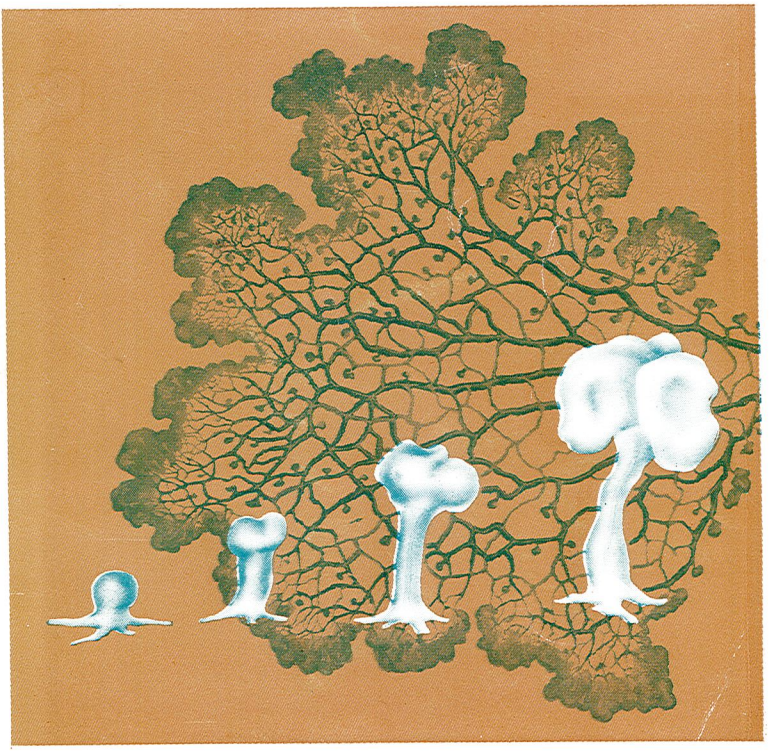
BSCS PAMPHLETS 13

Slime Molds and Research



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AMERICAN INSTITUTE OF BIOLOGICAL SCIENCES
Biological Sciences Curriculum Study

Slime Molds and Research

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SLIME MOLDS AND RESEARCH

By C. J. Alexopoulos and James Koevenig

Introduction

When man first appeared on the surface of the earth, about a million years ago, and began observing his environment in an intelligent fashion, he must have noticed that some substances grew larger day after day and others did not. The growing things were of two kinds: some were green, stationary, and never seemed to eat anything, whereas others were not green, moved from place to place, and ate the green things or . . . each other. Man himself seemed to have more in common with creatures of this second category than with those of the first, but, of course, he was very much superior—at least in his own thinking—to any of them.

Thus systematic biology must have begun, with man mentally classifying all living beings as plants or animals. Many changes took place in man and in his thinking in the interim between that first observation and the development of man's great civilization which flowered in Athens 2500 years ago, but the ancient Greek philosophers—Aristotle, Theophrastus, etc.—who were founders of biology, continued to classify all living things into these two great categories. By the time Carolus Linnaeus, the great Swedish naturalist, came into the picture, man had amassed much information concerning living organisms. The microscope had been invented and Leeuwenhoek, the Dutch lens grinder, had already discovered bacteria and other microscopic organisms which he called *animalcules* because they moved rapidly under his lenses and did not exhibit any green color. Nevertheless, in his famous *Systema Naturae* (1758), from which modern systematic biology begins, Linnaeus still classified all living beings into the same two great categories primitive man had conceived, plants and animals, which Linnaeus called "kingdoms."

It was not until 1866 that Haeckel, a German biologist, suggested that this old-fashioned system presented problems when it came to classifying certain types of organisms which did not seem to fit snugly into either category. He proposed a third kingdom, the *Protista,* in which troublesome organisms could be placed. The true slime molds, at the borderline between the plant and animal kingdoms, are among such organisms. One phase of their life cycle is definitely animal-like. It creeps in an ameboid fashion over the substratum, just like an animal, and "eats" solid food. It has no cell walls and no chlorophyll. All of these are animal-like characters. But give it some time, food, and proper conditions of moisture, light, and temperature, and one day it will change itself into a myriad of tiny, often brilliantly-colored balls, goblets, cones, or featherlike structures, which are solidly attached to a log or dead leaf, and which give off, with every gust of wind, thousands of microscopic spores, each surrounded by a cellulose wall. Stationary, spore-bearing, cellulose-containing! Certainly these are plant characters.

Plants or animals? Myxomycetes (slime molds) or Mycetozoa (fungous animals)? Think of them as you will, call them what you wish, but remember that the study of these organisms is beginning to give us answers to some fundamental biological questions. The Myxomycetes are no longer the property of a few devoted specialists. They are being widely used as experimental organisms by physiologists, taxonomists, developmental morphologists, geneticists, biochemists, and biophysicists. They are used as sources of living material for experimental work for the solution of broad biological problems such as protoplasmic streaming, cell movement, cell differentiation, photoreception, nuclear division, and other exciting and important subjects. We shall discuss a few of these problems after we look at the general life cycle of the true slime molds. To begin with, let us ask the question, what do true slime molds look like and where do we find them?

Where Do We Find True Slime Molds?

Everyone who has strolled through the woods has seen true slime molds or has passed by them, but few people recognize them. A hunter is wandering through the forest following a

warm autumn rain. He sits on a rotting log and places his hand on what appears to be brown hair about one inch long growing out of the wood. A lumberjack kicks a four-inch white mass in a wet sawdust pile only to discover when it is broken open that it looks like soot inside. A husband stumbles out in the early morning hours to mow the lawn before spending a day at the golf links. He bends down to remove a rock and notices a large bluish patch on the grass. A little girl runs through moist leaves in a wooded residential area. She picks up a leaf and turns it over to discover tiny metallic sausages on white stalks, comparable in size to pinheads. Unknowingly, all these people have discovered slime mold fruiting bodies. Within these fruiting bodies are spherical spores about 5-15μ in diameter with rigid walls. Each spore contains a quiescent protoplast.

Most of the 400-odd known species of true slime molds may be found all over the world in the proper habitat. Slime mold spores are usually distributed by the wind, which accounts for the widespread occurrence of most species. Nevertheless, certain ecological (environmental) factors may be important in restricting the distribution of a few species to the tropics or to the temperate zones of the earth. Moisture is probably most important. Even though we know that true slime molds do occur in desert regions, they are seldom seen there, and their occurrence is known only because they have been isolated and grown in the laboratory from material collected in the desert. For practical purposes, we can say that if you want to collect slime molds in the field you must look in moist places where organic matter abounds. Decaying logs and leaves in moist woods are the first places to look for slime molds during the warmer seasons of the year. If you look long enough and frequently enough you will find them!

If you confine your collecting to slime molds you see in the field, you will miss some of the more interesting and less commonly found species. In 1933, Professor G. W. Martin and his student Frank A. Gilbert wanted to show their botany classes the algae that grow on tree bark. They randomly collected bark samples from various trees around the campus of the University of Iowa and placed them in moist chambers (Petri dishes containing moistened filter paper discs) so as to revive the algae on

the bark. Several days later, upon examining the bark with a dissecting microscope, they found not only green algae but also fruiting bodies of some slime molds which had never been found in North America before! This technique is now widely used for the detection of minute slime molds, too small to see in the field, and for obtaining fruiting bodies, for class use or research any time of the year.

Fruiting bodies of true slime molds have been developed in moist chamber from such unlikely sources as dried cactus wood collected in the Arizona desert and from bark taken from trees in the winter when the temperature is as low as —30°C. Usually about a week is required before fruiting bodies develop on the bark or other material placed in moist chamber.

It is assumed that spores present on the substratum germinate and that the slime mold completes its entire life cycle in a week. This is a very short time, but not impossibly short. Sometimes, however, fruiting bodies appear on the bark in three or four days and, on rare occasions, in as short a time as 24 to 48 hours. It is hard to believe on such occasions that the complicated life cycle was completed from spore to spore in so short a time and some other explanation must be found to account for the sudden appearance of fruiting bodies.

The Life Cycle

One hundred fifty years ago, a Swedish botanist, Elias Fries, was out collecting fungi when he happened upon a slimy mass on some moist leaves. He placed the leaves in his hat, or so the story is told, and continued his collecting. Upon examining the mass later he found that it had spread all over his hat and had transformed into many small, cylindrical fruiting bodies. Magic? Hardly. Fries had simply observed the connection between two stages in the life history of these unusual organisms: the plasmodium and the fruiting bodies.

It was not until about 50 years later that the great German mycologist, Anton de Bary, germinated true slime mold spores and observed the remaining stages in the life history.

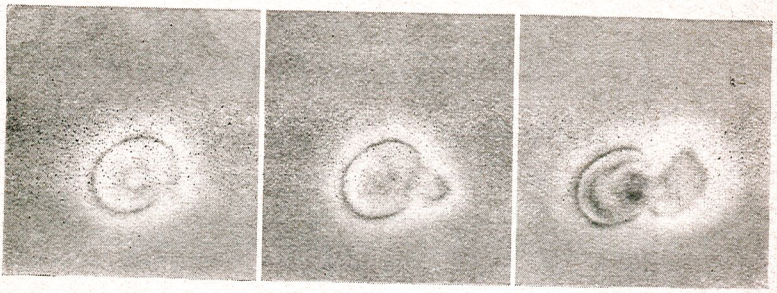
Let us now review the life cycle and follow these different stages in detail (see illustration on page 17).

