A STUDY OF THE NUTRITIONAL AND ENVIRONMENTAL FACTORS INFLUENCING THE GROWTH AND MAINTENANCE OF A DESERT STRAIN OF PHYSARUM PUSILLUM MARTIN.

by

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STATEMENT BY AUTHOR

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INTRODUCTION

The Myxomycetes (Mycetozoa) are an important phylogenetic group which exhibit characteristics of both the plant and animal world, and are considered by some to be a link between them. Studies of these organisms are needed in order that their biological process and their economic importance may be better understood and appreciated. They possess certain unique attributes such as synchronous mitosis (Daniel and Rusch (8)), initially unpolarized naked protoplasm (Cohen (6)), and a relatively brief life cycle which, it is believed, might render them potentially powerful experimental tools in biological research.

Although the slime molds were recognized as early as 1822 by Schweinitz (21), relatively little work has been done on this group of organisms. As late as 1938, Gray (13) pointed out that only a few species had been cultured, and that therefore their life processes were poorly understood. In the intervening years and especially in the past few years the situation has improved somewhat, but even today only a few species have been successfully cultivated in pure culture in the laboratory. Moreover, the majority of the nutritional and physiological studies have been concerned with a single species, Physarum polycephalum, possibly because it has been the only species available for purchase from the scientific supply companies. Simple techniques for culturing and

maintaining other species are therefore needed. It was the purpose of this investigation to contribute to this field and, for the first time, to utilize a species isolated from a desert area.

HISTORICAL REVIEW OF THE LITERATURE

In 1888, Lister (19) reported that the plasmodium of Badhamia utricularis would digest and assimilate the fruiting bodies of certain Hymenomycetes. As a result of this observation the first nutrient substratum for the growth of a slime mold was introduced. Although, grossly, these cultures appear to be two-membered, composed of plasmodium and fungal fruiting body, the fungal tissue was unsterilized and must have constituted a good culture medium for the growth of tremendous numbers of associated microorganisms which would also aid in the digestion of the fungus and probably serve as nutrient for the slime mold. In this case the culture must be considered multi-membered.

In 1901, Harshburger (14) observed that the plasmodium of Fuligo septica completely digested a piece of raw beefsteak and "well-nigh" digested coagulated egg white but for the same reasons as given above this must also be considered as an example of a multi-membered culture.

Howard (16 & 17) found the plasmodium of Physarum polycephalum feeding on the pilei of Pleurotus sapidus and proceeded to free the plasmodium from many of the associated organisms by permitting it to migrate over the surface of a sequence of non-nutrient agar plates. He attempted to grow it on sterile laboratory media and found that, of the various nutrient materials tested, oatmeal agar was most satisfactory.

Small fragments of the plasmodium contaminated with a "toruloid" yeast were found to thrive and greatly increase in mass when transferred to this agar. The plasmodium was found to remain active on this medium at temperatures between 7 and 32°C, but growth seemed most rapid at 20 to 26°C. It was also reported that the acidity of the medium could be increased to pH 4.8 and still be tolerated, but the buffer used in obtaining this value was not given.

In 1936, Camp (3) reported a modification of Howard's method for the cultivation of P. polycephalum that involved the inoculation of some of the oat-grown slime mold to a moist chamber with the subsequent sprinkling of a few grains of unsterilized rolled oats on the spreading plasmodium. The continued additions of small amounts of oats resulted in the development of a plasmodium of large size. If filter paper were applied to the sides of the moist chamber vessel, the plasmodium would soon migrate upward over this surface. It could then be removed and cut into the desired sizes for further inoculations and study. Since the plasmodium was not purified, the microorganisms growing in association with the slime mold probably contributed to the nutritional qualities of the oatmeal.

In 1938, Gray (13) reported the first instance of laboratory cultivation of Physarum compressum, Physarum tenerum, and Hemitrichia vesparium. Corn decoction agar was found to be more satisfactory than oatmeal for growing these plasmodia; however, since purification of the plasmodium was never accomplished growth on this medium may have been

due to the fact that it permitted development of various kinds of associated microbes.

Pure culture cultivation of Myxomycete plasmodia was first reported by Cohen (4) in 1939. Two methods were used in purifying the plasmodia of Badhamia foliicola, B. utricularis, Fuligo septica, Stemonitis axifera and Physarum polycephalum. In one instance, the plasmodium was permitted to migrate over washed non-nutrient agar until it had been freed of contaminating organisms. In the second, the plasmodium was fed a heavy suspension of living yeast streaked on non-nutrient agar. The first method was unsuccessful unless the plasmodia were smaller than 0.5 cm in fan diameter and unless the rate of migration over the agar surface was fairly rapid. The rate of migration was found to be a determining factor for the second method as well, but avoided the devitalization of the plasmodium due to the lack of food that was encountered in the first method. The yeast cells associated with the plasmodium after use of this enrichment technique were, in contrast to the original contaminating organisms, readily removed by the migration process. If no growth occurred after inoculation of the plasmodial samples into several types of nutrient liquid media, the plasmodium was considered pure. Attempts to maintain Physarum polycephalum and Badhamia foliicola in pure culture on media such as yeast extract, malt extract, oat agar, oat mash, pea mash, pea infusion, potatoes, carrots, bananas, and apples were unsuccessful. It was not possible to culture P. polycephalum in pure culture on oatmeal

even though this had proven to be the most satisfactory medium for culture in the impure state. The most satisfactory medium for the growth of purified plasmodia was found to be autoclaved Saccharomyces ellipsoideus streaked on the surface of non-nutrient agar buffered at pH 6.0 with M/75 phosphate buffer. It was observed however, that too much of the autoclaved yeast was inhibitory.

A qualitative analysis, undertaken by Cohen (5), of the microbial flora accompanying crude plasmodial cultures revealed no constant associations with the plasmodia studied. In two-membered cultures on oatmeal agar, the physical nature of the microbial colony often seemed to determine the feeding habits of the plasmodium. On slimy colonies the plasmodium appeared to suffer from an overly moist substratum that permitted them to feed only at the edges of such colonies. This was due, it was thought, to the smothering effects produced by the mucoid organism. With dry colonies, on the other hand, the plasmodium migrated over the entire colony until all of the nutrient was utilized. Oatmeal agar was the medium of choice since Pablum agar, glucose yeast extract agar, malt extract agar, and oatmeal mash favored an overly abundant microbial growth. Saccharomyces ellipsoideus was found to be the most desirable associated organism since the other yeasts used were either too slow growing, too glutinous or too small to be easily removed from the plasmodium by migration.

