The present results suggest that light emission in luminous bacteria is under the control of at least two genetic factors which, as far as one can tell, do not affect other reactions essential for the growth of the organism. Bacterial luciferase is a flavoprotein which can catalyze the rapid oxidation of reduced pyridine nucleotides by molecular oxygen,\(^1\) and it may have figured more prominently in the metabolism of primitive forms of luminous bacteria as well as other organisms. From an evolutionary standpoint it suggests the possibility that these biochemical reactions which support light emission may have had, in the past, some selective advantage for the survival of the organisms.

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**INTERACTIONS AMONG VARIANT AND WILD-TYPE STRAINS OF CELLULAR SLIME MOLDS ACROSS THIN AGAR MEMBRANES**

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The cellular slime molds are members of the phylum Myxomycophyta and the order Acrasiales. They display a complex developmental cycle\(^1\) beginning with the germination of spores into phagotrophic myxamoebae. Following a period of exponential growth, the individual cells stream radially toward centers of aggregation under the influence of special "initiator" cells\(^2\) \(^3\) and in response to the production of specific chemical agents.\(^4\) \(^5\) The aggregates are then transformed into organized multicellular structures called "pseudoplasmodia." Each pseudoplasmodium undergoes additional morphogenetic alterations, ultimately producing, in the genus *Dictyostelium*, a fruiting structure with a mass of spores at the top, a parenchymatous, cellulose ensheathed stalk below, and in one species a basal disk.

These developmental changes are extremely orderly and undoubtedly subject to genetic control. Irradiation of the spores and myxamoebae of three species with ultraviolet light has enabled the isolation of many stable variant strains displaying aberrant development.\(^6\) Among them is a class of morphogenetically deficient stocks which grow normally but cannot complete (and some cannot start) the morpho-
genetic sequence. Thus a number are aggregateless and remain as randomly dispersed myxamoebae after growth. Others (fruitless) can accomplish a part or the whole of the aggregation process, but all stop development short of the appearance of mature fruits.

It has been found that cell mixtures of many pairs of deficient strains can develop synergistically to produce complete fruiting bodies with viable spores, although neither can do so alone. This synergism could be explained on at least four bases: (1) syngamy or heterokaryosis between the deficient partners to produce morphogenetically competent cells; (2) exchange of diffusible metabolites between the partners so as to shunt the biochemical blocks responsible for the deficiencies; (3) exchange of nondiffusible agents by direct cell contact; (4) need for the complete array of cell types necessary to normal development. Together, the deficient partners can provide this assembly, but neither can do so alone.

Serious doubt has been cast upon the first interpretation by the findings that (a) clonal platings of spores from synergistic fruitings produced no recombinants but only the original deficient phenotypes and (b) cells taken from any intermediate stage of the synergistic development did not yield recombinant clones. Thus, if syngamy or heterokaryosis occurs at all, it must be extremely rare. The present communication describes the results of experiments designed to distinguish among the remaining interpretations. The deficient strains have been allowed to develop on either side of agar membranes of a thickness as little as 30 μ, or about two cell diameters. It has been found that stocks which respond synergistically when mixed cannot counteract each other's developmental deficiencies if separated by a membrane. This result is made especially meaningful by the fact that the chemotactic substances responsible for aggregation, which have been shown to be exceedingly labile, can pass through membranes as thick as 200 μ. It would appear, therefore, that the assumption of synergism through exchange of diffusible intermediates is untenable at least for the collection of deficient stocks now at hand.

METHODS

A. ORGANISMS

Two species of slime molds were employed: Dictyostelium mucoroides, strain S-2, and Dictyostelium discoideum, strain NC-4. Of the former, only the wild type was used; of the latter, the wild type and a number of variant stocks to be specified later. The organisms were grown on glucose-peptone agar in association with Aerobacter aerogenes.

B. PREPARATION OF CELLS FOR EXPERIMENT

After a stock had been grown for 48 hours at 22° C. and had reached the stationary growth phase, the myxamoebae were separated from the few remaining bacteria by differential centrifugation. After three washes with cold distilled water, the cells were suspended in salt solution and counted in a Levy chamber. Such suspensions can be dispensed on a washed agar-distilled water substratum so as to control both the number of cells and the population density. Under these conditions the cells remain viable but do not increase in number. The wild-type populations aggregate and differentiate to produce normal fruitings with viable spores, while the variants attain their own distinctive levels of development.
C. PREPARATION OF AGAR MEMBRANES

Stainless-steel cylinders, machined from 7/8-inch tubing, were employed in constructing the membranes. The cylinders have an outside diameter of 2.2 cm., a wall thickness of 1 mm., a length of 5 mm., and at one end an inside flange of 3 mm. Figure 1 shows the type of cylinder employed.