When sufficient moisture is present, the spore walls crack open and each spore liberates one or more naked cells called *protoplasts.* Depending upon environmental conditions, each of the protoplasts either develops two flagella used in locomotion or it remains in the ameboid form. Sooner or later two flagellated protoplasts (swarm cells), or two ameboid protoplasts (myxamebae), meet and fuse, producing a single cell with a double number of chromosomes. This is the zygote and is roughly comparable to the fertilized egg of most plants and animals. The zygote grows, feeding on bacteria. Its nucleus divides and the two-nucleated cell which results is called a *plasmodium.* At this stage the plasmodium is minute and can be observed only through the microscope. With continued growth its nuclei continue to divide so that a large mass of protoplasm is eventually produced, containing thousands or even millions of nuclei. Though this mass of protoplasm has no cell walls, it nevertheless assumes a recognizable shape. Portions of the plasmodium become organized into veinlike tubes which trail back from a fanlike sheet of protoplasm constituting the anterior edge of the plasmodium. It is in this anterior portion that most of the feeding takes place. The plasmodium flows around those solid food particles with which it comes in contact, engulfs them, and digests them. After several days to several weeks the entire plasmodium, under the influence of various environmental factors, changes into one large or many small fruiting bodies—depending on the species—in which the spores and other structures develop. Just before the spores are formed, the number of chromosomes is halved through a special type of nuclear division called *meiosis.*

The transformation from a jellylike, more or less shapeless, acellular mass of protoplasm into a large number of intricately organized fruiting bodies containing spores takes place in a matter of 12 to 24 hours and is one of the most spectacular phenomena in the world of living things.

Problems for Research

Spore Germination. The simple description of the life cycle presented so far suggests that the sequential events in this transformation are completely known, and that there is little impor-



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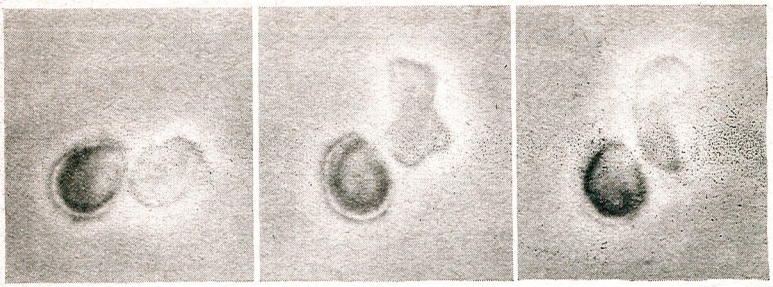
tant research left to be done. As a matter of fact, controversy and unsolved problems are almost characteristic of any study undertaken on the Myxomycetes. Even the simple process of spore germination has not been fully explained to the satisfaction of all biologists.

We say "when sufficient moisture is present, the spore walls crack open and each spore liberates one or more naked cells. . . ." What is "sufficient moisture"? If spores are placed in only a humid atmosphere, no germination occurs, although the spores may swell, indicating that some water has been absorbed. However, germination will occur if spores are placed in water or on a moist agar surface.

In this respect myxomycete spores are similar to plant seeds which also absorb water from a humid atmosphere and usually require liquid water for germination. In seeds the absorption of water depends in part upon seed coat permeability, which may change with time, or environmental conditions. The permeability of a myxomycete spore wall may also change. For example it has been suggested that bacteria aid in spore germination in some true slime molds. What effect do bacteria have on the permeability of the spore wall? Spores of most Myxomycetes will germinate immediately after they are formed, yet the percentage of germination may vary considerably after several months or years. Does this result from permeability changes in the spore wall? Is only permeability to water involved in spore germination? Seeds require varying amounts of oxygen in order to germinate. The respiration rate of dormant seeds, like that of hibernating animals, is quite low. The rate in seeds increases following absorp-



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Time-lapse sequence of a myxomycete spore germinating. Water is imbibed, the protoplast swells, the spore wall splits, and the protoplast becomes active, crawls out, and forms flagella. (× 1200)

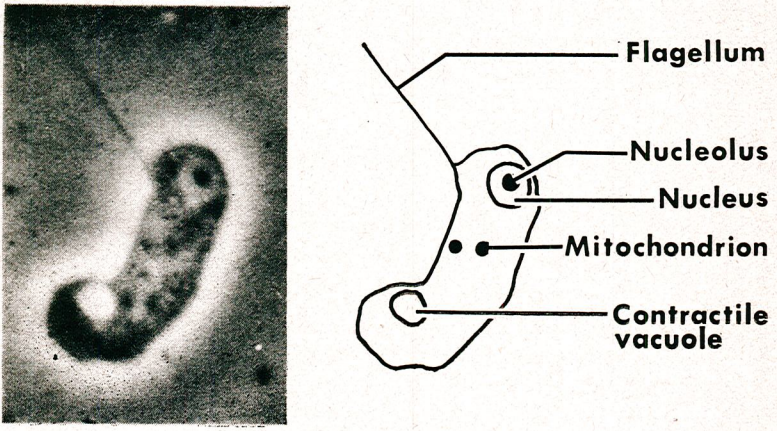
tion of water. The seed coat must usually be permeable to oxygen before germination is completed. Do myxomycete spores require oxygen in order to germinate? Is the respiration rate in dormant spores lower than that in germinating spores? These are just a few unanswered questions.

The next phrase in the sentence we are analyzing states "the spore walls crack open. . . ." This is true of some species but not of all. Studies by Jahn, a German biologist, and later by Gilbert and by Smart in the United States, have shown there are at least two methods of germination. The spores of some species (*Fuligo septica, Leocarpus fragilis,* etc.) do crack open, but spores of others (*Dictydiaethalium plumbeum, Stemonitis fusca,* etc.) seem to liberate their protoplasts through a minute pore which develops in the wall at the time of germination.

Is the mechanism similar in the two methods of germination? As early as 1905, Jahn presented the hypothesis that the protoplast absorbs water, swells and ruptures the spore wall similar to the rupture of the seed coat in a germinating lima bean. He postulated that activation of an enzyme which changed starch to sugar resulted in a greater uptake of water. Other biologists suggested that osmotic pressure caused spores to germinate by cracking open, and enzyme digestion of spore walls caused germination through a pore. Another hypothesis was that both osmosis and enzymes were involved in both methods of germination. Unfortunately, none of these hypotheses has yet been adequately

tested in the laboratory but we do know that temperature changes or the addition of an impure solution of the enzyme cellulase can change the method of germination.

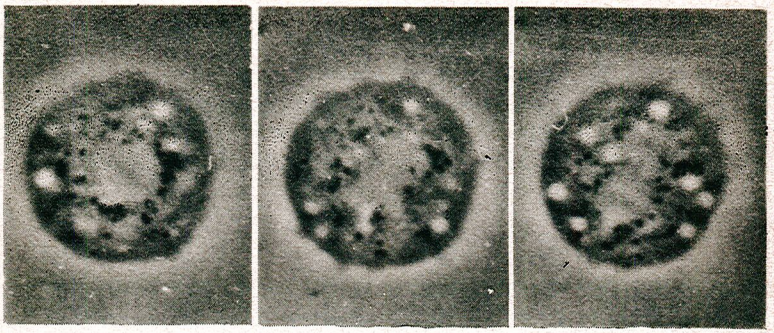
One of the most obvious approaches to the problem of germination is a study of the spore wall. Some photomicrographs and electron micrographs show that the spore wall is slightly variable in thickness. The distribution or simply the presence of thin places in the spore wall may be related to the method of germination. Composition of the spore wall may also be of considerable importance in this regard. The few investigations carried out so far simply show that cellulose is one of the constituents of some slime mold spores. Other compounds have not yet been identified. The employment of more modern analytic techniques, such as X-ray diffraction, will probably add greatly to our information of the chemical composition of this important structure. Much more work needs to be done before we have a clear understanding of either the physical structure or the chemical composition of the spore wall. Such knowledge is prerequisite to an understanding of the principles underlying spore germination.



The internal structure of a swarm cell is visible if one uses a phase microscope. (A swarm cell can change into a myxameba by losing its flagella. The myxameba, in turn, can reform flagella and become a swarm cell.) (× 2000)

Swarm Cells and Myxamebae. In our general description of the life cycle we said that "each spore liberates one or more naked cells. . . ." In 33 of 62 species investigated up to now, some spores give rise to more than one myxameba. This would indicate that the liberation of several protoplasts from a single spore is not uncommon. This may be of great significance in modern research on Myxomycetes most of which is based on clonal cultures. A clone is a genetically homogeneous population produced entirely by asexual means. The easiest way to obtain a clone in the Myxomycetes is to isolate a single spore in a container by itself and allow it to germinate and produce a population of haploid myxamebae through successive cell divisions. If a single spore liberates more than one protoplast, we cannot be certain that all the cells in a culture derived from a single spore are of the same genetic constitution since it is possible that two or more different protoplasts are enclosed within one spore wall. In at least one species (*Fuligo cinerea*) this has been tested by carefully observing the spores with a phase microscope. These studies revealed that only one protoplast and nucleus occurred in each spore, but they sometimes divided prior to germination, resulting in two emerging protoplasts. Assuming this division involved mitosis, then both of the cells are genetically identical.

We stated that "each of the protoplasts develops two flagella. . . ." However, some authorities have reported protoplasts with one flagellum while others report two, or even more. One reason for this disagreement may be that one of the flagella is often difficult to see; another reason is that certain protoplasmic extensions (pseudoflagella) of the swarm cell may be mistaken for true flagella. The time during which observations are made may also be a factor. For example, it has been shown that in *Didymium nigripes* each protoplast develops one flagellum soon after it emerges from the spore and a second flagellum sometime later. Thus, if an observer counts the flagella soon after germination, he may conclude that swarm cells are uniflagellate, but if he observed them several hours after germination he may conclude that they are biflagellate. Though these reports may seem to contradict one another, they may both be correct. The descriptions must simply be qualified with a statement of time elapsed after germination.