Sobels (22) used migration and enrichment methods similar to those of Cohen (4) for purifying plasmodia. She found that Licea flexuosa,

after growth on non-nutrient agar streaked with yeast, could be purified by a sequence of two or three transfers on non-nutrient washed agar. However, the addition of penicillin or streptomycin to the agar was found to improve the chances for obtaining pure plasmodia. Yeasts were found to be the only satisfactory nutrients for pure culture maintenance. Torulopsis laurentii was found to be more favorable than Saccharomyces cerevisiae for supporting the pure culture growth of Licea flexuosa. Isolations of organisms from the natural habitat of plasmodia revealed Torulopsis laurentii to be a common associate of the following Myxomycetes: Badhamia utricularis, Fuligo septica, Fuligo sp., Ceratiomyxa fructiculosa, and Physarum confertum. Although the plasmodium of Licea flexuosa was at first overwhelmed by the growth of this yeast on oatmeal agar, feeding would commence after about three weeks and result in the production of quantities of streaming protoplasm as great as that obtained with freshly collected impure cultures on the same medium. Badhamia utricularis in two-membered culture on oatmeal grew best at 20 to 24°C. At 30°C, growth was impaired by the rapid multiplication of the associated organisms, and at 35°C the plasmodium died quickly. Crude cultures on oatmeal however, were found to resist a temperature as high as 40°C but the duration of this resistance was not given. In general, for the other species tested, the optimum temperature for growth was found to be about 25°C, while 18°C proved to be more satisfactory for extended maintenance. The buffered agar used was prepared with diluted Sorensens' buffer at pH

6.0.

Sobels and Cohen (23) cultured 13 different slime molds in two-membered culture on oatmeal agar and found that 200 units penicillin and 10 mg streptomycin per ml of oatmeal agar was sufficient to purify five of the plasmodia tested by one migration over this medium. Only one plasmodium succumbed during the purification period and the others that were not purified by the first transfer remained impure even after further transfer and subsequent migration. The optimum temperature for the culture of the majority of plasmodia was said to be between 20 and 23°C. They noted that although feeding and growth were slowed at 16°C, this temperature was found to be suitable for storage of many plasmodia. However, temperatures a degree or two below 16°C, as well as prolonged exposure to temperatures higher than 27 to 30°C, were stated to be generally harmful. In their experience the vegetative plasmodium did not survive freezing. It was noted that most plasmodia preferred a mildly acid media at about pH 6.0 and that many Myxomycetes could withstand diffuse room light successfully although direct exposure might be damaging.

Cohen and Sobels (7) pointed out that completely satisfactory routine methods for measuring plasmodial growth was lacking. They found that measurements of the increase in area of the plasmodial fan was useful in recording the relative growth, and that the rate of migration was an indication of the vigor of the slime mold. Starving plasmodia were observed to move slowly and show an irregularly developed fan.

Hok (15), using Physarum polycephalum as one of the test organ-

isms, utilized the migration method of Cohen (4) and improved his enrichment method for purifying plasmodia by using a thin suspension of living yeast which, in his experience, permitted the plasmodium to migrate rapidly without devitalization. The enrichment method, however, was found to yield very few microbe-free plasmodia, and the migration method often terminated in plasmodial death or sclerotial formation. Non-nutrient antibiotic agar was then tried. The specific agent and the concentrations in units/ml of media that were tolerated by P. polycephalum are listed below:

penicillin	--	1000	units
streptomycin	--	100	units
bacitracin	--	10	units
tyrothricin	--	0.1	mg
subtilin	--	5	units

The most successful technique found was to allow the plasmodium to migrate first over washed non-nutrient agar and then over an antibiotic agar containing both penicillin and streptomycin in a final concentration of 20 units per ml. of medium. Migration on the latter was accompanied by a flooding of the agar with an aqueous penicillin-streptomycin mixture for a period of about five hours after which the excess moisture was poured off and the plasmodium transferred to a satisfactory nutrient medium. Bakers yeast was found to be the most satisfactory nutrient for culturing the purified plasmodia although the autoclaved yeast produced poor growth in comparison to that obtained with the use of the living agent. He concluded that heat and the resultant autolysis produced by autoclaving destroyed, to varying degrees, the growth-promoting properties of the live yeast.

This destructive effect was avoided by the use of ultra-violet light or nitrogen mustard as the killing agent. Results with yeast so treated were as good as those obtained with the living yeast. The optimum temperature for growth of the plasmodium was found to be between 19 and 25°C. Growth was retarded at 16°C and mortality was high at 30°C, while no cultures survived 34°C for more than two days. The optimum pH for the culture of P. polycephalum was found to be approximately 6.0 M/45 acetate buffer was used for pH values ranging from 3.5 to 5.5 and M/45 phosphate buffer was used for those between 5.5 to 8.0. Direct transfers from a pH of 6.0 to either a pH of 3.5 or a pH of 8.0 were found to be lethal although the plasmodia could be adapted to these values with gradual changes in the pH. In any case growth at pH 3.5 and pH 8.0 were said to be poor. M/30 acetate or phosphate buffers were shown to be tolerated as well as M/45 and M/75 buffers. It was also observed that the reaction of the agar (pH 8.0 or pH 3.5) shifted toward neutrality after the plasmodium had grown on the media for one week.

In 1959, Alexopoulos (1) cultured Stemonitis flavogenita on corn meal agar and oatmeal agar. He concluded that the plasmodium probably subsisted on the bacteria present in the culture so that most likely almost any media supporting a moderate growth of bacteria would be suitable for the culture of this slime mold.

More recently, Daniel and Rusch (8) found that 1000 units of penicillin as well as 100 units of streptomycin had no obvious harmful effect

on the development of Physarum polycephalum but these compounds were found to be unnecessary for purification since four or five migrations over sterile phosphate agar at pH 4.3 was sufficient to free the plasmodium from its associated organisms. Plasmodial purity was checked by allowing the plasmodium to migrate over nutrient agar plates for two transfers. The purified plasmodium was then inoculated to nutrient agar streaked with sterile rolled oats and whole yeast and finally to a 500 ml Erlenmyer flask containing sterile moistened rolled oats. Growth on the autoclaved rolled oats was reported to be rapid and profuse. They also developed a nearly synthetic soluble medium for plasmodial growth in shaken liquid culture. Considerably less growth was obtained in the shaken plasmodial cultures when phosphate buffer was used than when a calcium carbonate buffer was employed. These cultures were also incubated in the dark to prevent a possible retardation of growth by prolonged exposure to ordinary room lighting.