In order to make a membrane, a cylinder was held with forceps at the unflanged end and dipped into molten 2 per cent agar at 50°–60° C. When lifted out, a thin film of agar adhered to the flanged end. The assembly was then held perpendicularly for a time to allow the excess agar to drain away from the membrane, and this was removed by touching against the lip of a beaker. When the film had hardened, the assembly was placed, flanged end upward, in a paraffin embedding jar over water-saturated filter paper. This jar was sealed with lanum and maintained at 22° overnight, after which the assembly was used for the experiments to be described.

A membrane made in this fashion is exceedingly thin. When thicker membranes (i.e., in excess of 100 μ) were required, a cylinder was placed flanged end downward on a glass slide. A measured volume of molten agar was pipetted within the flanged area. When the agar had hardened, the cylinder was pushed off the glass plate, the membrane adhering to the flange. The assembly was placed in an imbedding jar and treated as previously described.

D. MEASUREMENT OF MEMBRANE THICKNESS

Thickness determinations were performed with the aid of a microscope fine adjustment whose angular displacement had been calibrated in terms of the perpendicular displacement of the objectives. The distance through which the low-power objective had to travel to be focused first upon cells atop the membrane and then upon cells on the underside was taken to be the membrane thickness at that spot. The accuracy of the calibration was checked by measurement of a known distance (the height of the counting chamber in a Levy hemocytometer).
RESULTS

A. IMPREGNABILITY OF THE MEMBRANES

In order to test the membranes for the presence of holes through which organisms might pass, specimens were prepared from glucose-peptone agar under sterile conditions. An appropriately diluted suspension of *D. discoideum* spores, free of bacteria, was dispensed on one side of a membrane and a drop of *A. aerogenes* broth culture on the other. Such systems were incubated for as long as five days. Controls consisted of membranes where bacteria and spores had been inoculated on the same side. In addition, sterility controls to test for the presence of bacteria were run on the spore suspensions.

On control membranes, the growth was that to be expected from previous experience using agar plates. On the experimental membranes, the spores germinated, a process known to occur in the absence of bacteria; but growth of the myxamoebae was observed in only one membrane of the first ten tested, and this only after appreciable delay. It was surmised that air bubbles in the molten agar had produced this imperfection, and, when membranes with air bubbles were culled, no subsequent failures were encountered.

B. TESTS OF MORPHOGENETICALLY DEFICIENT STRAINS

In the original demonstration of synergistic development, washed cell suspensions of the deficient stocks in salt solution were mixed in paired combinations and in varying cell ratios and dispensed on a washed agar–distilled water substratum. Table 1 summarizes the types of synergistic structure observed. To test for ex-

<table>
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<th>Strain</th>
<th>Extent of Development When Alone</th>
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<th>Extent of Synergistic Development</th>
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<tr>
<td>Fr-1</td>
<td>Loose aggregates</td>
<td>Fr-4</td>
<td>Immature fruits</td>
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<td>Agg-53</td>
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<td>Agg-53A</td>
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<td>Agg-59</td>
<td>Mature, normal and thick-stemmed fruits</td>
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<td>Agg-204</td>
<td>Immature, and bushy fruits†</td>
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<td>Fr-2</td>
<td>Tightly knit but aberrant aggregates</td>
<td>Agg-59</td>
<td>Mature, normal and bushy fruits†</td>
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<td></td>
<td></td>
<td>Agg-204</td>
<td>Mature, normal and bushy fruits†</td>
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<td>Fr-3</td>
<td>Pre-pseudoplasmodia</td>
<td>Agg-53</td>
<td>Mature, normal fruits</td>
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<td></td>
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<td>Agg-53A</td>
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<td>Agg-204</td>
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<td></td>
<td>Agg-206</td>
<td>Mature, normal fruits</td>
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*See text for experimental details. The term "mature" refers to the fact that the fruits possessed terminally developed spore and stalk cells. "Normal" indicates that the fruitings were indistinguishable from those of the wild type.

