NUTRITION OF CELLULAR SLIME MOLDS

II. Growth of *Polysphondylium pallidum* in Axenic Culture

HANS-RUDOLF HOHL and KENNETH B. RAPER

Departments of Bacteriology and Botany, University of Wisconsin, Madison, Wisconsin

Received for publication 15 August 1962

Abstract

Hohl, Hans-Rudolf (University of Wisconsin, Madison) and Kenneth B. Raper. Nutrition of cellular slime molds. II. Growth of *Polysphondylium pallidum* in axenic culture. J. Bacteriol. **85**:199–206. 1963.—Several strains of *Polysphondylium pallidum* were grown on a liquid soluble medium in axenic culture. The medium contained embryo extract, serum albumin, Tryptose, dextrose, vitamins, and salts. The final cell yield was about 6–11 $\times$ 10^6 cells/ml, depending on the strain. The generation time was usually about 5 to 6 hr. The myxamoebae were grown for over 125 generations on this soluble complex medium without decrease in growth vigor or loss of their capacity to form normal fructifications when removed to an appropriate surface (e.g., agar). Thus the whole life cycle of this species was completed in the absence of any bacteria or bacterial products. Other species of the *Dictyosteleaceae* grew less well or failed to grow in the liquid medium described.

Many reports on the cellular slime molds, published within the past quarter-century, have dealt primarily with the latter phases of their life cycle, i.e., cell aggregation and fructification. Only a few studies have given special attention to the growth and multiplication of the myxamoebae (Raper, 1937, 1939; Sussman and Bradley, 1954; Gerisch, 1959, 1960), and these have centered almost exclusively on *Dictyostelium discoideum* as a test organism. No method has been developed for growing the cellular slime molds in the absence of bacteria or bacterial products. It is a goal much to be desired.

Exploratory investigations in our laboratory, directed toward this objective, have included a number of cellular slime molds, some of which seemed to offer special promise for obtaining axenic cultures. In the preceding paper (Hohl and Raper, 1963) we described techniques for growing these microorganisms on living and dead bacteria in liquid culture; in developing these techniques we explored various parameters that affect the growth of the myxamoebae, including such environmental factors as pH, temperature, osmotic pressure, and the presence of toxic substances.

This report describes, for the first time, the successful cultivation of one of the cellular slime molds, *Polysphondylium pallidum*, in a soluble medium free of any bacteria or bacterial products. Many nutrient combinations were investigated in our efforts to develop such a medium, and the present paper records our progress toward this goal. To date, primary emphasis has been directed toward achieving maximal vegetative growth of the myxamoebae. Studies on the morphology and subsequent morphogenetic movements leading to fructification by such axenically grown myxamoebae are in progress and will be reported later.

Materials and Methods

P. pallidum WS-320 was employed generally as the test organism, and the results reported herein relate to this culture unless otherwise indicated. The following procedures were employed for preparing the inoculum. Spores of the slime mold grown on agar plates with *Escherichia coli* (Raper, 1937, 1951) were transferred to tubes containing 5 ml of an autoclaved suspension of bacteria (*E. coli* strain B/r, 10^{10} cells/ml in 0.016 M Sörensen's phosphate buffer at pH 6.0). The tube cultures were incubated at 25 C on a rotary shaker at 250 rev/min. After 1 day, they were checked by transferring samples to tubes containing peptone-yeast extract nutrient broth; contaminated cultures were discarded. After 2.5 days, the myxamoebae had usually consumed the bacteria, the suspension had cleared considerably, and a slightly yellow color had developed. The myxamoebae were then centrifuged, washed in buffer solution, counted, and properly diluted,

usually to 5×10^5 cells/ml; 0.1 ml of this suspension was inoculated into tubes containing 5 ml of the medium to be tested, thus giving a concentration of 10^4 cells/ml of medium. Stirred flasks (Hohl and Raper, 1963) were substituted for the rotated tubes if larger quantities of myxamoebae were required.