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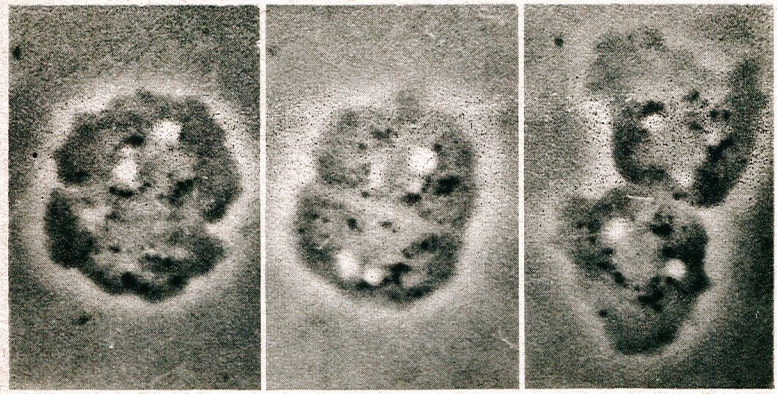
You may ask: "What difference does it make whether there is one or more flagella?" It depends what the scientist is interested in. If he is studying cell movement, it is essential that the motion of all the flagella be analyzed. If he is primarily interested in evolutionary relationships, the type of flagellation may be an important clue to the relationships he is seeking—at least according to current concepts. There is, for example, another group of organisms, called the cellular slime molds (Acrasiales), whose naked protoplasts never develop flagella. Most authorities believe the Acrasiales are not related to the Myxomycetes because they lack flagella. If the spores of certain Myxomycetes are germinated on agar whose surface is relatively dry, the emerging protoplasts do not develop flagella. Does this mean that the Myxomycetes and Acrasiales are more closely related than is generally supposed? Not at all, because the capability to produce flagella is still present in the Myxomycetes, even though flagella formation has been suppressed by manipulating the environment. None of the Acrasiales is capable of producing a flagellated stage under any known conditions.

Sexual Fusions and Mating Types. Continuing our examination of the life cycle as summarized, "Sooner or later, two flagellated . . . or two ameboid cells . . . meet and fuse to produce [a zygote]." Some of the questions that can be raised regarding this statement include: Exactly when does this meeting take place? What determines fusion? Can any two cells fuse? Are the cells



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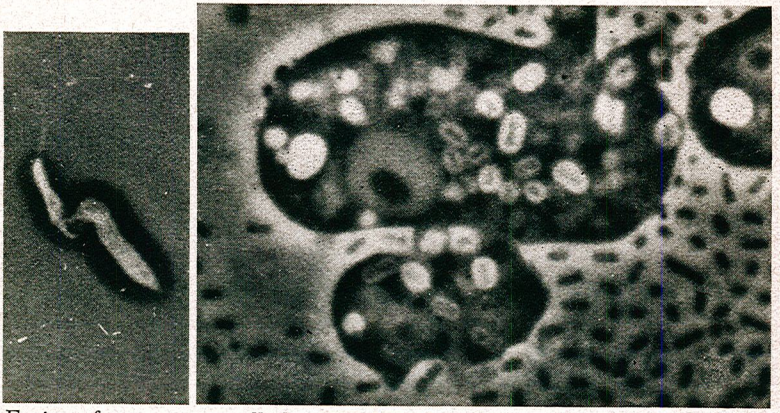
A clone results from successive divisions of myxamoebae similar to the division shown in this time-lapse sequence. A. Metaphase, B. Anaphase, C. Telophase, D. Cleavage beginning, E. Cleavage completed, F. Daughter myxamoebae move apart. (× 3000)

attracted to one another? Do flagellated cells fuse in some species and ameboid cells in others?

It has been known for some time that if two swarm cells or two myxamebae of any species are placed together in a culture dish they may not fuse. Recent evidence suggests that before fusion occurs the number of myxamebae or swarm cells must reach a certain level. Even when the clone reaches a critical density, relatively few cells fuse, if the number of zygotes or minute plasmodia that are formed is any indication of the number of fusions. Is it possible that there is some initiating chemical substance which has to reach a certain concentration before fusion can occur? Does it accumulate as the population increases, or is its release triggered by the age or density of the population? Does it cause morphological and physiological changes in cells? We do not know.

Another fundamental question is: May any two cells fuse? We have some answers to this question. If we isolate single spores of *Fuligo cinerea,* allow them to germinate, then isolate single protoplasts and wait until large clones (in which all cells are genetically alike) are formed, plasmodia develop in nearly all cultures. If we assume that fusion of cells is prerequisite to plas-



Fusion of two swarm cells begins when they attach at their posterior ends (left). In several species the fusing cells must be of different mating types. (× 900). (Right), The zygote which results from sexual fusion differs slightly from a myxameba. It is usually larger, more granular, and has a larger nucleus. (× 3000)

modial formation, we shall have to conclude that, in this species, all cells are sexually compatible, *i. e.* that any two cells in the population may fuse. Most species of slime molds which have been investigated (only a small percentage of the total) behave in this way.

Contrarywise, when we isolate and grow cells of *Didymium iridis*, we find that plasmodia do not develop in such clonal cultures. However, when we mix cells from different clones, we find that there are two types of cells with respect to fusion. Cells of type "A" fuse with type "B" cells, but "A" cells will not fuse with each other, nor will "B" cells fuse with each other. Some kind of mating system appears to be involved. When plasmodia resulting from fusions between "A" and "B" cells are induced to produce fruiting bodies and a large number of individual spores are then isolated, we find that the two mating types are present in approximately equal numbers. This suggests that the mating types are genetically controlled. Each of the diploid nuclei in the plasmodium possesses both of the genes controlling mating. The genes are segregated from one another during meiosis which precedes spore formation. The spore (and resulting myxameba) nucleus is haploid and possesses only one gene controlling mat-

ing. Isolation of single spores of different geographical strains of *Didymium iridis* has revealed six additional mating types all of which appear to be controlled by genes located at the same locus on a chromosome. It is probable that many additional mating types exist.

These discoveries raise the question, why are some cells compatible, whereas others are not? We can observe no differences when we examine living or dead cells of different mating types. Is there some chemical difference between mating types that will explain their mating behavior? Maybe our clue will come from similar situations in other organisms. In certain yeasts, for example, and in the green alga *Chlamydomonas,* cells of one mating type have a protein on their surface while cells of the other mating type have a complex sugar on their surface. During fusion these different compounds interact. Perhaps there is a similar mechanism in the slime molds. In order to analyze chemically the cells, their secretions, or byproducts, we must have pure cultures (cultures in which only one species of living organism is present) grown in a defined medium (one in which we know precisely the identity and amounts of the ingredients).

Much of what we know about the biology of bacteria and fungi has been learned by painstaking observations and experiments in the laboratory with organisms growing in artificial culture, usually on agar to which nutrients have been added. The same is true with Myxomycetes. But whereas no bacteriologist or mycologist would consider working with anything but a pure culture, the "myxomycetologist" has had to be content to work with the crudest type of culture—one in which bacteria, fungi, protozoa are mixed together with the slime mold he is studying. Suffice it to say that only about 10% of the known species of slime molds have been grown in culture under any conditions and that not a single species has been grown in culture from spore to spore without bacterial cells present in the culture!

This is probably the greatest stumbling block at the present time in slime mold research, and must be solved before we can make significant headway in investigating the biology of these intriguing plant-animals. For this reason many research efforts are being devoted to this problem and considerable progress has already been made.

Laboratory Culture. Myxomycete swarm cells or myxamebae can be grown in two-membered culture with known species of living bacteria, or in pure culture (with no other living organisms present) with dead bacterial cells. Two methods may be used to accomplish this, depending on the species of slime mold being investigated.

With slime molds whose myxamebae are active and move rapidly, we may spread the spores on the surface of plain agar, which does not support bacterial growth, and allow them to germinate. The myxamebae will then crawl away from the spores and leave the bacteria behind. We can then isolate the pure mxyamebae, transfer them to an agar medium that will support only moderate bacterial growth and add a few drops of a suspension of living cells of *Escherichia coli, Aerobacter aerogenes, Serratia marcescens,* or other bacterial species. We then incubate the culture at 20-25°C. The weak medium and low temperature will permit only enough bacterial growth to provide food for the myxamebae. Eventually, zygotes and plasmodia will develop, and sporulation will occur in a two-membered culture. The same technique may be used to obtain pure cultures by feeding the myxamebae with killed bacteria instead of living bacteria. However, it is necessary to kill the bacteria, not with heat but with formalin or some other chemical. If we add bacteria killed by heat, the myxamebae will not grow. Heat apparently destroys some substance in the bacterial cells necessary for the growth of the myxamebae.

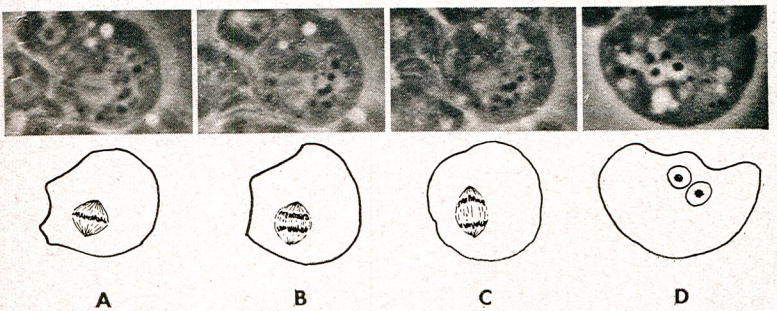
For slime molds whose myxamebae do not move away from the empty spore cases rapidly enough to free themselves from the bacteria, we must start our cultures with spores which we have first treated with a germ killing agent to eliminate the bacteria. We must be careful, however, to use concentrations that do not injure the slime mold spores.

Some progress has been made recently toward culturing slime mold myxamebae in the absence of bacteria. Partially defined media (containing some ingredients of unknown chemical composition) have been developed in which myxamebae grow and multiply in the absence of bacterial cells living or dead. Myxamebae fuse on such media and develop zygotes which grow into minute plasmodia, but the plasmodia soon die. The nutrient re-

quirements of the plasmodia are different from those of the myxamebae of the same species. This suggests that the presence of a double set of genes in the nuclei somehow changes the nutritional requirements, but the mechanism for this change remains completely unknown.