The formation of a brittle resistant structure (a sclerotium) from plasmodia subjected to drying, that when remoistened will reform an active plasmodium, has long been known. Probably the earliest investigations of this phenomenon were made by DeBary (9). Recently, Jump (18), using Physarum polycephalum as the test organism, made an extensive study on the factors inducing sclerotial formation. He found that while a brittle structure was produced by drying, it was also possible to obtain a highly plastic non-dessicated form of resistant structure by lowering

the temperature to about 5°C. A similar phenomenon occurred when the plasmodium was subjected to high osmotic pressure, sub-lethal concentrations of certain heavy metals, certain contaminating fungi, or high acid concentrations. Starvation was usually found to produce death or fruiting rather than a sclerotium. Microscopically the two types of resistant structures were reported to be the same in that both contained numerous microscopic spherical to polyhedric structures that reconstituted plasmodia in a similar way. It was felt that the plastic type of structure was sufficiently like the well known brittle type to warrant considering both as sclerotia but, since a sclerotium is immediately thought of as a dried brittle body, he considered it desirable to propose a new terminology. The designation, cystosorus, was applied to either resistant structure and the microscopic bodies of which they were composed were designated as macrocysts. He suggested that the plastic sclerotial stage might be significant to the survival of Myxomycetes under conditions of low temperature. It has been said that spores have retained their viability through 63 years of storage (Elliott (10)), while dried sclerotia appear to be viable for no more than a few years at the most. The latter have been the major means of storing plasmodia since they are formed and reconstituted with considerable reliability and since they can be stored at room temperature. Jump outlined a method by which the plasmodium can be readily converted to the brittle type of cystosorus by allowing it to migrate from two-percent non-nutrient agar on to cellophane on which it is dried slowly.

However, it was indicated that the cystosorus formed at refrigeration temperatures was not adapted to storage since it is reconstituted to an active plasmodium on returning it to room temperature.

MATERIALS AND METHODS

Physarum pusillum (Martin (20), the Myxomycete used throughout this investigation, was obtained from the bark of a living mesquite tree (Prosopis juliflora) located in the Yuma, Arizona, area. To obtain the active plasmodium, bark samples were placed on filter paper in several large 15 cm petri dishes and were flooded with tap water. Twenty-four hours later the excess was poured off but a moist state was maintained. In two to five days, plasmodia were observed on the bark in each of the plates. The moist chamber method for obtaining plasmodia from dry natural materials was first used successfully by Gilbert and Martin (12).

To obtain the plasmodia on artificial media, bark samples with plasmodia were removed from the moist chambers and placed on 2 % non-nutrient agar medium in standard 10 cm petri dishes. Usually, within 24 to 48 hours, the plasmodium migrated from the bark to the agar suface. To free the plasmodium from contaminating microorganisms, an agar block with the intact plasmodium was then transferred to another petri plate containing non-nutrient agar. After two or three such transfers with subsequent migration, the plasmodium was transferred to one of the following nutrient media in an attempt to culture this Myxomycete on mycological or bacteriological substrates:

Bacto nutrient agar

Bacto Czapeks agar
Bacto corn meal agar (without glucose)
Stokes agar (24)
Malt-yeast extract agar (25)
Oatmeal agar (17)

Each of the first five media was used full strength and, since Cohen and Sobels (7) had found that full strength laboratory media was inhibitory to some plasmodia, each was diluted with non-nutrient agar to concentrations of 75, 50, 25, 6, and 3 %.

Since none of the laboratory media, diluted or undiluted, supported plasmodial growth, another method was tried which had been utilized by Cohen (4), and Sobels (22), and Hok (15) for the culture of other slime mold species. Plasmodial transfers were made to 2 % non-nutrient agar plates streaked with either living or autoclaved cells of a laboratory strain of Saccharomyces cerevisiae. Preparation of the autoclaved cells involved growth in 500 ml Erlenmyer flasks containing 100 ml of malt-yeast extract broth (25). The broth was then poured off and the cells were resuspended in distilled water. Further washing of the cells was accomplished by repeated centrifugation and resuspension in fresh distilled water. After three such washings, the net wet weight of the cells was obtained by weighing the centrifuge tube with and without the cell pack. A 20 % w/v suspension of yeast was then made by adding the appropriate amount of distilled water. The yeast suspension was dispensed in 55 ml screw cap tubes and autoclaved at 121°C for 15 minutes. About 0.25 ml. of the sterilized yeast suspension was transferred to each of the non-nutrient

agar plates which were then evenly streaked with the suspension over the entire agar surface. Living yeast cells, grown on malt-yeast extract agar slants, were similarly streaked on non-nutrient agar plates.

Both the autoclaved and living yeast cell preparations were found to be satisfactory for supporting the growth of the plasmodium but, since autoclaved yeast cells appeared to be as effective as the living agent and since maintenance of the plasmodium free from living organisms was one of the major objectives of this investigation, it was decided that the killed cells of Saccharomyces cerevisiae should be used as nutrients in further studies and should also serve as a control in determining the usefulness of other substrates as plasmodial nutrients.

The plasmodia were grown and maintained in a thrifty state in the laboratory on the autoclaved yeast medium described above as long as weekly transfers of the plasmodium were made to fresh media of this type. Since the transferring process was performed with the aid of a flat nichrome needle, which served to cut out the agar block as well as transfer it, the transferrable size of the plasmodium in its intact state was limited. It was therefore frequently necessary to divide the plasmodium by cutting it and the underlying agar with the flattened needle. Using this technique, large numbers of active plasmodia could be obtained, but before investigations could be performed on the nutritional and environmental conditions favoring growth it was necessary to 1) develop a method for securing uniform plasmodial inocula and 2) devise techniques for measuring plasmodial

growth. The first of these objectives was accomplished using a modification of the technique describe above. The plasmodium was grown on the autoclaved yeast with subsequent transfer and growth on fresh yeast medium until as many plasmodia was needed had been obtained, and then, since cutting the plasmodium had no obvious adverse effects, the plasmodia were cut to the size desired and transferred to the test medium. The techniques utilized to meet the second objective were suggested from the following observations. It was noted that the plasmodium grew on the non-nutrient agar streaked with autoclaved yeast in a fairly consistent manner. The small plasmodia (less than 6 mm sq.) migrated from the transfer block onto the yeast agar surface where they commenced feeding and increased in size to approximately 6 mm sq. Beyond this point however, the plasmodial fan began extending in a linear fashion so that the width remained at approximately 6 mm while the length of the fan continued to increase (see plate I). This suggested a method for obtaining simple measurements of relative plasmodial sizes which, if recorded over a period of time, would give information on plasmodial growth. In this investigation the extent of the peripheral fan development after four days on the test medium, as measured in millimeters, was used as an indication of the effect of a given experimental situation on the growth of the plasmodium. To supplement these observations, a direct photographic technique was used in some instances. The culture, in a coverless petri dish, was centered on F-3 Kodak photographic paper (4 by 5) and was

exposed for eight seconds to light emitted from a Leitz substage microscope lamp the aperture of which had been reduced to a size of approximately 3 sq mm. The light was mounted on a ring-stand 50 cm. above the specimen. After exposure the paper was processed in the usual manner.

With methods available for obtaining fairly uniform inocula and for measuring growth, it was possible to study the effect of various nutritional and environmental changes on the growth of this desert strain.