The growth of the myxamoebae was measured by duplicate hemacytometer cell counts. Turbidity measurements of the suspension proved to be inadequate for growth determinations, especially in the lower concentrations.

Protein determinations were made by the Folin method. The myxamoebae were suspended in 4% trichloroacetic acid in 50% acetone, and the precipitated protein was dissolved in 0.4 N sodium hydroxide prior to the colorimetric measurements. Crystalline bovine serum albumin was used as a standard.

Hydrogen-ion concentrations were measured with a Beckman model G pH meter at the beginning and at the end of the experiments, and with pHydrion papers during the periods of cell growth. All experiments were run in triplicate.

The medium finally adopted contained the following components: 2% bovine embryo extract, 4% bovine serum albumin, Eagle's vitamin mixture (Eagle, 1955) in double strength, 1% Tryptose, 1% dextrose, and a modified Hank's salt solution (Paul, 1960). Before being incorporated into the medium, each of the components was tested separately for toxicity by adding it to cultures of myxamoebae growing in a suspension of dead bacteria in an appropriate buffer solution (Hohl and Raper, 1963). The detailed composition of the medium is given in Table 1.

The following stock solutions (sterile) were kept in the refrigerator prior to use: Difco TC Bovine embryo extract, 100%; Difco TC Bovine serum albumin, 5%; Difco TC Vitamins Eagle, 100 ×; modified Hank's salt solution, 10 ×. The desiccated embryo extract was reconstituted by adding salt solution and was used within 10 days.

To make 10 ml of medium, 100 mg of Tryptose and 100 mg of dextrose were dissolved in 1 ml of salt solution (10 ×) and 0.6 ml of distilled water. After autoclaving for 20 min at 121 C, 0.2 ml of embryo extract (100%), 8 ml of serum albumin (5%), and 0.2 ml of Eagle's vitamin solution (100 ×) were added aseptically prior to inoculation. The tubes were incubated at 25 C on a rotary shaker at 250 rev/min.

TABLE 1. *Composition of complex medium developed for growing myxamoebae of Polysphondylium pallidum in axenic culture*

Constituent	Amount
	mg/100 ml
Bovine serum albumin	4,000.0
Bovine embryo extract (100%)	2.0 ml
Tryptose	1,000.0
Dextrose*	1,000.0
D-Biotin	0.048
Folic acid	0.088
Niacinamide	0.024
Ca-pantothenate	0.048
Pyridoxal-HCl (USP)	0.040
Thiamine-HCl	0.068
Riboflavine	0.008
Choline chloride	0.028
NaCl	80.0
KCl	40.0
$CaCl_2 \cdot 2H_2O$	10.0
$MgSO_4 \cdot 7H_2O$	10.0
$MgCl_2 \cdot 6H_2O$	10.0
$Na_2HPO_4 \cdot 7H_2O$	6.0
KH_2PO_4	6.0

* Whereas dextrose showed no stimulation of growth in media yielding low cell populations, it was recently observed that it has a slight but definite activity in combination with the complex medium finally adopted. It is now routinely used, but all experiments reported in this paper refer to the medium without dextrose.

If the medium was to be solidified, agar was added prior to autoclaving to give a final concentration of 1%. The other components were added when the autoclaved solution was still warm but not hot enough to precipitate the albumin. The mixture was poured into petri dishes as a thin (2 mm) layer. Usually, 2% rather than 4% serum albumin was used for the solidified medium.

RESULTS

Growth on complex medium and influence of different components. Typical growth curves for *P. pallidum*, strains WS-320 and Pan-17, on the soluble complex medium are presented in Fig. 1. The final concentration of myxamoebae for strain Pan-17 almost equaled the concentration obtained with the controls (WS-320) grown on living bacteria, whereas the final concentration of amoebae in strain WS-320 was about one-half to one-third that of the controls. The end concentration of myxamoebae obtained varied con-