† "Bushy" refers to the appearance of a variant strain of NC-4 (Sussman and Sussman, *Ann. N.Y. Acad. Sci.*, 56, 949, 1953). Agg-204 and Agg-206 were isolated from this stock.

change of diffusible materials, the stocks which had responded synergistically when paired as described above were washed and suspended in salt solution. Dilutions containing 10⁶, 10⁶, and 10⁶ cells/cc. were prepared, and 0.01-cc. aliquots were placed
on washed agar–distilled water membranes. After a few minutes the cells had lodged upon the agar surface, and the excess fluid could be drained away by decantation without disturbing the cells. Then the maneuver was repeated on the other side of each membrane with a suspension of myxamoebae from a second deficient strain. The synergistic pairs were tested in all possible cell ratios, as given above. In addition, all strains were tested against the wild type NC-4. The results can be categorized as follows:

1. **Wild Type versus Fruitless; Fruitless versus Fruitless.**—The wild type produced normal fruits; the fruitless variants, able to aggregate but incapable of further development, reached levels of development identical to those attained when the strains were incubated alone. The juxtaposition of another stock did not in any way affect the specific morphogenetic deficiencies.

2. **Wild Type versus Aggregateless; Fruitless versus Aggregateless.**—The wild type and fruitless strains developed precisely as they do when alone. The aggregateless cells were found to concentrate in small numbers directly above or below the centers of aggregation of the wild-type or fruitless forms.

3. **Fr-3 versus Any Other Strain.**—The fruitless stock Fr-3 differs from the other fruitless clones in that colonies on growth medium can proceed considerably beyond the aggregation stage. In contrast, if the cells are washed and placed on washed agar–distilled water medium, they aggregate but develop no further. When Fr-3 cells were dispensed on a washed agar membrane and opposed by any other strain, including aggregateless varieties, they were stimulated to equal the extent of development that they could achieve on growth medium. Thus, while passage of diffusible materials must have occurred in this instance, the result is rendered trivial by the nonspecificity of the response and the fact that the development was no more complete than on the growth medium.

### C. The Passage of Highly Labile Chemotactic Agents Across the Membrane

The failure of the deficient stocks to develop synergistically when separated by a membrane is not of itself a crucial datum, since one might argue that the substances passed are extremely labile and are destroyed before penetrating the barrier. No answer to this objection can be conclusive, because, while there is a practical limit to how thin one can make the barrier, there is no limit to how labile one may imagine the substances to be. One can only hope to use as thin a membrane as possible and to demonstrate that agents known to be exceedingly labile can traverse the required distance. In order to satisfy the preceding requirement, a study was made of the ability of the chemotactic agent or agents associated with aggregation to pass through the membranes. Recently Shaffer has succeeded in obtaining active preparations of the aggregative principles by leaching them from *D. discoideum* and *Polysphondylium violaceum* pseudoplasmodia. His results indicate that the half-lives of these agents at room temperature are of the order of 1 minute.

The agents, given the generic name “acrasin” by Bonner, are produced by cells at the aggregative center and along the cell streams which converge upon it. Runyon by covering myxamoebae spread on agar with a Visking sausage casing and placing other myxamoebae on top, was able to demonstrate the passage of a diffusible agent through the membrane, since the aggregative centers above and below coincided exactly. The same phenomenon has been explored in greater
detail by the writers, using agar membranes of different thicknesses. Washed wild-type myxamoebae at a concentration of $10^7$ cells/cc. of salt solution were dispensed in 0.1-cc. aliquots on both sides of membranes of varied thicknesses. When aggregation was well under way, microscopic observation revealed degrees of attraction between aggregates above and below the membranes which could be categorized as follows: (1) strong attraction: the centers absolutely coincided, and the cell streams and branches thereof were precisely duplicated (see Fig. 2); (2) weak attraction: the centers coincided, but many of the streams and branches were not duplicated or, at best, were only approximately imitated above and below the membranes; (3) no attraction: the centers did not coincide, nor did the radially convergent streams.

When a pair of aggregates was found which could be indisputably relegated to one of the above categories, a determination was made of the membrane thickness at that point. Figure 3 summarizes the results obtained when myxamoebae of D. discoideum wild type opposed each other across the membranes and when similar

Fig. 2.—Strong chemotactic attraction between aggregates separated by a membrane. Cell streams atop the membrane are in focus. Coincident streams below the membrane are out of focus. ×200.

experiments were performed with D. mucoroides wild type. Regarding the first-named species, a strong aggregative attraction was observed at distances up to 140 μ, a weak response to 205 μ, and no attraction beyond 230 μ. The D. mucoroides system appears to be only slightly more sensitive. It was of interest to note that, when interspecific appositions were made between D. discoideum and D. mucoroides,
the coincidence of the heterologous aggregates followed the same pattern as shown in Figure 3, the sensitivity of response being no less than those of the homologous systems, a finding in agreement with the results obtained by Raper and Thom, using cell mixtures of the two species.