The first investigation was suggested by the work of Sobels (22) who found that a yeast, Torulopsis laurentii, isolated from the natural habitat of the slime mold, Licea flexuosa, produced much better growth of her Myxomycete than did cells of Saccharomyces cerevisiae. Therefore, it was felt that possibly better results might be obtained using organisms isolated from the natural habitat (mesquite bark) of the strain of Physarum pusillum used herein. To test this possibility, 16 different microbes including ten bacteria, three yeasts, two molds, and two streptomycetes were isolated by plating and dilution techniques from the bark itself and from the crude plasmodial cultures. The malt-yeast extract medium of Wickerham (25) and Bacto nutrient agar were the media used. The colonies growing on these media were subjected to purification procedures and preserved on appropriate media in the refrigerator. Each organism was grown in shaken broth culture of the same nutrient composition as the original isolation medium after which it was treated in the manner described above for the preparation of the autoclaved cells of Saccharomyces cere-

cerevisiae. Non-nutrient agar plates were streaked with the autoclaved organisms in 20 % w/v suspension following the method utilized for the yeast. Four plates of each type of organism were prepared and inoculated with a 9 mm plasmodium measured as described previously. Because preliminary studies had shown that both 20 and 25°C were apparently equally favorable growth temperatures for this slime mold, duplicate plates of each organism used as a nutrient source were incubated at each of these temperatures in an attempt to determine if differences in growth at these two temperatures could be brought out under conditions of varied nutrient. Plain non-nutrient agar and non-nutrient agar streaked with the autoclaved cells of Saccharomyces cerevisiae served as controls.

The results of this experiment indicated that the cells of an autoclaved yeast, isolate # 1 from the mesquite bark, produced much better plasmodial growth than did the killed cells of S. cerevisiae and was more readily handled than a pink mold-like yeast, isolate #3, that yielded comparable results. Isolate # 1 was therefore used for the subsequent experiments reported herein.

The 20 % w/v suspension utilized for streaking onto non-nutrient agar was arbitrarily chosen as being a suspension of suitable concentration. It was therefore of interest to examine the possibility that other concentrations might produce a more satisfactory growth of the plasmodium. Autoclaved yeast suspensions of 40, 32, 24, 16, and 8 % were prepared. Four plates of each concentration were made by streaking 0.25 ml of the

appropriate yeast suspension on the non-nutrient agar surface. After inoculation of the plasmodium the plates were incubated in duplicateat 20 and at 25°C.

Although oatmeal agar has previously failed to support the growth of this species, Cohen (4), Sobels (22), and Hok (15) had been successful in growing several Myxomycete species on oatmeal agar in association with yeast (i.e., two-membered culture). To determine if Physarum pusillum would also react favorably in similar two-membered culture, the oatmeal agar of Howard (17) was prepared in 250 ml Erlenmyer flasks and inoculated with plasmodia. Live yeast (isolate # 1) were inoculated in a circular fashion around the transfer agar block. The plasmodium then migrated off the agar block and commenced feeding on the yeast which had visibly grown by the time the plasmodium reached it (in about 24 hours). The flasks were incubated at 20°C.

The range of temperatures permitting growth and survival of this desert slime mold were investigated using the methods described previously for yeast plate preparation and plasmodial inoculation. Duplicate plates of the plasmodia were subjected to the following temperatures: -10, 5, 20, 25, 30, 32, 35, 37, 45°C.

For the determination of the pH optimum for plasmodial growth, non-nutrient agar was buffered with varying amounts of Na_2HPO_4:12 H_2O and KH_4PO_4 (Sorenson's buffer). The phosphate concentration used was maintained at M/75 as suggested by Cohen (4) and media of pH 5.3, 6.1

6.4, 6.6, 6.8, 7.1, 7.4, and 7.5 were prepared. The yeast nutrient was added and inoculation was carried out in the usual manner. Duplicate plates were incubated at 20 and 25°C. Non-nutrient agar streaked with yeast was used as a control.

Preliminary experiments with greater phosphate molarities had indicated that this Myxomycete was affected by increases in the buffer concentration. To examine this effect more closely M/50, M/30 and M/15 phosphate buffered non-nutrient agars of pH 6.0 and pH 7.0 were prepared. They were then streaked with yeast in the usual manner, inoculated with plasmodia, and incubated in duplicate at 20°C.

Although brief exposures to fluorescent laboratory lighting did not appear to have a harmful effect on the plasmodium, it was felt that continuous light might produce obvious effects since Cohen (4) and Sobels (22) had noted that continuous light was harmful to the plasmodia used in their research. Duplicate yeast plates with plasmodia were prepared in the usual fashion and subjected to continuous light as supplied by a G. E. 40 watt fluorescent light fixed about 60 cm above the specimens. A similar set of plates was placed in the dark. Both sets were incubated at 25°C.

In the earlier stages of this investigation it was possible to purify this plasmodial species by several migrations over plain non-nutrient agar. Later however, air contaminants were encountered which were less easily removed. Certain other workers such as Sobels (22) and Hok (15)

had used antibiotic (usually penicillin and streptomycin) supplemented non-nutrient agar to purify plasmodia and had noted the tolerance of the slime mold for these microbial metabolic products. Non-nutrient antibiotic agar was therefore prepared and inoculated with contaminated plasmodia to determine if antibiotics might serve as a dependable means of purifying this species as well as to observe any effects the antibiotics might produce. The following types and concentrations were used: Penicillin (Crysticillin - Squibb) at 300, 150 and 75 units/ml of media and Streptomycin-sulfate (Squibb) at 5, 1, 0.5,0.25 mg/ml of media. Mixtures of antibiotics were not used. The purity of the plasmodia was tested by inoculation of small plasmodial samples to malt-yeast extract broth and agar and to Bacto nutrient broth and agar. The media were incubated for four days at 25°C before final readings were made.

During the course of experiments concerning the growth and survival temperatures for the plasmodium, it was observed that at temperatures of 5 and 37°C, this plasmodial strain formed resistant, plastic cellular structures which were similar to the structures Jump (18) reported to be formed by Physarum polycephalum at 5°C. He considered this process to be an encystment. Since no observations into the resistance of these plastic encystments had as yet been made and since their formation might be useful in the development of a storage method for this Myxomycete, a series of experiments were performed to consider these aspects.

To determine how long these structures would remain viable at 5°C, after having been formed at that temperature, 30 yeast plates with plasmodia were prepared in the usual manner. The plasmodia were allowed to migrate off their transfer agar blocks and the plates were then refrigerated. Five plates were removed at the end of one week and held at room temperature for 24 hours, or up to five days if necessary, to permit plasmodial reconstitution. Similar tests were made at intervals of 7, 21, 35, 56, 84, and 112 days.

A similar experiment was done to determine how long the structures would remain viable at 37°C after having been formed at that temperature. Six plates were incubated at 37°C and two were removed at three day intervals.

An experiment was then performed to investigate the resistance of the structures formed at 5°C to freezing temperatures. Two sets of plates in triplicate were subjected to -10°C for 24 hours. One set of plates was placed immediately at room temperature while the other was placed at 5°C for 24 hours before bringing it to room temperature. Two other such plates were left at -10°C for 25 and 41 days before thawing.