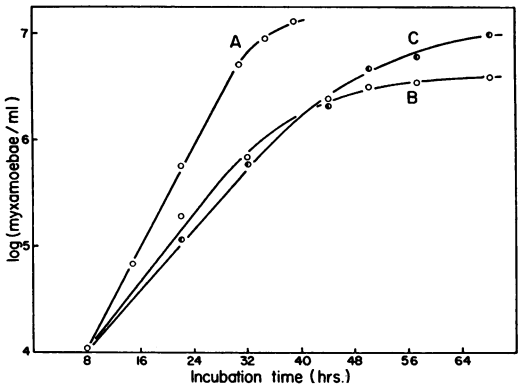

FIG. 1. *Typical growth curves of two strains of Polysphondylium pallidum on the soluble complex medium (curve* B: *WS-320; curve* C: *Pan-17). For comparison, the growth of strain WS-320 on living bacteria is also included (curve* A).

siderably. For strain WS-320, this ranged between 4 and 8 $\times$ 10^6 cells/ml; for strain Pan-17, between 8 and 14 $\times$ 10^6 cells/ml. The comparison is based on the number of cells produced. We shall subsequently see that cells grown on the complex medium are, on the average, larger than myxamoebae grown on bacteria. Growth of the myxamoebae, measured as increase in protein, therefore usually equals or even exceeds the amount found in bacteria-grown cultures. The pH rises from 6.0 to about 7.2 during the 3-day growth period.

There was a difference in the rate of growth between cultures grown on bacteria and in the complex medium. During the phase of logarithmic growth, the myxamoebae of *P. pallidum* WS-320 had a generation time of 2.5 hr when grown on living bacteria, 3.5 hr on dead bacteria (Hohl and Raper, 1963), and 5 to 6 hr in the complex medium. Figure 1 also demonstrates the comparatively long period of transition from the end of the log phase to the termination of growth.

When we consider the influence of each of the main components of the medium, it becomes apparent (Table 2) that no component alone is an absolute requirement (except for serial transfers), but that a combination of all of them gives the highest cell yield. The most important factor is the embryo extract, since it not only allows some growth in the absence of the other components, but it also limits the growth to a large extent when omitted from the whole medium. The embryo extract seems to act as a growth factor (Fig. 5) or as a macronutrient. This latter possibility appears reasonable from the fact that embryo

TABLE 2. *Influence of different components on growth of Polysphondylium pallidum WS-320*

Salts	Trypsin	Vitamins	Serum albumin	Embryo extract	No. of generations	Final concn (cells/ml)
+				+	6.3	7.8×10^5
+	+	+		+	7.0	1.2×10^6
+	+	+	+	+	9.4	6.6×10^6
+	+		+	+	8.9	4.5×10^6
+			+	+	8.4	3.3×10^6
+	+	+	+		5.4	4.3×10^5

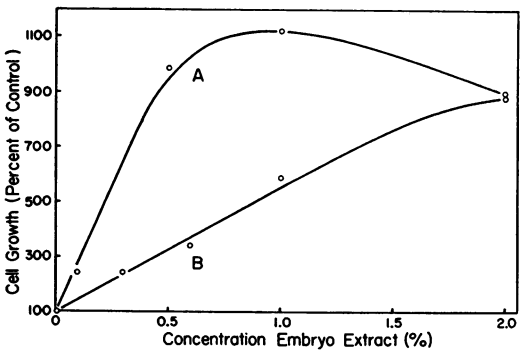

FIG. 2. *Influence of embryo extract on the growth of Polysphondylium pallidum WS-320 on a medium with 4% serum albumin (curve* A) *and on a medium with 2% serum albumin (curve* B), *measured after 3 days of growth.*

extract contains about 0.6 mg of protein per ml (in the 2% solution used), compared with about 1 to 2 mg of protein in the myxamoebae that are able to grow in 1 ml of medium. It can be further demonstrated by experiments (Fig. 2) in which the concentration of the embryo extract is varied. When the normal medium with 4% serum albumin is used, the yield increases rapidly at low concentrations and then tends to level off. The embryo extract acts here mainly as a growth factor. If the amount of serum albumin is reduced, or if it is omitted completely, the cell yield increases proportionally to the amount of added embryo extract, which demonstrates that it acts as a macronutrient in this latter case.