Zygote Formation and Plasmodial Development. Earlier in this discussion we stated that the zygote is roughly comparable to the fertilized eggs of animals and green plants. This is true only in that it initiates the diploid phase of the life cycle. But there are some fundamental differences. In the slime molds there is no egg and sperm as such. The two fusing cells are structurally identical, and each contributes approximately equal quantities of cytoplasm. In the more complex animals and plants virtually all the cytoplasm in the zygote comes from the egg, the sperm contributing mostly nuclear material and very little, if any, cytoplasm.

Let us now look at the mechanics of zygote formation in the slime molds. When two compatible myxamebae or swarm cells come together, their membranes somehow break down at the point of contact and the cytoplasm fuses into a single protoplast. The general assumption has been that the two nuclei come together and fuse, and this has been repeated in the literature so many times that we sometimes forgot there was no actual proof that this was so. To be sure there was some supporting evidence. Many investigators killed and stained populations of myxamebae



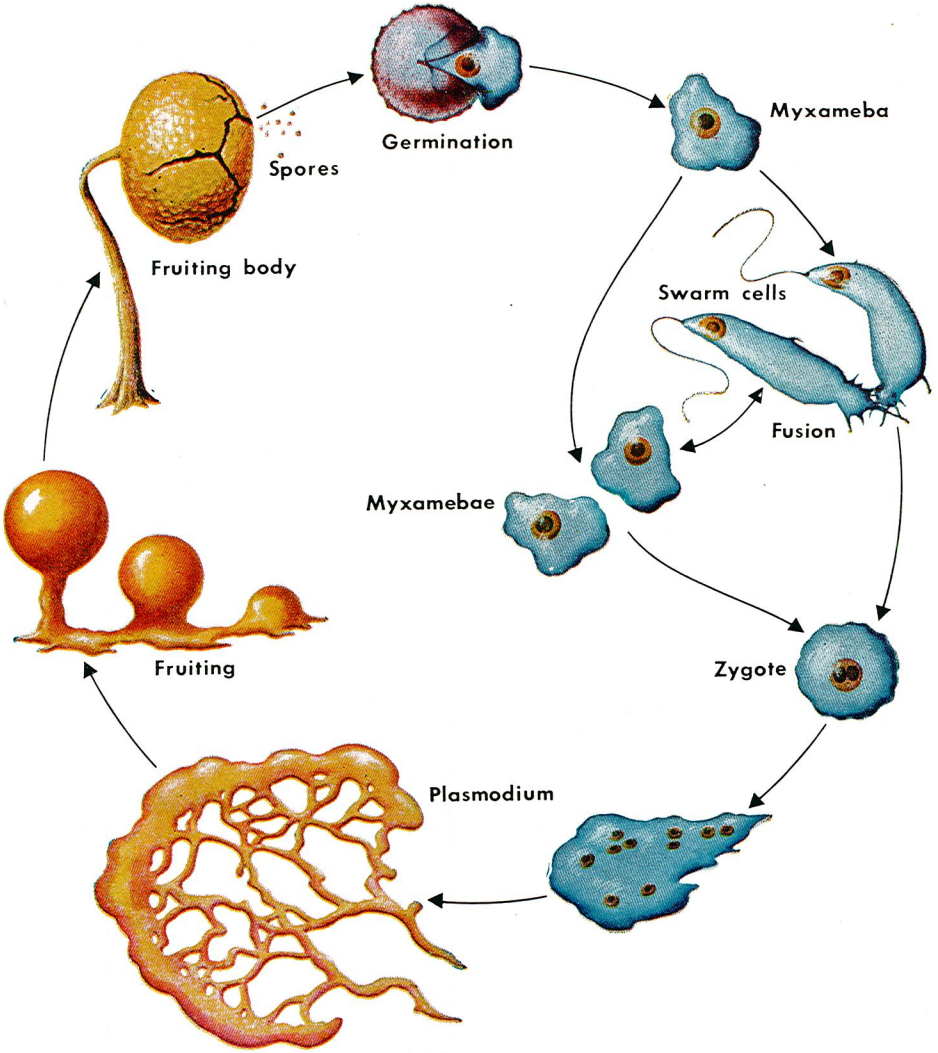
The zygote may develop into a two-nucleus plasmodium by division of its nucleus as shown in this time-lapse series. A. Metaphase, B. Anaphase, C. Late anaphase—early telophase, D. Two nuclei. (× 1750)

and found cells with two nuclei in close contact which they believed to be in the process of fusing. But this was not proof. Could not these nuclei be the products of mitosis in a dividing myxameba? How then obtain proof? *Only by observing the living nuclei actually fuse,* the sticklers for proof insisted. But the nuclei are very difficult to see under the ordinary microscope. Even when the phase microscope was perfected and the nuclei could be easily distinguished, it was necessary to find two fusing cells and then watch them for hours in order to actually see nuclear fusion. This question was finally resolved when a motion picture camera was placed on a phase microscope and pictures taken every few seconds actually recorded the fusion of nuclei in a zygote.

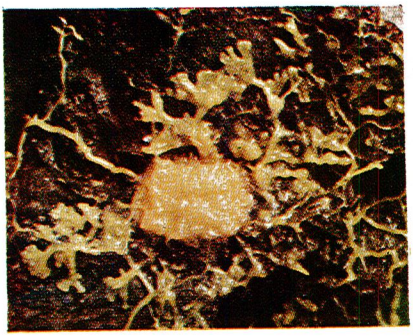
In higher plants and animals the zygote develops into a mature organism by successive cell divisions. In the slime molds we have said that the situation is comparable and that the zygote develops into a plasmodium, which is the body of the slime mold. How do we know this is true? The evidence comes through the observation that when a single pair of mating cells is isolated in a separate Petri dish, a plasmodium will eventually develop. This takes place by growth of the zygote and by nuclear division unaccompanied by cell division. The zygote feeds on bacteria and grows in size just as the myxamebae do. But unlike the myxamebae which divide many times and form a population of cells, the zygote does not divide. Only its nucleus divides, the two daughter nuclei remaining together in the cytoplasm. The binucleate protoplast thus formed is the first stage of the plasmodium. The two nuclei may divide again soon. Again there is no cytoplasmic division and the resulting protoplast is quadrinucleate.

The formation of the plasmodium from a zygote was discovered in the latter part of the 19th century by killing, fixing, and staining methods which had revealed two simultaneous mitotic divisions in a single protoplast. This constituted the best proof possible short of actually watching the nuclei go through the whole division process. Again the time-lapse movie camera provided additional proof by filming the first two divisions of the diploid nucleus in the zygote and the first stages in the formation of the plasmodium.

But some scientists believe that, in certain slime molds, many zygotes, produced in a given population of myxamebae, are at-

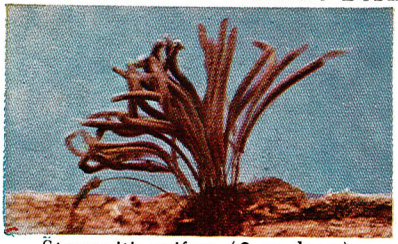


Above: Diagram of a generalized life cycle of a slime mold. The spore germinates giving rise to a cell which is either a myxameba or swarm cell depending on whether flagella are present. Flagella may be lost under certain environmental conditions, like evaporation of the surrounding water, and may be re-formed under other conditions, like replacement of surrounding water.



Right: Mature plasmodium of *Physarum polycephalum* which is beginning to change into fruiting bodies.

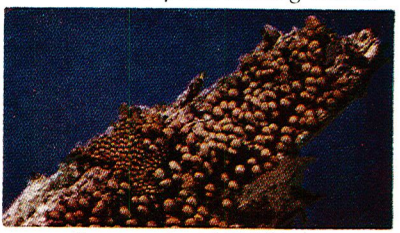
Diversity of Fruiting Bodies in Some Common Slime Molds



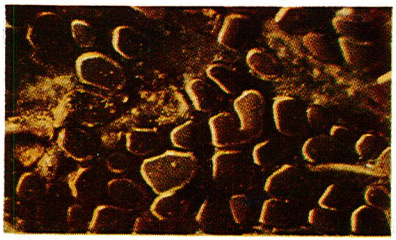
Stemonitis axifera (2 cm long).

Diachea leucopodia fruiting bodies.

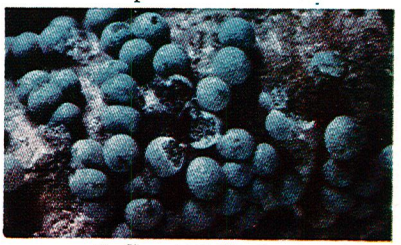
Physarium viride fruiting bodies.



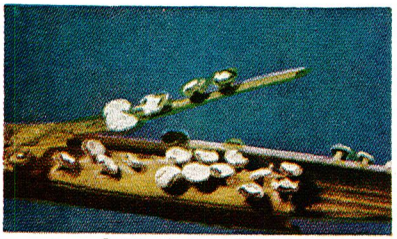
Two species of *Trichia*.



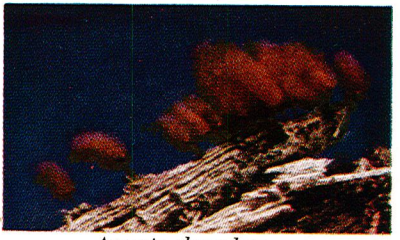
Perichaena depressa fruiting bodies.



Badhamia foliicola.

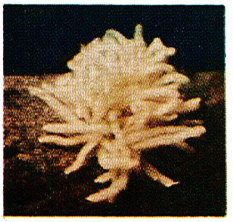


Diderma hemisphaericum.

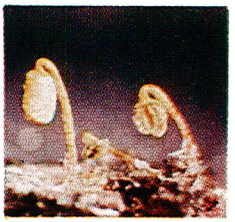


Arcyria denudata.

A *Fuligo septica* fruiting body is a limy mass of tubes, 20 cm wide.