To determine if the encystments produced at 37°C would resist exposure to 45°C, two sets of duplicate plates with the cellular structures were placed at 45°C. One set was removed after 24 hours to room temperature and another after 48 hours.

Apparently the structures formed at high temperatures were similar

to those formed at low temperatures but it was thought that the resistance they demonstrated might be specific for the condition bringing about their formation. To test this possibility, duplicate plates of the encystments produced at 5°C were placed directly into the 37°C incubator for 48 hours before bringing them to room temperature, and duplicate plates of the structures produced at 37°C were placed directly into the refrigerator for one week before attempting reconstitution at room temperature.

It has long been known that, by drying some plasmodia, a brittle structure (a sclerotium) that is resistant to various environmental factors is formed. Jump (18) pointed out certain microscopic similarities between the structures brought about by drying and those produced by low temperatures. Since the plastic nature of the latter form seemed to set it apart from the brittle type, it was thought that if the plastic type might be dried successfully without destroying its viability the differences between these two structures would be less obvious and the similarities even more apparent. To demonstrate this, encystments produced at 5°C were transferred with their underlying agar to plates lined with dry filter paper. These were then placed again in the refrigerator for two weeks after which the dried agar and the desiccated encystments were transferred to non-nutrient agar plates and incubated at room temperature.

From preliminary studies on this plastic dormant stage there was reason to believe that it would be useful for storing and maintaining slime molds in stock cultures that could be used to quickly and dependably

reconstitute the plasmodium and to serve as a source of inocula when several plasmodia of similar past history were needed. Since a convenient method for storing the plasmodium that would avoid agar dessication and contamination was desired, a procedure utilizing screw cap tubes as storage containers was tested for its usefulness. Slants of non-nutrient agar in 55 ml screw cap tubes were streaked with 0.25 ml of autoclaved yeast cells (isolate #1). The tubes with loosened caps were placed in a reclining position to allow an even dispersal and drying of the yeast suspension on the slant. Inoculation with plasmodia was accomplished in the usual manner. The plasmodium was allowed to migrate from the transfer agar block and the block was removed. After about one week of refrigeration, encystment of the plasmodia had occurred and the caps were tightened. Two tubes were removed to room temperature after 10 days and the caps were again loosened. Four others were similarly treated after 30 and 60 days of refrigeration.

RESULTS

The plasmodium of Physarum pusillum was obtained from the bark of four different mesquite trees located within the same general area and appeared to be the only slime mold obtainable from this environment. Twenty bark samples were collected all of which yielded this slime mold in moist chamber culture. The plasmodium found on the bark proved to be of the typical physaroid type described by Alexopoulos (2), having irregular and rather thick strands of protoplasm with a typical fan in most cases. However, the plasmodium on non-nutrient agar tended to be somewhat more delicate than many of the phaneroplasmodia thus far described and in no case did it attain the giant size so characteristic of certain members of the genus.

Although the purification of the plasmodium was accomplished by numerous migrations over non-nutrient agar with and without autoclaved yeast cells, it was found that antibiotic media would shorten the purification process. For example, only migration over streptomycin supplemented non-nutrient agar was required to purify the plasmodium. Table I summarizes the results obtained with antibiotic media and indicates the concentrations of the agents that were effective as well as the apparent effects on the plasmodium

Plasmodia inoculated to Bacto nutrient agar, Bacto Czapeks agar, Bacto corn meal agar, Stokes agar, malt-yeast extract agar, and

oatmeal agar failed to grow and either fruited or died within two weeks. However, none of the media seemed to produce an inhibitory effect in that the plasmodia behaved much like those migrating on plain non-nutrient agar.

The autoclaved cells of Saccharomyces cerevisiae were found to produce satisfactory plasmodial growth. However, certain organisms isolated from the natural environment of the slime mold proved to be more effective. The results of this investigation are listed in Table II together with a brief characterization of each organism. Only two of the sixteen isolated nutrient organisms (isolate #1 and #3) maintained each of the plasmodia cultures for the entire two week period. These plasmodia obtained fan sizes in four days that were significantly greater than those achieved by any of the other plasmodia in this test including the S. cerevisiae control. Isolate # 11 (a gram negative rod) was superior to other bacteria and molds and produced results that were somewhat comparable to isolate # 2 (Rhodatorula sp.). Another gram negative rod (isolate # 8) and a Streptomyces sp. (isolate # 6) produced maintenance results similar to isolates # 11 and # 2, but produced considerably less growth than either # 11 or # 2. Isolates number 7, 9, 10, 15 and 16 produced results that were no better than the non-nutrient control. Isolates number 5, 13 and 14 were only slightly more satisfactory, while isolates number 4 and 12 maintained two plasmodia each for the full 14 day period and for the most part produced larger plasmodial fans than those obtained on the non-nutrient

agar control. From the point of view of maintenance and of plasmodial growth the two yeast isolates (# 1 and #3) were definitely superior to the others. Since the mold-like nature of isolate # 3 made it difficult to spread evenly over the non-nutrient agar surface, the mucoid yeast (# 1) was chosen for subsequent work. The mucoid nature of this yeast was not evident after the cells had been washed several times. Plasmodial growth on each of these two yeast substrates was recorded by the direct photographic method developed during the course of this study. A daily sequence of growth on plates streaked with autoclaved isolate # 1 is shown in plate I and the growth sequence on isolated # 3 is given in plate II. The latter was prepared in a slightly different manner to reveal the positive chemotactic effect of the yeast on the Myxomycete. The yeast suspension was allowed to run at random over the agar surface. The plasmodium migrated only in areas containing yeast (# 3) and followed precisely the pattern made by the yeast.

Another purpose of this phase of the investigation was to determine if differences in plasmodial growth at 20 and at 25°C could be observed as a result of varying the type of nutrient. No significant changes were noted and the overall growth patterns observed at the two temperatures appeared to be similar.

The results of the study undertaken to determine the effect of yeast concentration (isolate # 1) on the growth of this slime mold at 20 and at 25°C are summarized in table III. The best growth was obtained

on plates streaked with the 40 % suspension. Although the overall trend of plasmodial development seemed to increase as the yeast concentration increased, good growth was obtained with the 20 % w/v suspension already prepared and its use was continued. No consistent temperature effects were noted.

In the early stages of this investigation, oatmeal agar was not found to support the growth of this Myxomycete but an attempt to grow the slime mold on this agar in two-membered culture with yeast was undertaken since it had been shown to be a successful technique for the culture of other plasmodial species by Cohen (4), Sobels (22) and Hok (15). The plasmodium grew on oatmeal agar in association with living cells of isolate # 1 very successfully for about one week but at the end of this period the mucoid nature of the yeast seemed to overwhelm the plasmodium so that in approximately three weeks no sign of plasmodial activity could be found. Continued incubation of these cultures did not allow the plasmodium to recover. In general, plasmodial growth was not better than that obtained with the autoclaved yeast.