In the early experiments, when it was still uncertain whether the myxamoebae would grow in the absence of particulate food, a suspension of autoclaved lactalbumin (Difco, 3 to 10%) was used. Later, when it was shown that this nutrient supported equally good growth after passage through a Seitz filter, lactalbumin was replaced

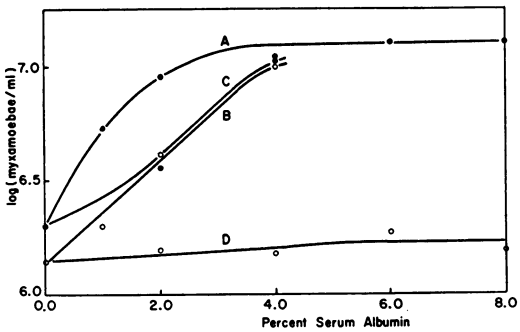

FIG. 3. *Influence of different concentrations of serum albumin from two different sources on the growth of two strains of Polysphondylium pallidum: strain Pan-17 on serum albumin from Calbiochem (curve* A) *and from Difco (curve* C), *and strain WS-320 on serum albumin from Difco (curve* B) *and from Calbiochem (curve* D).

by the more desirable soluble product, bovine serum albumin. In other experiments, bovine, rabbit, and chicken sera, instead of serum albumin, were included in the medium at concentrations between 5 and 20%. In the absence of embryo extract, rabbit serum allowed an increase of the myxamoebae up to about 1.5×10^6 cells/ml, whereas with bovine serum the cell concentration reached only 1.1×10^5 cells/ml. In the presence of embryo extract, concentrations of 3.5×10^6 cells/ml were obtained with either serum. Comparative tests of bovine serum albumin and bovine serum ultrafiltrate (Difco) showed that the activity was entirely in the albumin fraction. Consequently, this was substituted for serum, and at proper concentrations (4%) gave yields twice as high as 20% serum.

Several factors oppose the simple assumption that the serum albumin serves as a main energy source. To obtain optimal results, 4% albumin must be added to the medium (Fig. 3), i.e., 40 mg of protein/ml of medium compared with only 1 to 2 mg of protein/ml contained in the myxamoebae grown therein. Two of many possible explanations are (i) that the favorable action of serum albumin results from its physical properties (adsorption of toxic metabolites, changes in the viscosity of the medium) or (ii) that possibly only one specific part of the whole molecule is used (e.g., a single amino acid or a peptide). However, these two possibilities do not explain the fact that bovine serum albumins from different sources have different activities. Whereas bovine serum albumin from Difco Laboratories possessed excellent growth activity for all strains of *P. pallidum* tested (e.g., WS-320 and Pan-17 in Fig. 3), a bovine serum albumin from Calbiochem (25% in Tyrode's solution) had almost no activity for any strain except Pan-17, in which organism growth was considerably improved (Fig. 3, curve A). At the moment it seems that impurities may be partly responsible for the growth response, although it must be added that a twice-crystallized preparation of plasma albumin (Calbiochem) supported growth of strain WS-320 up to 3.5×10^6 cells/ml.

Serum and serum albumin tend to decrease the rate of growth, compared with controls without these substances. In other words, addition of serum or serum albumin increases the final yield substantially, but at the same time also increases the generation time of the myxamoebae (from about 4 hr to 5 to 6 hr with serum albumin). The serum albumin is also responsible for the increase in the average size of the myxamoebae, as revealed by comparative measurements of cells in the presence and absence of this component. The same effect is exerted by whole serum, as one might anticipate.

Many compounds and combinations of media have been tested for improvement of growth. Among them, a mixture of vitamins (Difco-Eagle) and 1% Tryptose were chosen and included in the final medium, since they had some additional positive effect (Table 2). Media used for animal tissue cultures, such as Eagle's Medium L, Medium NCTC 107, and Medium 199 (all from Difco Laboratories), had to be diluted too much (because of their high sodium chloride concentration) to give appreciable cell yields, even in the presence of lactalbumin or serum. Other components tested included yeast extract, peptone, casein hydrolysate, malt extract, beef extract, Casamino Acids, neopeptone, and trace elements. No combinations better than that listed in Table 1 were found.