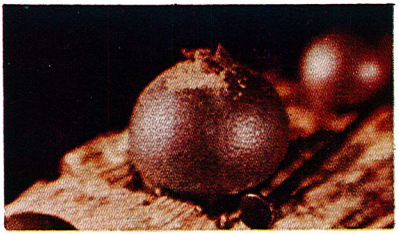


The long drooping yellow structures are nets of *Arcyria nutans*.



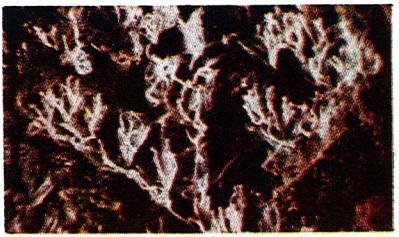
Physarella oblonga fruiting bodies. One has been cut in half.

Leocarpus fragilis resembles tiny (4 mm tall) golden eggs.

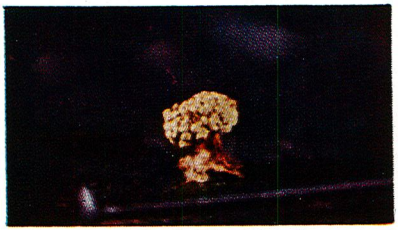


Lycogala epidendrum fruiting bodies forming *globose* bodies.

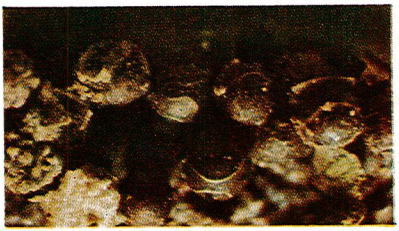
In *Hemitrichia serpula* the plasmodia veins become the fruiting body.



Ceratiomyxa fruticulosa spores appear to be attached to the surface of stalks.



Physarum polycephalum has been used in most physiological research studies.

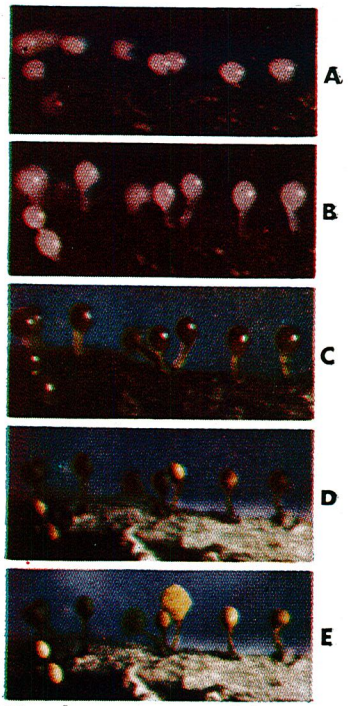


These 1 mm baseballs are fruiting bodies of *Perichaena corticalis.*

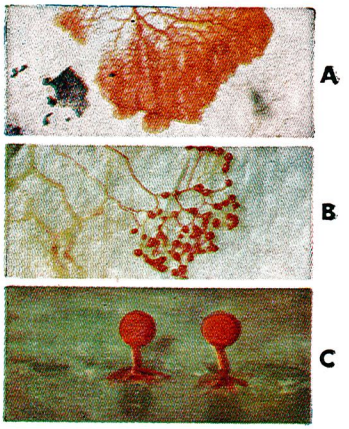
The outer covering of a *Hemitrichia stipitata* fruiting body breaks as an inner net expands.

Physarum rubiginosum has lime deposits within the fruiting bodies.

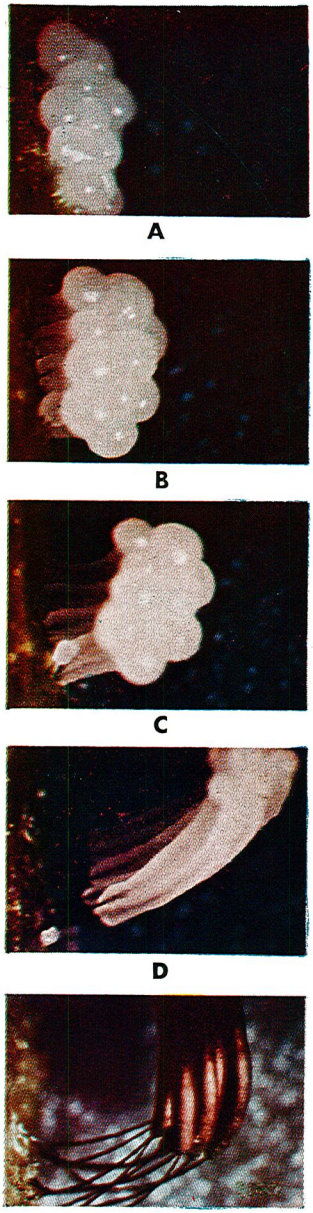
Many fused tubes form a *Dictydiaethalium plumbeum* fruiting body.



Time-lapse sequence of fruiting in *Hemitrichia stipitata*. The entire change takes 6–12 hours.



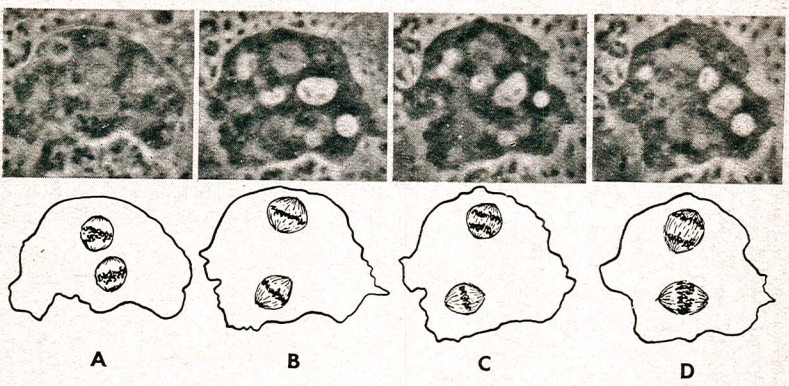
(A) Fanlike tip of mature *Physarum roseum* plasmodium. (B) Same plasmodium 8 days later. (C) Greater magnification.



A time-lapse sequence of fruiting in *Stemonitis fusca* shows a noncellular stalk forming inside the transforming protoplasm.

tracted to one another, aggregate, and form one large mass. The membranes then break down and the mass fusion of protoplasts results in a multinucleate plasmodium. This is certainly a possibility, and many observers believe they have visual evidence of this method of plasmodium formation. Certainly we know that small plasmodia unite and pool their multinucleate cell with a uninucleate cell, presumed to be a zygote, but rigid, undisputed proof of plasmodium formation by the coalescence of many zygotes is still lacking.

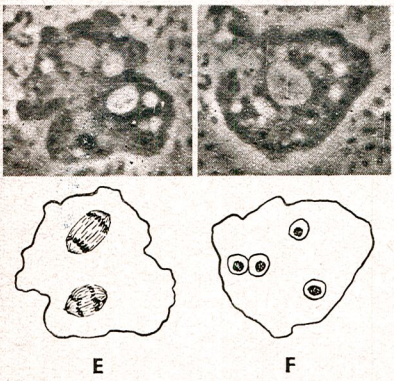
We have already seen how a single zygote develops into a multinucleate plasmodium, but surely the big mass of protoplasm easily visible to the naked eye results from more than fusion of microscopic cells or zygotes. In most living organisms development involves cell division, growth, and differentiation. Since the plasmodium, contrary to the cell theory, does not consist of cells but is acellular, no cell division is involved in its development although the nuclei divide many times. What about growth? We can actually see that from day to day a plasmodium in culture covers a larger area of the agar in a Petri dish. We must remember, however, that a plasmodium has no cell walls; no confining boundaries. When it comes in contact with food, it clumps over the food particle and may appear small, whereas in the absence or scarcity of food particles, the plasmodium has a tendency to spread into a thin sheet of protoplasm over a large area. Might we be confusing the spreading of a plasmodium with growth? If we define growth as an increase in weight, then we should be able to weigh our organism at different intervals as you might weigh yourself to see if you are growing. It is not difficult for you to step on the scales, but it is difficult to remove a plasmodium from the substratum. Scientists at the cancer research laboratory of the University of Wisconsin decided they would have to grow pure cultures of a plasmodium in a liquid medium before they could harvest and weigh it accurately. This had never been done before. As a matter of fact no one knew whether plasmodia could obtain food in liquid form. Growing a plasmodium in liquid meant establishing a suitable carbon and nitrogen source, and supplying the necessary minerals, vitamins, and perhaps some unknown growth factors. The first successful Wisconsin medium had several ingredients of unknown chemical



composition such as tryptone and chick embryo extract, but by careful and patient research a completely defined medium was finally developed. It now became possible for the first time to grow the plasmodium of one slime mold—*Physarum polycephalum*—in pure culture, in a liquid medium of known chemical composition.

The weighing problem was not completely solved, however. The plasmodium fragmented and grew as small globules in the liquid medium. All of these pieces could not be removed, weighed, and placed back into the medium without injuring or contaminating the culture, hence samples had to be withdrawn. A method of weighing had to be established. If you weigh yourself, drink several glasses of water, and reweigh yourself, you will gain weight, yet this increase is not due to growth. Perhaps dry weight might be more accurate. An even more accurate method would be to measure the amount of protein present. Of the three basic types of compounds (carbohydrates, fats, and proteins) proteins alone contain nitrogen. If the plasmodium was grown in liquid culture, a precise amount of sample could be withdrawn, and the amount of nitrogen calculated. This would indicate the amount of protein present and, therefore, the protoplasmic mass of the sample. The way was thus opened for nutritional studies which were not possible before, and for the study of growth.