The attractive potency of aggregates of deficient stocks when opposed by other deficient varieties or by the wild type was also observed. In general, the aggregates of any fruitless strain could be shown to coincide with those of any other fruitless or the wild type across thin membranes (30–100 μ). Attraction at greater distances was not studied. The most interesting result was obtained when aggregateless myxamoebae were opposed by fruitless or wild-type cells across thin membranes. As mentioned previously, the aggregateless myxamoebae clustered directly above or below the opposed centers of aggregation. The areas within which the aggregateless cells were so affected were very small, being limited to a diameter of perhaps 0.2 mm. (to be compared with 3.0 mm., which is the approximate diameter of a wild-type aggregate, including the cell streams under the conditions employed). Subsequent study of more sensitive aggregateless stocks has shown that some cluster not
only at the site of a wild-type center but, to a lesser extent, at the outlying streams as well. This is consistent with the results of Bonner,\(^1\) which indicate that acrasin is produced both by the center and the streams. It has been shown\(^2\) that the aggregateless strains can respond to the aggregative stimulus imposed when mixed with wild-type cells. It would seem, therefore, that while they may be sensitive to chemotactic agent, the aggregateless cells must be incapable of producing it, since the production of acrasin by large numbers of sensitized cells would inevitably be accompanied by the formation of ramified cell streams over extensive areas and presumably the concerted movement of these cells toward the center.

**DISCUSSION**

It has been shown that, while synergistic development cannot be accomplished by deficient stocks separated by as little as 30 \(\mu\) of agar, at least one extremely labile, diffusible substance produced by the wild type can penetrate as much as 200 \(\mu\). It is the opinion of the writers that any interpretation of the synergistic morphogeneses based simply upon exchange of diffusible metabolites when considered in the light of these facts becomes highly improbable. One might argue that this is not the only requirement but that, in addition, specific geometric orientations must exist between the participating cells before effective exchanges can be accomplished. This extension of the argument at the level at which it must be applied seems no different from the assumption of exchange by direct cell contact. In any event, had diffusible substances any role in the synergistic process, it is reasonable to suppose that at least some interactions should have been observed, albeit with aberrant results.

Regarding the general applicability of the agar membrane system, it should be noted that the membranes are quite simple to prepare and manipulate. They might conceivably prove useful in many instances where interactions between cell populations must be observed on a solid substratum.

**SUMMARY**

Morphogenetically deficient variants of the slime mold *Dictyostelium discoideum* cannot complete the normal developmental sequence when alone but can do so synergistically when mixed in paired combinations. Such partners have been placed on opposite sides of agar membranes possessing thicknesses as little as 30 \(\mu\), or about two myxamoeboid cell diameters. With one exception, synergistic development could not be accomplished under these conditions. In contrast, a substance which has already been shown to be exchanged between the cells during development and is known to be both diffusible and highly labile could penetrate the membrane to a distance of at least 200 \(\mu\).

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\(^1\) K. B. Raper, "Developmental Patterns in Simple Slime Molds," *Growth (Third Symposium)*, 5, 41, 1941.


Previous investigations\(^1\) have established that the anomalous action of the \(A^b\) "allele" from Ecuador,\(^2\) which is associated with purple plant and aleurone and dominant brown pericarp pigmentation, is ascribable to a gene complex of closely linked components which are separable by crossing-over. Thus the rare occurrences of the \(A^4\) or dilute-acting derivatives among gametes from \(A^b\) \(a\) plants are, in almost all cases, associated with crossing-over at the locus. Analyses of the \(A^4\)-bearing crossover strands in these experiments indicate that the \(A^4\) (designated \(\alpha\)) component is the leftmost or proximal member of the complex. The dominant brown pericarp effect of \(A^b\) is attributable to this member of the complex. Certain technical difficulties have hindered the isolation and characterization of the rightmost component (designated \(\beta\)), but recent preliminary experiments indicate that it may be isolated on the reciprocal crossover strand. The \(\beta\) element, like \(A^b\) from which it derives, produces a purple plant and aleurone but differs from it in its determination of red pigment in the pericarp. Using the \(C\) notation for the centromere, the order of the members of the \(A^b\) complex is \(C\alpha\beta\).

More recent studies\(^3\),\(^4\) indicate that most of the \(A^4\)-derivatives from \(A^4/A^b\) individuals also occur in association with crossing over. On the basis of this observation and additional evidence on the derivatives from certain special compounds involving \(A^b\), it was concluded that \(\alpha\) and \(\beta\), or the segments in which they reside, are members of an adjacent duplication in which the genetic materials are ordered in the