Size of inoculum. The influence of the size of the inoculum on the growth of the myxamoebae is demonstrated in Fig. 4. There seem to be two major influences. First, it may be seen that reduction of the number of cells in the inoculum results in an improved generation time; e.g., with an inoculum of 10^3 cells/ml the generation time was about 3.5 hr, as compared with about 10 hr with 10^6 cells/ml. Second, the final concentration of myxamoebae attains a substantially higher level where the heavier inoculum is used: about

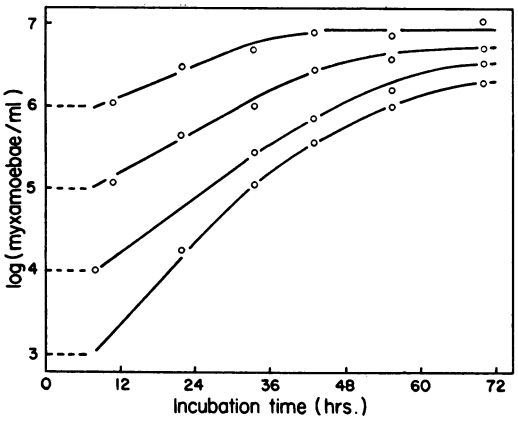

FIG. 4. *Influence of size of inoculum on the growth of Polysphondylium pallidum WS-320 in a soluble complex medium.*

10^7 cells/ml, in contrast with 2.5×10^6 cells/ml where the starting population was 10^3 cells/ml. These differences in growth rate have been observed in media with serum albumin or lactalbumin. In media containing 20% bovine serum, the situation is different. There the growth rate is the same with all amounts of inoculum tested, and the generation time for all cultures is 7.5 hr.

Continuous subculture. To test the ability of soluble complex media to support indefinite growth of the slime mold, the myxamoebae, after being washed, were serially transferred to fresh medium. Figure 5 shows the result obtained with a medium containing lactalbumin, Tryptose, vitamins, and amino acids. This medium, inoculated with 10^4 cells, supported growth to about 1.5×10^6 cells/ml in the first culture with or without added embryo extract, but in the absence of embryo extract it lost this capacity rapidly and the myxamoebae failed to grow after about 20 generations. If embryo extract was present, the growth rate even improved during the experiment, as indicated by a decline in the generation time from 7.5 to ca. 5.0 hr during a sequence of seven transfers. The medium with serum albumin, as finally adopted, showed similar characteristics. The medium with embryo extract has now supported growth for over 125 generations without decrease in growth vigor or fruiting capacity. Without the embryo extract, growth continued through only two subcultures for a total of 14 generations and then ceased. It seems, therefore, that for prolonged cultivation of the myxamoebae in the absence of bacteria or bacterial products a growth factor is necessary, which may be supplied by adding embryo extract.

Serial transfers may also be made in petri dishes on a solidified medium (see below) by transferring spores to fresh plates, that is, by following the same procedure usually employed when the myxamoebae are grown together with bacteria.

Growth and fructification on solidified medium. Three methods of obtaining fruiting structures from myxamoebae grown on the complex medium were employed. In the first method, myxamoebae were grown in liquid medium, then washed and transferred to plates containing water-agar (1.5%) or thin hay agar (Raper, 1937). Care has to be taken to avoid too long a cultivation (more than 4 to 5 days) of the myxamoebae in the liquid medium, since they tend increasingly to round up and become incapable of fruiting. The plated cells start to aggregate after 15 to 20 hr and fruit normally. It may be mentioned that the fruiting structures of strain WS-320 are symmetrical (Hohl and Raper, 1963, Fig. 3), with regularly spaced whorls of side branches, whereas those of strain Pan-17 are often irregular in pattern, with twisted sorophores. These differences, however, represent strain characteristics, for the most part, since they recur independently of the type of culture employed.