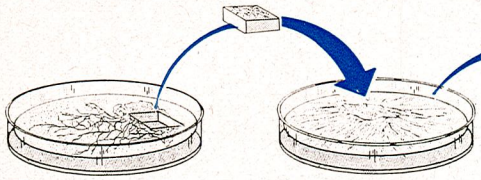
The development of a liquid culture technique brought forth some other interesting discoveries. It has been known for a long



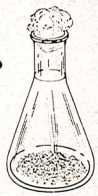
Nuclei within a plasmodium divide synchronously. This time-lapse sequence shows two nuclei dividing to form four. A. Late prophase—early metaphase, B. Metaphase, C. & D. Anaphase, E. Telophase, F. Four nuclei. (× 1750)

time that the nuclei in a growing plasmodium divide synchronously. Now it was found that in a chemically defined liquid medium, under strictly controlled conditions, this synchrony is nearly absolute. All the nuclei in a single plasmodial globule are usually at the same stage of division at exactly the same time. However, nuclei of different plasmodial globules growing in the same flask have different mitotic rhythms. When the globules from a single flask are harvested and allowed to spread on moist filter paper, they coalesce bringing together in the same cytoplasmic matrix nuclei with different mitotic rhythms. About 7½ hours following the fusion of the plasmodial globules all the nuclei divide synchronously!

Synchronous nuclear division or mitosis enables scientists to study large numbers of nuclei all in the same stage of division. One study used radioactive DNA (deoxyribonucleic acid) precursors to study DNA synthesis. It was found that DNA synthesis occurred immediately after mitosis with the DNA level remaining constant until the next division. This is in contrast to results from studies on other organisms. When the plasmodium was exposed to ionizing radiation, nuclear division was delayed or stopped, but DNA synthesis was apparently not hindered. These results suggest that although DNA must be synthesized before mitosis occurs the DNA synthesis does not trigger the division and may occur without the nuclei dividing. Synchronization may result from some system of communication between nuclei, possibly through some chemical substance which is carried through

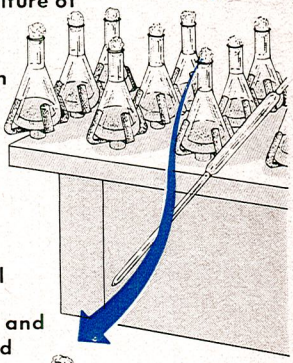


1. Elimination of contaminants by successive transfer of plasmodial fragments (usually 5) to sterile agar plates

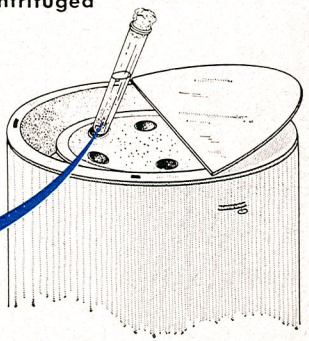


2. Transfer to sterile oat flakes

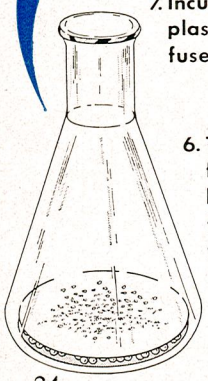
3. Determination of liquid defined medium



4. Routine culture of plasmodia in liquid medium on shaker in dark for 66 hrs.

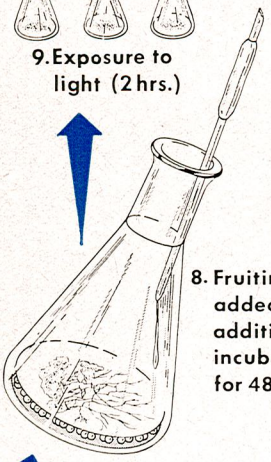


5. Plasmodial fragment harvested and centrifuged

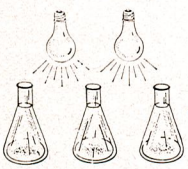


6. Transfer to moist filter paper (glass beads prevent paper from becoming too moist)

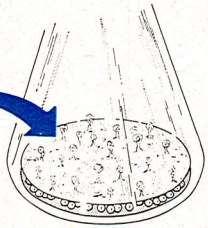
7. Incubation in dark, plasmodial fragments fuse (4 hrs.)



8. Fruiting medium added, additional dark incubation for 48 hrs.



9. Exposure to light (2 hrs.)



10. Fruiting (sporulation)

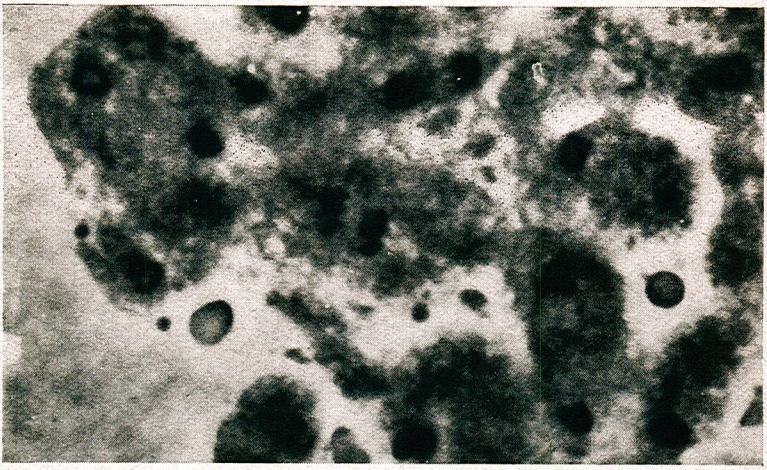
the cytoplasm but is unable to travel through the surrounding medium and affect the nuclei in another protoplast. What type of substance is this? Does it have to reach a certain concentration before the next nuclear division? Where and how is it formed? These questions, when answered, may have a bearing on the study of growth in all living organisms including man.

Differentiation is also involved in the development of a plasmodium. In most species the young plasmodium is a round, flattened, protoplasmic mass. Slowly it differentiates into the type of plasmodium characteristic for the species. Studies on different slime molds show that although the mature plasmodia of different species may be quite different, the young plasmodia are very similar. Why does one plasmodium remain small while another becomes large? Why does one develop an ameboid fan-shaped anterior end, while another becomes tubelike or lacelike? The general shape of a plasmodium is thought to be genetically controlled. But how do the genes regulate the development into one or the other? What effect does the environment have on the ultimate form? These questions are quite complicated, and we have just begun to find a few answers.

Sporulation. After a plasmodium grows to a certain stage under the proper environmental conditions, it becomes converted into one or more fruiting bodies according to species. Much of the research now in progress in various biological laboratories concerns itself with answering the questions: What is that "certain stage" which the plasmodium must reach before the mechanism which initiates fruiting is put into motion? What are the "proper environmental conditions"? Why do not all species of Myxomycetes follow the same pattern? Are there a number of morphogenetic patterns into which the slime molds fit?

Certain movements occur during the formation of fruiting bodies. Some of these are universal. For example, the plasmodium of one of the most common and widely distributed slime molds, *Ceratiomyxa fruticulosa*, grows inside the wood of decaying logs. One never sees it until it is ready to fruit, at which time

The technique of growing a plasmodium in pure culture on a defined medium and induction of fruiting took many years of determined and concentrated research.

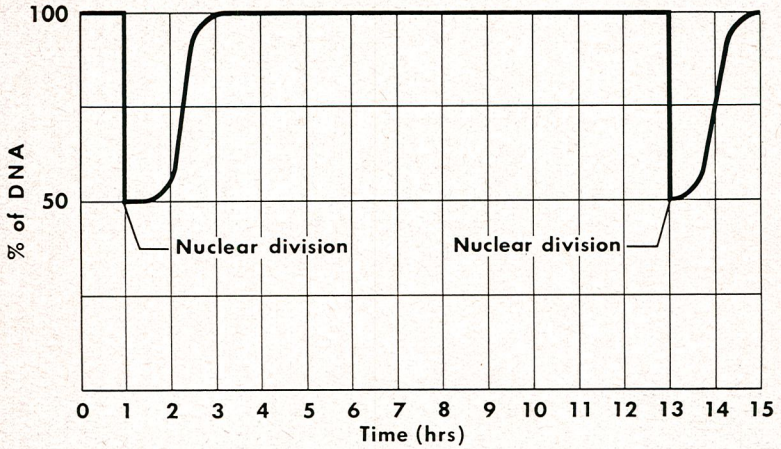


Synchronous division of nuclei in a mature plasmodium can be stained with chemicals which are specific for nucleic acids. (× 3500)

it emerges on the surface of the log and changes into a large number of fructifications. *Ceratiomyxa* simply does not fruit inside the wood. If it did, its spores could not possibly be dispersed and it would certainly not be as widespread as it is. This emergence just prior to fruiting is common among Myxomycetes. What is it that compels the plasmodia to come to the surface?

The rhythmical back and forth streaming of the cytoplasm within a plasmodium is also nearly universal. This movement continues until the fruiting bodies are formed and the cytoplasm divides into spores. Although the cytoplasmic streaming within a developing fruiting body appears to be random, the gross external movements appear to be quite specific. The clumping, elongating, shortening, pushing out, pinching in, twisting, interweaving, and fusing determine the characteristic external shape for each species, although these movements and the shape may be modified by environmental conditions. What controls the specific movements? If the control is nuclear, how can the nuclei which are carried back and forth, control specific units of protoplasm?

Most organisms exhibit polarity sometime during development. Even in the cellular slime molds or Acrasiales, where the fruiting body is formed by many aggregating amebae, polarity occurs.

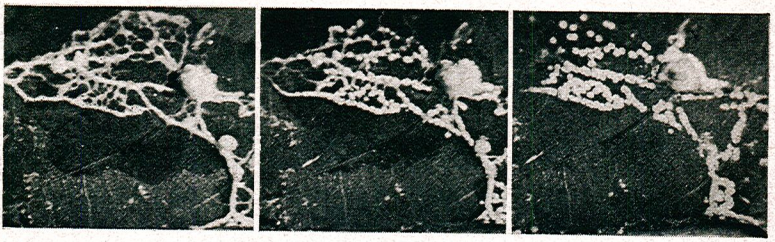


Measurements of DNA synthesis within a plasmodium were made with radioactive tracers. Results showed that DNA synthesis occurred immediately after rather than before mitosis, in contrast to what happens in other organisms which have been investigated.