The effects of temperatures from -10 to 45°C on plasmodial development are noted in table IV. After 24 hours, at -10°C, plates were removed from the freezer and permitted to thaw at room temperature. Macroscopically the plasmodium appeared to have been frozen in situ, but microscopically they were greatly disrupted appearing to be in a shredded state. Viability was not restored during a two weeks observation period.

At 5°C, the results were considerably different. Observations (made with a Leitz microscope installed in the cold room) revealed that the plasmodium continued to stream normally for six minutes, after which the rate of streaming appeared to slow. Fifteen minutes later all streaming in the fan area had discontinued while streaming continued in the veins. At this time, the plasmodium began to take on a very white appearance. After two hours, the plasmodium seemed to adapt somewhat to the new environment so that, in the larger veins, streaming continued at a fairly normal rate for seven days. However, during this time, the size of the plasmodium was greatly reduced even though yeast cells were present. By the seventh day the plasmodium appeared grossly to be only slightly different from the streaming mass observed the day before. However, microscopic observations revealed the constricted protoplasm to be organized into numerous spherical to polyhedric sub-units similar to those reported by Jump (18) to be formed by Physarum polycephalum under similar conditions. The area that had ceased streaming within the first six minutes had apparently been killed during this time since it did not organize into the cellular structures (i.e., the "cysts" described by Jump). When the encysted mass was removed to room temperature, the plasmodium was reconstituted within 24 hours.

Good growth was obtained at both 20 and at 25°C. At 30°C, plasmodial growth on the autoclaved yeast was less than that obtained at 20 and at 25°C. The plasmodium was maintained at 30°C for twelve days

but no observations were made beyond this period. Although growth occurred at 32 and at 35°C, it was less than that observed at 30°C. No observations on maintenance beyond the four day test period were made for these two temperatures.

At 37°C the sequence of events was considerably different than that occurring at 35°C. Observations made after 24 hours incubation revealed the plasmodia as yellowed plastic clumps. Since the plasmodia appeared to be dead they were removed from the incubator and placed at room temperature with the idea of discarding them. However, in 24 hours, before the plates were discarded, they were re-examined and found to contain abundant active plasmodia. To examine this phenomenon more closely, freshly prepared plates were placed at 37°C and, within 24 hours, the plasmodia had again clumped. On microscopic observation the cellular structures that had been observed to form at 5°C were also found to be present in these yellowed clumps. After 24 hours at room temperature, these also re-formed into plasmodia.

At 45°C, the plasmodia produced even more markedly yellowed masses within 24 hours. However, plasmodia were not re-formed at room temperature even after two weeks. Since, on microscopic observation, no cellular bodies could be seen, these plasmodia were assumed to have been killed by the higher temperature.

The effect of the pH of the medium on the growth of this plasmodium is given in table V. The best growth on media buffered with M/75

phosphate was achieved by plasmodia growing at pH 7.1 and incubated at 25°C. In general, good growth was obtained on agar buffered at pH 6.8 to 7.4 and appeared to be somewhat better at 25°C than at 20°C. The large variation in the values obtained at 20 and at 25°C at pH 6.4 could not be explained. However, the non-buffered control also produced better growth at 25°C in contrast to previous results.

Table VI gives the results obtained for determining the effect of the buffer concentration on plasmodial growth. The overall growth at pH 6.0 and 7.0 at M/75 and M/50 phosphate buffer was better at pH 7.0 using the M/75 concentration. However, at M/30 a marked reduction in growth was observed and all the plasmodia clumped and appeared to be dead by the fifth day. Microscopically, no cellular bodies were observed. At M/15 PO_4 all of the plasmodia were apparently dead in 24 hours. From these and the previous tests on hydrogen ion concentration the optimum pH for growth of this species, using phosphate buffer, was found to be approximately 7.0, but only the M/75 phosphate buffer produced results comparable to those obtained with non-buffered agar.

Incubation in continuous light during plasmodial growth (table VII) was definitely harmful. The fan sizes obtained after four days growth in continuous light were significantly less than those obtained in cultures grown in darkness.

Table VIII shows the length of time that the encysted plasmodium remained viable under conditions of regrigeration and the length of time

required for reconstitution to occur after each storage period. The plasmodia refrigerated for seven days formed cysts by the sixth day. These encystments reconstituted plasmodia within 24 hours that were nearly as large as the original specimens. Similar results were obtained for the five slime molds stored for 21 days at 5°C. However, after this period the ability to reconstitute quickly appeared to be altered. One plasmodium failed to revive after 35 days and the other were slower to reconstitute than the previous specimens, but in all other cases the encysted plasmodia recovered even though reconstitution was not as rapid as in the 7 or 21 day encystments. The test was originally planned to terminate on the 112th day but to enable the test to continue beyond this period only one culture was removed, and proved to be viable. Further observations are still to be made.

The results of a similar experiment performed at 37°C are summarized in table IX. The viability of the cysts was apparently lost between the tenth and ninth days of incubation at 37°C.

It is evident from table X that plasmodia encysted at 5°C could tolerate freezing temperatures although this temperature killed the active plasmodium within 24 hours. The cysts were found to survive freezing for at least 41 days. Gradual thawing at refrigeration temperatures increased the number of viable cultures obtained.

While active plasmodia were killed within 24 hours at temperatures of 45°C, plasmodia encysted at 37°C were able to withstand this

temperature. Upon removal to room temperature all of the cultures reconstituted plasmodia. However, no reconstitution occurred in cysts incubated at this temperature for 48 hours, and cysts place at 50°C for 24 hours also failed to revive.

The results given in table XI indicate the interchangeability of resistance by the temperature-conditioned cysts. Those formed at 37°C were able to tolerate 5°C for the length of time employed and those formed at 5°C were able to tolerate the 37°C temperature for at least two days.

Plasmodia dried under refrigeration conditions were found to be viable when they were brought to room temperature. Reconstitution required about three days.

The effect of refrigerator storage on encysted plasmodia in screw cap tubes is given in table XII. All of the cultures so prepared remained viable throughout the test period. However, the cultures refrigerated for 60 days took about four days for reconstitution of the plasmodium. It was found that the encysted plasmodia were revived simply by bringing the stock cultures to room temperatures or cultures could be removed with a loop and transferred to either non-nutrient agar or yeast media for subsequent reconstitution. It was also found that the active plasmodium itself could be successfully transferred in this way since the scraping process involved in such loop transfers was not particularly damaging, although several hours were required for the plasmodium to regain full vigor.