In the second method, the myxamoebae are transferred to roller tubes, as described by Gerisch (1960). Within a few minutes, the cells clump together and form very small agglutinates, which constantly increase in size during the following hours. If, after 8 to 16 hr, these agglutinates are put on an agar surface, they initiate

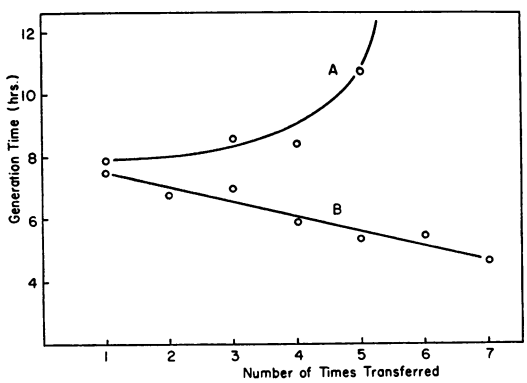

FIG. 5. *Continuous subculture of Polysphondylium pallidum WS-320 in the presence* (B) *and absence* (A) *of embryo extract.*

fructification within a few hours. Depending upon the size of the agglutinate, one or several sorocarps may develop. No detailed investigations have yet been made to determine the exact time relationships between fructification by normal aggregation and fructification by induced agglutination under controlled conditions.

In a third method, the myxamoebae are cultivated directly on the solidified medium. The amount of growth desired can be regulated by the amount of medium supplied. If too much nutrient is available, the myxamoebae cannot exhaust it, waste products presumably reach a concentration inhibitory to cell aggregation, and only microcysts are formed. Therefore, the plates should be poured thinly to a depth of about 2 mm. With an inoculum of five cells/mm^2, sparse fructification is obtained on the medium after about 2 days, even in the complete absence of serum albumin. With 2% serum albumin, the number of fruiting structures is much increased, but their formation is somewhat retarded and starts after about 3 days. This is partly due to the longer growth period, but perhaps also to the slight decrease in growth rate in the presence of serum albumin, as shown in the liquid cultures. The agar itself may have some stimulatory effect upon the growth rate. If a small amount (0.1%) of agar is added to the liquid medium and the cultures are grown on the rotary shaker, the slope of the growth curve is steeper than that of the control without added agar. However, the final concentration of myxamoebae remains unchanged, showing that the agar affects only the rate of cell growth. The myxamoebae grown on the surface of the agar-solidified complex medium are more uniform in size and smaller than the myxamoebae grown in liquid culture, where they reach unusually large dimensions.

Size of the myxamoebae. During the course of these studies, it became clear that the average size and the behavior of the myxamoebae may vary depending on the kind of medium used. To measure the relative size of the cells, they were suspended in a saturated solution of ethylenediaminetetraacetic acid (EDTA) to round them up, and their diameters were determined.

In our studies of slime mold growth in liquid cultures, three main types of myxamoeba populations were observed (Fig. 6). The first, which we may consider normal, is obtained by growing the slime mold on dead bacteria and is characterized

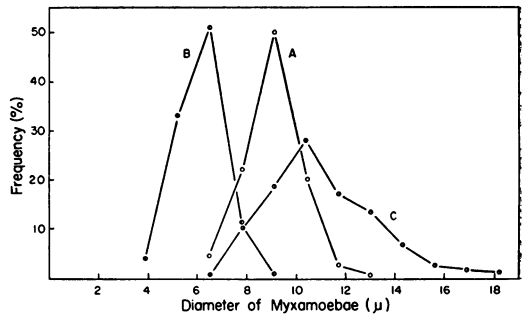

FIG. 6. *Size distribution of cells of Polysphondylium pallidum grown on different media. Number of cells measured:* (A) *117*, (B) *133*, (C) *169*.

by the uniform size of its cells (curve A), which flatten easily when transferred to a surface, e.g., agar.