The plasmodium of the Myxomycetes also exhibits a certain degree of polarity. When a certain stage of maturity is reached, the plasmodium forms a frontal fan and always moves in its direction. Recent research has also shown that the front and rear portions of a plasmodium differ in chemical composition.

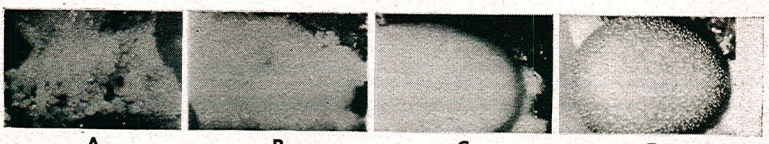
Differentiation may be defined as chemical and morphological or observable change. Early in the development of most animals the embryo undergoes certain chemical changes so that if the embryo is cut in half an incomplete embryo or adult results. In plants some chemical differentiation is evident even though it is reversible. In the Acrasiales some chemical change in the amebae occurs very early in the life cycle. Some amebae will form the stalks of the fruiting bodies whereas others will form the spores. In the myxomycete plasmodium, however, the chemical differentiation is apparent only during the actual formation of the fruiting bodies. After fruiting has been initiated in a plasmodium, portions of this plasmodium can be cut away. Each portion, no matter how small, will usually develop into one or more fruiting bodies. Even pieces of a developing fruiting body will often form totally functional, although aberrant fruiting bodies.

Considerable information is available on the morphological



A B C

In *Diderma testaceum* the external changes involved in fruiting are slight. The clumped plasmodial veins become the fruiting bodies. (× 1)

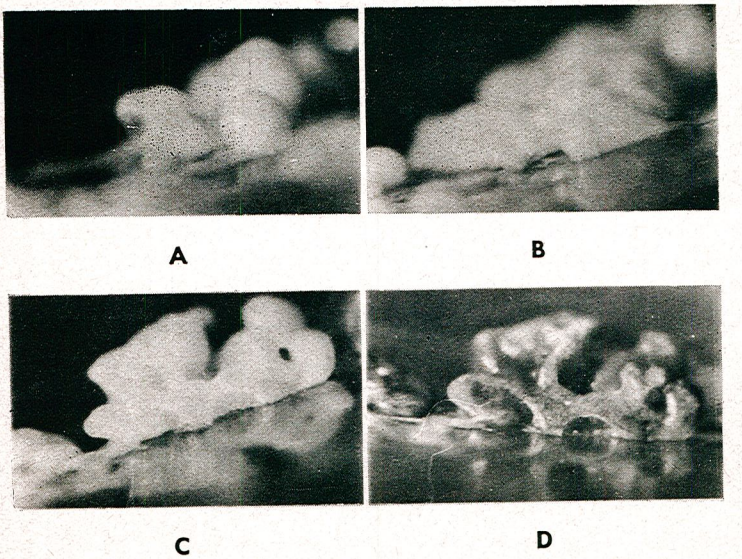


A B C D

In *Dictydiaethalium plumbeum* many papillae fuse to form one large fruiting body. (× 1)

changes that result in the formation of the fruiting bodies of various Myxomycetes and the various structures that compose them, and in the formation of the spores. Detailed descriptions, stained slides, drawings and photographs of the gross structures are available. But we still have not determined all of the exact factors which influence fruiting and trigger this phenomenon. Nevertheless, our knowledge is constantly growing, particularly as regards species which can be grown in culture and can be induced to sporulate under observation. *Physarum polycephalum,* for example, has been and is being studied in many laboratories and these studies have yielded a wealth of important data.

Back in 1936 William Seifriz pointed out that fruiting has often been attributed to the tendency of plants to save themselves from extinction as a result of dryness. Certainly slime mold spores are more resistant to dryness than the plasmodium, swarm cells, or myxamebae. But, as Seifriz pointed out, dryness usually causes the swarm cells and myxamebae to encyst and the plasmodium to form a dry resting stage called *sclerotium.* The production of



In *Physarum gyrosum* several papillae shorten, twist, pinch and push out to form one or more irregular shaped bodies. (× 8)

fruiting bodies on a wet surface disproves the hypothesis that dryness is necessary for fruiting in slime molds.

It has been postulated by some biologists that depletion of nutrients stimulates fruiting. Others claimed this hypothesis was wrong. An English biologist found the age of the plasmodium affected fruiting. All of these observations were open to question because the plasmodia were not grown in pure culture and all conditions were not controlled.

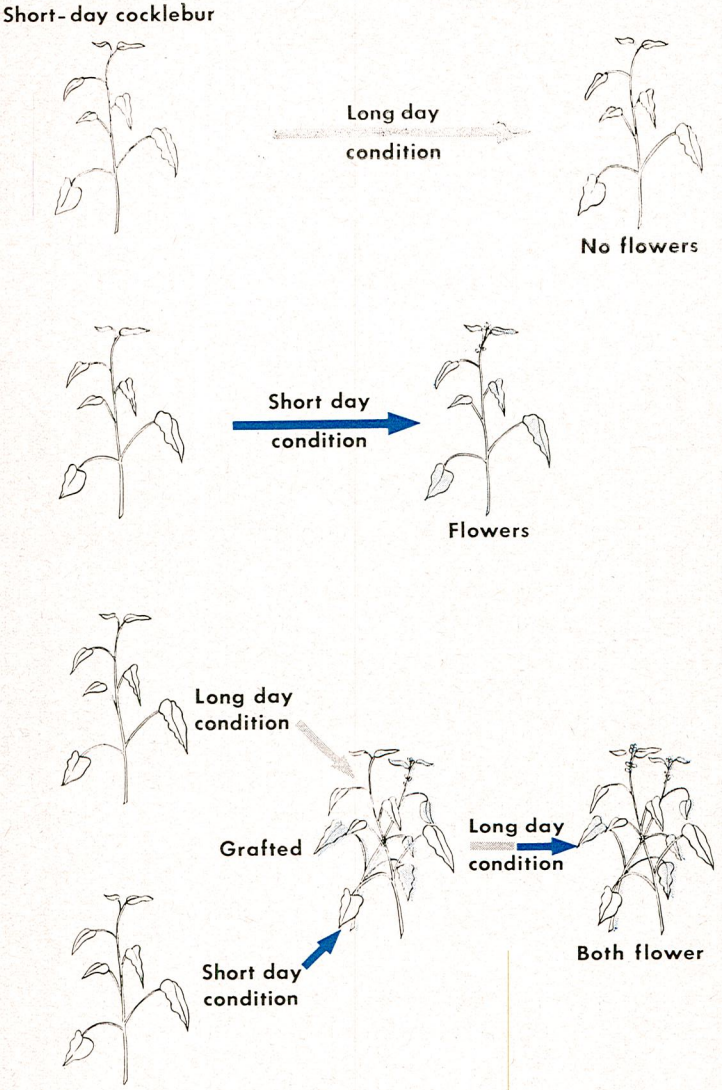
From the Wisconsin studies of *Physarum polycephalum* in pure culture, referred to earlier, we now know that nutrition and other factors do play important parts in sporulation. The organism will not fruit (form spores) if it is kept on the same medium on which the plasmodium grows so well. To fruit, the plasmodium must be supplied with niacin*, DPN or TPN (or certain

* Niacin or nicotinic acid is a vitamin essential to man. It is a part of DPN (Diphosphopyridine nucleotide) and TPN (Triphosphopyridine nucleotide), two coenzymes involved in respiration.

substitutes). In addition it must be incubated in the dark for a certain length of time and must then be exposed to certain wavelengths of light. But first of all, the plasmodium must have reached a certain age before spore formation can be induced.

The effect of light on sporulation is a particularly intriguing subject. In 1938 a series of experiments at Ohio State University demonstrated that light was a necessary prerequisite to fruiting in four species of slime molds with pigmented plasmodia (including *Physarum polycephalum*), but not in ten species with nonpigmented plasmodia. Various investigators have confirmed this observation many times since then. This indicates that the plasmodial pigments act as light receptors absorbing the energy required for fruiting. Unfortunately we do not know what these pigments are. Even though they have been extracted and analyzed by various chemical methods, there is complete disagreement as to their identity.

But how do we know that a pigment is actually involved in the reception of light necessary for the initiation of the fruiting process? Light is made up of different wavelengths. After the pigment has been extracted, we can pass light through a solution containing the pigment and see what wavelengths are absorbed and to what extent they are absorbed. A plot of the absorbed light is the absorption spectrum. The wavelengths necessary for initiation of fruiting can be determined by exposing plasmodia to known wavelengths of light at known intensities for certain durations of time. We can then plot the spectrum effective in fruiting (the action spectrum). If we compare the action spectrum and the absorption spectrum and find that they coincide (at least in part) we can theorize that the pigment may be involved in the absorption of light. Several investigators have done this and have found that not all of the extracted pigments qualify as photoreceptors effective in inducing sporulation. It should be noted, however, that the absorption spectrum of extracted pigments may be different from the absorption spectrum of pigments in a plasmodium. The pigments may be changed by the extraction. They may be combined with other compounds in a plasmodium. It should also be noted that not all of the pigments which absorb light may be involved in the triggering of sporulation.