DISCUSSION

The finding that the plasmodium of Physarum pusillum was the only type observed on the bark of each of four living mesquite trees is interesting and recalls similar observations made by Evenson (11) who noted that in seven moist chamber preparations of clippings from the tips of seven different plants of cane cholla, Badhamia macrocarpa was the only species of slime mold encountered. Various factors could be involved in such associations, if they exist, such as 1) the type of microbes living on the vegetation and serving as nutrient for the slime mold, 2) nutrient factors introduced by the plant itself, and 3) toxic factors. For instance, it was found that the yeasts isolated from the bark of the mesquite produced good plasmodial growth and that most of the microbes isolated supported some degree of growth. It is possible that the bark of the tree and its associated microbes provided a selective habitat for a certain species of Myxomycete. Toxic products produced by the Myxomycete or its associated organisms might also exert a regulatory effect on the type of slime mold, or slime molds, encountered. It was found in the course of this study that this species was quite sensitive to the antibiotic streptomycin (Squibb) in that 5 mg per ml of medium was sufficient to kill the plasmodium within 24 hours. Therefore, if organisms capable of producing compounds of this type were present in the given

micro-ecological niche, it might be an unfavorable environment for this strain although other types might thrive quite satisfactorily. Physarum polycephalum for example, as reported by Hok (15) and Daniel and Rusch (8), is apparently a great deal less sensitive to streptomycin.

In her investigation of the nutrition of Licea flexuosa, Sobels (22) found that a yeast isolated from the natural habitat of the slime mold produced much better growth than laboratory stock cultures of yeast. Two-membered oatmeal cultures with the yeast isolate were also successful although the yeast at first outgrew the plasmodium. However, the slime mold eventually recovered and became the predominant member. Physarum pusillum behaved similarly in oatmeal culture with a mucoid yeast (isolate # 1) but unlike Licea flexuosa it did not recover.

While certain autoclaved specimens of bacteria, mold, and Actinomycetes produced growth of P. pusillum, yeasts were found to produce the most satisfactory results. Cohen (4), Sobels (22), and Hok (15) also found this to be the case. None of the soluble laboratory media employed were found to be satisfactory for growth but they were not observed to be inhibitory as Sobels and Cohen (23) reported. Since Daniel and Rusch (8) have shown that at least one Myxomycete (Physarum polycephalum) could be grown in a soluble liquid medium and since Sobels (22) had shown that Licea flexuosa could be maintained on an yeast extract medium, it was felt that the absence of growth on laboratory media in the case of this strain of P. pusillum was not due to an inability to take up food in

solution. This point was to be further considered with the use of fractionated yeast cells but time did not permit this phase of the investigation. Alexopoulos (1) found that either corn meal agar or oatmeal agar was satisfactory for the crude culture of Stemonitis flavogenita. Neither of these media were found to be satisfactory for either pure or crude cultures of Physarum pusillum.

To determine the optimal conditions for the growth of the plasmodium, it was necessary to have some means of measuring and comparing growth under different conditions. The appearance of the slime mold growing on autoclaved yeast suggested a modification of the method utilized by Cohen and Sobels (7) who estimated the increases in area of plasmodial fan as an indication of growth. Feeding on plates streaked with nutrient organism proceeded in a way that produced an elongated fan which increased in length as the slime mold grew. Since this characteristic appeared to be fairly constant, it was felt that a linear daily measurement of the fan periphery would be an adequate representation of the relative growth of individual specimens and, if uniform inocula were used throughout a test series, these sizes could be used as a means of comparing the rates of growth between specimens. It was not felt that a measurement involving the entire fan area would provide a more accurate comparison of growth since this technique is more subject to errors involved in plasmodial spreading which is not a result of growth processes. Since the plasmodium is a highly plastic structure and is therefore capable of

surface expansion and contraction, it must be admitted that any method involving a direct measurement of size is greatly limited in its application. Only the trends of growth may be determined and only large consistent differences in size may be considered significant.

Studies concerned with the environmental factors affecting plasmodial growth demonstrated certain differences existing between this desert plasmodium and the plasmodia investigated by other workers. For example, Sobels and Cohen (23) found that, in their experience, temperatures above 27 to 30°C resulted in poor plasmodial growth and eventually in death while Hok found mortality to be high at 30°C and no survival for more than two days at 34°C. Although growth was not, in our studies as satisfactory at 30°C with autoclaved yeast as a substrate, cultures of Physarum pusillum could be maintained successfully for at least 12 days. At 35°C the plasmodium grew more slowly and could be maintained for at least four days. The fact that this plasmodium seemed to survive more successfully at temperatures above 30°C than has been reported for the plasmodia of most species, may be a result of its existence in a desert area. However, it was also interesting to note that the best growth was obtained at temperatures of 20 to 25°C and in this respect was similar to the slime molds investigated by Cohen (4), Sobels (22) and Hok (15). These workers, in considering the hydrogen ion content favorable for growth, had found that an acid environment (pH 6.0) seemed to be most

satisfactory. The strain used in this study appeared to prefer a neutral to slightly alkaline environment. There is a possibility that adaptation to the alkalinity of the desert soils may in part explain this difference. Hok (15) had found that Physarum polycephalum grew as well on M/30 phosphate buffer as on the lower buffer concentration. P. pusillum was was definitely harmed by this concentration and death occurred in all cases within five days. M/15 phosphate buffer killed the plasmodium within 24 hours.

The harmful effects of direct continuous light on the growth of the plasmodium is apparently similar to that demonstrated by other Myxomycetes thus far studied. Continuous light must interfere with the normal metabolic processes of this slime mold but the mechanism of the phenomenon is not understood.

Possibly the encystment that occurred when the plasmodium was incubated at 37°C is an expression of the individuality of this desert strain since this type of reaction to high temperature has not been previously reported for any species. Jump (18) noted this phenomenon when Physarum polycephalum was held at 5°C but did not report its occurrence at high temperatures. Possibly the production of these structures is an important aspect of the survival of this species and others in the desert. Jump considered the encystments occurring in response to low temperatures and to various chemical agents to be analogous to sclerotial formation, a type of encystment brought on by drying and resulting in a brittle

as opposed to a plastic structure. He felt that the microscopic similarities of the two types were great enough to apply the same terminology to both. However, since the sclerotium, in mycological usage, implied a hard, brittle formation, he proposed that "cystosorus", not having this implication, be used for both and that the functional units making up the cystosorus be called "macrocysts". Although this new terminology appears to be desirable, it may be a source of confusion. The addition of prefixes to the words, cystosorus and macrocyst, indicating the conditions inducing their formation, might help to clarify the problem. For instance, macrocysts formed by low temperatures could be called psychromacrocysts and the gross structure containing the cysts a psychrocystosorus. It then follows that the cysts formed by high temperatures should be called thermomacrocysts with thermocystosorus representing the overall structure. The term sclerotium could then be used only in referring to those structures produced by drying or would by synonymous with sclerocystosorus. The microscopic units of sclerotia have in the past been called spherules but since this word has a different connotation in medical mycology, macrocysts, or more specifically scleromacrocysts, might be used in preference. Since all of the sclerotia described prior to the investigations of Jump were apparently of the dessicated type, no confusion should result from the use of the term, sclerotium, interchangeably with sclerocystosorus.