The second type consists of cells that are, on the average, smaller than normal myxamoebae (curve B); it is found in media giving low cell yields, that contain no serum albumin or serum. The cells tend to round up, especially towards the end of the growth period, and do not flatten when plated on a surface. Often, small parts of such cells are separated from the main bodies by constriction, and these may or may not adhere to the parent cells.

The third type is typical for the complex medium described in this paper. The myxamoebae are, on the average, larger than normal cells and have a marked tendency to become giant. Yet there are not two different cell types—giant and normal—but a continuous transition between the smallest and the largest cells (curve C).

Phase-contrast microscopy reveals that some of the larger myxamoebae are multinucleate. Other cells seem to have several arms, each containing one nucleus; but here it is often very difficult to decide whether this complex consists of a single cell or of several cells clumped tightly together, or of cells connected by anastomoses (Huffman, Kahn, and Olive, 1962; Olive, Dutta, and Stoianovitch, 1961). By adding EDTA to the surrounding fluid, the myxamoebae are forced to round up and thus reveal their individuality. This whole problem of cell size and cell behavior, as influenced by nutrition and other conditions of culture, will be further investigated in the hope that it may be resolved.

Growth of different strains and species. To test the ability of the complex medium to support

TABLE 3. *Comparative growth of various species and strains of Acrasieae on the complex medium*

Species	Strain	Final concn (cells/ml)	Increase (×)	No. of generations
Polysphondylium pallidum	Pan-17	1.1×10^7	1,100	10.1
	Salvador	8.4×10^6	840	9.8
	WS-320	6.9×10^6	690	9.5
	Campbell	6.0×10^6	600	9.3
	FR-47	4.2×10^6	420	8.7
P. violaceum	Shaffer's "Founder"	8.0×10^4	8	3.0
	V-6, P-6, V-9	1.0×10^4	0	0
Dictyostelium mucoroides	S-2	8.2×10^4	8.2	3.0
	Singh	6.0×10^4	6.0	2.6
	WS-47	5.5×10^4	5.5	2.5
	WS-278	3.5×10^4	3.5	1.8
D. purpureum	WS-321	7.7×10^4	7.7	3.0
	V-1	2.0×10^4	2.0	1.0
	3645	1.0×10^4	0	0
D. sphaerocephalum (?)	FR-14, WS-331	1.0×10^4	0	0
D. lacteum	Purdue-7a	1.0×10^4	0	0
D. discoideum	V-12	2.3×10^4	2.3	1.2
	NC-4 (type), Gerisch's V-12/Ml	1.0×10^4	0	0

the growth of different strains of *P. pallidum*, and of other cellular slime molds as well, a series of experiments was run that included about 25 strains of our stock cultures. Some of the results are summarized in Table 3. To date, the results show that all strains of *P. pallidum* grow well on this medium, but that all other species either fail to grow or grow only to a very limited degree. It is thus clear that the nutritional requirements for the cellular slime molds vary from species to species, and that additional factors (nutritional and environmental) must be considered as we attempt to devise optimal liquid media for the cultivation of a wide variety of species and genera.

DISCUSSION

This study demonstrates that at least one species of the *Acrasieae*, or cellular slime molds, is capable of completing its whole life cycle (vegetative growth and multiplication, aggregation, and fructification) in the absence of any bacteria or bacterial products. Cell populations kept for over 125 generations in their vegetative state by serial transfers are still able to fruit when removed to an appropriate environment, thus confirming quantitatively the results obtained earlier by Raper (1940) for *Dictyostelium discoideum* and other species grown on living bacteria in conventional agar-plate cultures.

It is further shown that the myxamoebae do not have to feed on particulate food, but can grow and multiply in soluble liquid media. The mechanism of food intake seems to be similar in either case: particles or liquid droplets are engulfed and the contents of vacuoles so formed are digested. Whereas the process of ingestion and digestion of bacteria has been studied (Raper, 1937; Mercer and Shaffer, 1960; Gezelius, 1961), the process of pinocytosis has yet to be analyzed.

