which already possessed one or more.

One possible method of distinction would appear to lie in the examination of center formation by small numbers of wild type myxamoebae when mixed with "aggregateless" mutants, capable of responding to the initiating mechanism but not

of producing it. A number of aggregateless variants have been isolated and are being investigated with this end in view.

One may inquire into the genetic mechanism by which the aggregators and aggregatees arise. Previous findings (Sussman, 1951) have indicated that the differentiation observed could equally well arise via stable genic mutation and appropriate selection pressures, segregation or zygote formation within the clone, or by easily reversible genic or cytoplasmic modifications. At present, it does not appear that the results reported here can be construed to support or militate against any of these possibilities. However, it is hoped that with the information now at hand, a fruitful examination of this question will be possible.

The relation between the center-initiating mechanism and the production of acrasin, the chemotactic principle, may be examined in the light of the reported results. Observation of the aggregative process (Bonner, 1944) has indicate that the myxamoebae at the periphery of the aggregation pattern do not in all cases move radially toward the center but often migrate toward previously established streams of aggregating amoebae before taking up a radial direction. This would indicate that the production of acrasin is not a property possessed only by individuals at the aggregating center but also can be accomplished by outlying cells. It is therefore likely that the capacity to initiate a center of aggregation is not identical with the ability to produce acrasin. One may speculate that the center-initiating agency may supply a co-factor necessary to the production of acrasin and thereby exert its effect.

Summary

1. Examination of the aggregation stage in the development of the Dictyosteliaceae has revealed that the number of aggregative centers formed is a function of the population size and density. At population densities which do not limit the formation of centers, the number of such centers is proportional to the number of myxamoebae present. For *D. discoideum,* the number of centers/cell was found to be 4.73×10^{-4} and for *D. purpureum,* 3.3×10^{-3}. These values correspond to one aggregation for about 2100 and 300 cells, respectively. The values remain constant throughout the aggregation process.

2. Analysis of the distribution of centers within small, replicate, population samples of myxamoebae indicates that the capacity for center initiation is possessed by only a small proportion of cells. The values for the number of centers/cell achieved by this method agreed well with those previously cited.

3. The implications of these findings are discussed.

LITERATURE CITED

Bonner, J. T., 1944. A descriptive study of the development of the slime mold, *D. discoideum*. *Amer. J. Botany,* **31**: 175–182.

Bonner, J. T., 1947. Evidence for formation of cell aggregates by chemotaxis in the development of *D. discoideum*. *J. Exp. Zool.,* **106**: 1–26.

Raper, K. B., 1940a. Communal nature of the fruiting process in the Acrasieae. *Amer. J. Botany,* **27**: 436–448.

Raper, K. B., 1940b. Pseudoplasmodium formation and organization in *D. discoideum*. *J. Elisha Mitchell Sci. Soc.,* **56**: 241–282.

Raper, K. B., 1941. Developmental patterns in simple slime molds. *Growth,* **5**: 41–76.

Sussman, M., 1951. The origin of cellular heterogeneity in the slime molds, Dictyosteliaceae. *J. Exp. Zool.,* **118**: 407–418.