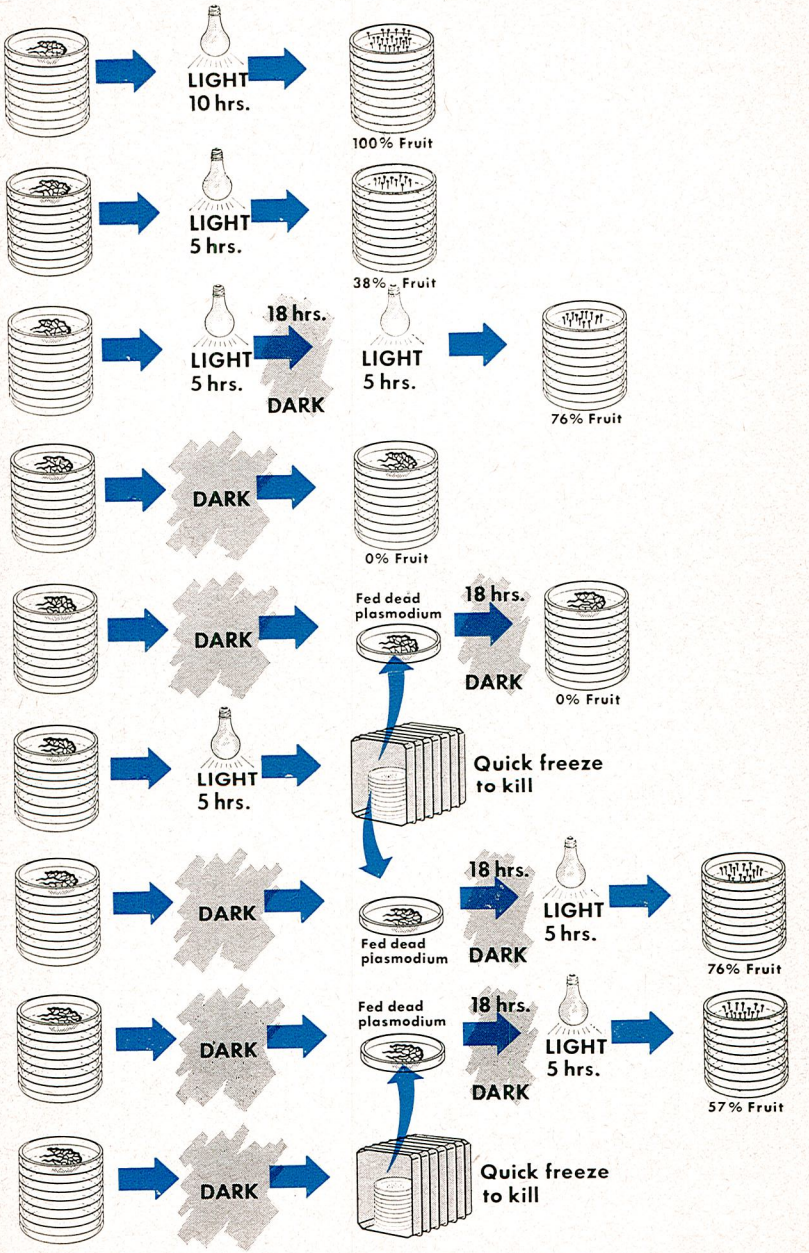
An experiment on the induction of flowering in a short-day cocklebur plant showed that a flowering stimulus can be transferred across a graft to a second plant.

A comparison of preliminary action spectra for the effect of light on fruiting in *Physarum polycephalum* and the effect of light on flowering in barley, cocklebur, and soybean plants shows that blue violet is effective to some extent in all organisms, but yellow and red light are effective only in flowering plants. This similarity and difference may be due to the presence of one common pigment in both, plus additional pigments in flowering plants, or to the presence of entirely different pigments in both. The results of isolation and identification of pigments from flowering plants and slime molds are indefinite. Preliminary work reveals that certain compounds such as the pteridines may be present in both groups. If they are proven to be effective in both slime molds and flowering plants, the results could be interpreted two ways: (1) the compounds have evolved along a similar line in two different groups, or (2) the two different groups may have had a common ancestor in their very early evolutionary history. If it is determined that different pigments are involved, then no evolutionary hypotheses can be drawn.

Flowering plants are usually classified into three main categories, based on their response to duration of illumination. The "short-day" or "long-night" plants flower when the daily exposure to light is shorter than a certain duration (usually between 12-15 hours), provided the "dark period" is not interrupted by light. "Long-day" plants flower only when the daily exposure to light exceeds a certain duration (usually 10-14 hours). Interruptions of the dark period do not affect the response. "Day-neutral" plants flower under a wide variation of day lengths. Some plants are apparently extremely sensitive to light and one exposure of favorable duration to light and darkness will induce the production of flower buds.

It was mentioned earlier that exposure to light is necessary for fruiting to occur in slime molds with pigmented plasmodia. It has also been suggested that the day length influences the duration of time required for fruiting. Recent work on photoperiodism in flowering plants indicates that it is the length of

An experiment, somewhat comparable to the cocklebur, showed that some kind of fruiting stimulus could be transferred from a dead plasmodium to another plasmodium which feeds on it.


LIGHT
10 hrs.
100% Fruit
LIGHT
5 hrs.
38% Fruit
LIGHT
5 hrs.
18 hrs.
DARK
LIGHT
5 hrs.
76% Fruit
DARK
0% Fruit
DARK
Fed dead
plasmodium
18 hrs.
DARK
0% Fruit
LIGHT
5 hrs.
Quick freeze
to kill
DARK
Fed dead
plasmodium
18 hrs.
DARK
LIGHT
5 hrs.
76% Fruit
DARK
Fed dead
plasmodium
18 hrs.
DARK
LIGHT
5 hrs.
57% Fruit
DARK
Quick freeze
to kill

the dark period which is important. The Wisconsin experiments on Myxomycetes indicate that a dark period of incubation prior to illumination is necessary to induce fruiting in *Physarum polycephalum*. The length of this dark period is critical. Is it possible that fruiting in the slime molds is regulated by the duration of light and dark as in the flowering plants?

Scientists at the California Institute of Technology found that flowering plants will not bloom if all leaves are removed prior to exposure to light. When two short-day cocklebur plants were exposed to long-day conditions, no flowering occurred. When one leaf of one plant was exposed to short days, that plant flowered. Apparently, some flowering stimulus is formed in that leaf and moves through the plant. In another experiment two short-day plants were grown under long-day conditions. One plant was briefly exposed to short-day conditions, was returned to long days and then grafted onto the other plant. Both plants flowered. The flowering stimulus produced in one plant is apparently transferred across the graft.

In 1954, a German scientist performed a somewhat comparable experiment with a slime mold. He found that a plasmodium of one species, (*Didymium nigripes*) would fruit under white, violet, or ultraviolet light, but not under green light or in darkness. The time of required illumination varied with intensity, within certain limits. He then grew some plasmodia in complete darkness and exposed some plasmodia to light. When he killed the illuminated plasmodia by freezing and fed them to the others, he found that the time of illumination required for fruiting could be reduced considerably. Apparently, some fruiting stimulus had been manufactured in the illuminated plasmodium which was transferred to the second plasmodium through the protoplasm.

Numerous compounds have been applied to flowering plants prior to, during, or after exposure to light in an attempt to gain insight into the mechanism of photoperiodism. Now that the factors necessary for fruiting in *Physarum polycephalum* have been partially identified, it should be possible to vary these factors and use various other compounds in an attempt to determine the mechanism of fruiting. Perhaps the mechanism in slime molds is similar to the mechanism in flowering plants; perhaps it is not. Only careful comparisons will give us the answer.

Ward, at the 4th International Congress of Biochemistry in 1958, stated that "The main approach to the problem of morphogenesis (origin of change) has been, and still is, concerned with experimentation involving a high level of biological organization. . . ." He goes on to point out the need for research based on an intracellular or molecular approach and raises the problem of separating biochemical changes involved in differentiation from those changes involved in growth. One of the few groups of organisms in which the growth phase is separated from the developmental or morphogenetic phase is the slime molds.

Recently, researchers at Temple University have quantitatively measured changes in amounts of respiratory enzymes in plasmodia and spores. The results indicate that the respiratory pathways change during fruiting. It was also found that sporulation could be triggered by inactivating certain chemical groups (-SH or sulfhydril groups) found on protein molecules. What effect do these groups have on fruiting? Does the exposure of certain pigments to light inactivate these groups? Answers to these questions may provide greater insight into the overall problems of development.

Conclusions

The true slime molds or Myxomycetes are among those organisms which are neither entirely plant or animal. Although their life cycle has been fairly well worked out, many fundamental questions concerning it remain unanswered. The answers have implication for solution of many broad biological problems.

Progress, at the moment, is being impeded by our inability to grow any of the true slime molds in pure culture on a defined medium from spore to spore. Success in growing the plasmodium of at least one species in pure culture, in a liquid medium of known chemical composition, has already led to important facts and interpretations on the nuclear cycle, synthesis of DNA, mitotic synchronization, photoreception, and the role of respiratory enzymes in differentiation. The primary immediate objective of myxomycete research should be to obtain all stages of the life cycle in pure culture on a defined medium. The attainment of this objective may unfold new avenues of research important to all mankind.

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Courtesy of Bureau of Audio-Visual Instruction, State University of Iowa
- pp 6, 7, 8, 10, 11, 12, 15, 22, 23, 28, 29
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Courtesy of Mrs. C. J. Allen, Jr., Riverton, New Jersey
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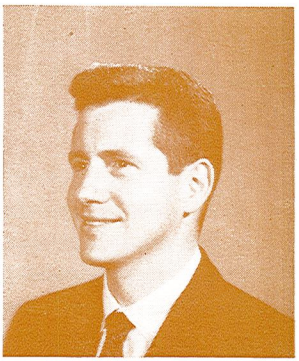

JAMES KOEVENIG

The slime molds are intriguing "plant-animals." Some scientists catalog them as Myxomycetes (slime molds), and some as Mycetozoa (fungus animals). Call them what you wish, the study of these organisms is beginning to provide answers to some fundamental biological questions. In this book the authors discuss the life history, nuclear cycle, and physiology of Myxomycetes. They show how these organisms are used as tools in biological research for the study of mitosis, protoplasmic streaming, morphogenesis, photobiological reactions, genetics, and other biological phenomena.

Dr. Alexopoulos is presently Professor of Botany at the University of Texas. He is the author of many technical articles on Myxomycetes and other fungi. He has been President of the Botanical Society of America (1963).

A former student of Dr. Alexopoulos, Dr. Koevenig is now Consultant with the Biological Sciences Curriculum Study. He has done research on Myxomycetes and produced a number of related educational films.

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