The growth of *P. pallidum* on media containing embryo extract and serum, or serum albumin, suggests that the nutrition of these cells is related to that of animal cells, as in tissue cultures, rather than to the nutrition of plant cells. Yet the high increase in cell numbers (up to about 1,000 ×, or ten generations, within 3 days) makes necessary higher concentrations of food material than are employed for animal tissue cultures, where the increase in cell numbers is normally much less. However, the salt concentration has to be lowered relative to media used in animal tissue culture, because of the sensitivity of the myxamoebae to osmotic pressure (Hohl and Raper, 1963).

Although both *P. pallidum* WS-320 and *Physarum polycephalum*, a plasmodium-forming slime mold, need embryo extract for their continuous growth, the active factors hemin or hemoglobin, which can replace embryo extract for the growth

of *Physarum* (Daniel, Kelley, and Rusch, 1962), are not sufficient to replace embryo extract in our medium.

It is to be hoped that further analysis of each component of the complex soluble medium now in use may eventually lead to a completely defined medium for *P. pallidum*, and that the present medium may serve as a basis for the axenic cultivation of other species of the cellular slime molds which seem to require additional factors. Investigations directed toward these ends are now in progress.

ACKNOWLEDGMENTS

We wish to thank Marianne Hohl for invaluable technical assistance. The work was supported in part by the Research Committee of the Graduate School from funds provided by the Wisconsin Alumni Research Foundation, and in part by research grants from the National Science Foundation (G-3375) and the National Institutes of Health (C-2119), U.S. Public Health Service.

LITERATURE CITED

DANIEL, J. W., J. KELLEY, AND H. P. RUSCH. 1962. Hematin-requiring plasmodial myxomycete. J. Bacteriol. **84**:1104–1110.

EAGLE, H. 1955. Nutrition needs of mammalian cells in tissue culture. Science **122**:501–504.

GERISCH, G. 1959. Ein Submerskulturverfahren für entwicklungsphysiologische Untersuchungen an *Dictyostelium discoideum*. Naturwissenschaften **46**:654–656.

GERISCH, G. 1960. Zellfunktionen und Zellfunktionswechsel in der Entwicklung von *Dictyostelium discoideum*. I. Zellagglutination und Induktion der Fruchtkörperpolarität. Arch. Entwicklungsmech. **152**:632–654.

GEZELIUS, K. 1961. Further studies in the ultrastructure of Acrasieae. Exptl. Cell Res. **23**: 300–310.

HOHL, H. R., AND K. B. RAPER. 1963. Nutrition of cellular slime molds. I. Growth on living and dead bacteria. J. Bacteriol. **85**:191–198.

HUFFMAN, D. M., A. J. KAHN, AND L. S. OLIVE. 1962. Anastomosis and cell fusion in *Dictyostelium*. Proc. Natl. Acad. Sci. U.S. **48**:1160–1164.

MERCER, E. H., AND B. M. SHAFFER. 1960. Electron microscopy of solitary and aggregated slime mould cells. J. Biophys. Biochem. Cytol. **7**:353–356.

OLIVE, L. S., S. K. DUTTA, AND C. STOIANOVITCH. 1961. Variation in the cellular slime mold *Acrasis rosea*. J. Protozool. **8**:467–472.

PAUL, J. 1960. Cell and tissue culture. The Williams & Wilkins Co., Baltimore.

RAPER, K. B. 1937. Growth and development of *Dictyostelium discoideum* with different bacterial associates. J. Agr. Res. **55**:289–316.

RAPER, K. B. 1939. Influence of culture conditions upon the growth and development of *Dictyostelium discoideum*. J. Agr. Res. **58**:157–198.

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

RAPER, K. B. 1951. Isolation, cultivation and conservation of simple slime molds. Quart. Rev. Biol. **26**:169–190.

SUSSMAN, M., AND S. G. BRADLEY. 1954. A protein growth factor of bacterial origin required by the cellular slime molds. Arch. Biochem. Biophys. **51**:428–435.