Other experimenters, including Sobels and Cohen (23), involved in the resistance of the plasmodium to temperature extremes, have pointed out that the plasmodium does not survive freezing. In this investigation it was found that while the active plasmodium could not survive freezing the encysted phase survived the condition for at least 41 days and it is felt that this may be extended to much longer exposure periods. Similarly, the active plasmodium was killed within 24 hours incubation at 45°C while the encysted slime mold survived. From these and other observations on the resistance of the encysted plasmodial phase, it appeared that these dormant structures could be utilized in the development of a storage technique. Since the encysted plasmodium could be adapted to storage by refrigeration in screw cap tubes, a technique used for many bacteriological and mycological cultures, it was considered that this procedure might be an acceptable maintenance and storage technique for this slime mold and possibly others. However, since time did not allow for a storage period longer than 60 days for tube cultures and 112 days in the case of plates, the length of the survival period under these conditions is not known. It may be that encysted plasmodia can be stored for even longer periods under conditions of freezing than are possible under refrigeration. In any case the use of the plastic form of plasmodial encystment for storage has certain advantages over the dessicated, brittle type so frequently used in that 1) it is conveniently produced and may be stored in the purified state with other stock cultures, 2) may be readily

divided and transferred with a loop to fresh media before reconstitution and 3) is conveniently and rapidly reconstituted. Although it is true that this type of encystment unlike the sclerotium may not be stored at room temperature it does not appear that this is particularly disadvantageous.

SUMMARY

A desert strain of Physarum pusillum Martin was obtained in moist chamber culture from mesquite bark.

The plasmodium was purified by migration over non-nutrient agar and agar containing antibiotics. Growth of the microbe-free plasmodium did not occur on oatmeal agar or on various types of bacteriological or mycological media. Good growth was obtained when the autoclaved cells of S. cerevisiae were used as the substrate but was improved when a yeast isolated from the natural habitat of the Myxomycete was used.

While growth appeared to be best at temperatures of 20 to 25°C, growth occurred at 35°C. Media buffered at neutrality with M/75 phosphate buffer was found satisfactory. Plasmodial growth under the influence of continuous light was reduced.

A type of encystment was observed to occur at both 5 and 37°C and appropriate nomenclature for this structure is suggested. The resistance of the encysted plasmodium to various temperatures was determined and on the basis of the findings, a convenient method of storage was devised.

TABLE I

The Effect of Antibiotics on the Plasmodium and Purification Procedure

Name of antibiotic	Conc. per ml medium	Purity after one migration*	Other effects observed
Streptomycin-sulfate (Squibb)	5 mg	---	Death in less than 24 hours
	1 mg	Accomplished	Slow rate of migration, fan highly reticulated
	0.5 mg	Accomplished	Rapid migration rate, fan reticulated
	0.25 mg	Not accomplished	No obvious effects
Crysticillin (Squibb)	300 units	Not accomplished	No obvious effects
	150 units	Not accomplished	No obvious effects
	75 units	Not accomplished	No obvious effects

* Determined by the presence or absence of growth in malt-yeast extract or Bacto-nutrient media after inoculation with bits of the plasmodium.

TABLE II

The Effect of the Autoclaved Cells of Organisms Isolated from the Natural Habitat on the Growth of *P. pusillum*

Isolate No.	Isolate Type	Description	Temp. in degrees centigrade	Fan size after four days (in mm)** Plate A	Plate B	Ave.	No. of cultures maintained for 14 days* Plates remaining
1)	Yeast	Non-spore forming, colorless, mucoid	20	48	60	54	2
			25	48	27	38	2
2)	"	*Rhondatorula* sp.	20	36	24	30	2
			25	26	39	33	1A
3)	"	Pink, mold-like	20	51	36	44	2
			25	72	42	57	2
4)	Mold	*Stemphyllium* sp.	20	6	24	15	1A
			25	12	24	18	1B
5)	"	*Rhizopus* sp.	20	18	21	20	1A
			25	18	15	17	0
6)	Actinomycete	*Streptomyces* sp. dark grey	20	9	12	11	2
			25	6	9	8	1B

*Transfers to fresh media streaked with the same organism were made every 4 days.

**The fan size of the inoculum was approximately 9 mm.

TABLE II (continued)

The Effect of the Autoclaved Cells of Organisms Isolated from the Natural Habitat on the Growth of *P. pusillum*

Isolate No.	Isolate Type	Description	Temp. in degrees centigrade	Fan size after four days (in mm)** Plate A	Plate B	Ave. age	No. of cultures maintained for 14 days* Plates remaining
7)	Actinomycete	*Streptomyces* sp.	20	12	9	11	0
		light grey	25	10	21	16	0
8)	Bacteria	Mucoid, gram neg.,	20	18	0	9	1A
		fat rod.	25	9	6	8	2
9)	"	Gram neg., pleo-	20	15	6	11	0
		morphic rod.	25	9	3	6	0
10)	"	Waxy, yellow, gram	20	3	0	2	0
		neg., fat rod.	25	15	6	11	0
11)	"	Yellow, mucoid,	20	30	12	21	2
		gram neg. rod	25	45	18	33	1B
12)	"	Bright yellow,	20	18	24	21	0
		gram neg. rod.	25	19	15	17	2

*Transfers to fresh media streaked with the same organism were made every 4 days.

**The fan size of the inoculum was approximately 9 mm.

TABLE II (continued)

The Effect of the Autoclaved Cells of Organisms Isolated from the Natural Habitat on the Growth of *P. pusillum*

Isolate No.	Isolate Type	Description	Temp. in degrees centigrade	Fan size after four days (in mm)** Plate A	Plate B	Ave. age	No. of cultures maintained for 14 days* Plates remaining
13)	Bacteria	Light, yellow	20	24	27	26	0
		gram neg. rod	25	18	21	20	1A
14)	"	Grey, translucent	20	6	12	9	1B
		gram neg. rod	25	24	1	13	0
15)	"	white, gram pos.,	20	9	9	9	0
		short rod	25	6	0	3	0
16)	"	yellow, large	20	12	6	9	0
		gram pos. cocci	25	12	6	9	0
0)	Control	*S. cerevisiae*	20	15	42	29	2
			25	6	24	15	2
00)	Control	Non-nutrient agar	20	2	11	7	0
			25	9	6	8	0

*Transfers to fresh media streaked with the same organism were made every 4 days.

**The fan size of the inoculum was approximately 9 mm.

TABLE III

The Effect of Various Concentrations of Autoclaved Yeast Cells on Plasmodial Growth

Yeast concentration w/v	Temperature in degrees centigrade	Fan size after four days in mm. A	B	Average
40 %	20	54	100	77
	25	48	96	72
32 %	20	60	78	69
	25	84	30	57
24 %	20	54	51	53
	25	75	30	53
16 %	20	39	60	50
	25	72	45	69
8 %	20	72	46	69
	25	54	24	39
0	20	0	2	1
	25	6	2	4

TABLE IV

The Effect of Temperature on the Growth and Survival of P. pusillum Using Autoclaved Yeast Cells as Nutrient

Temperature in degrees centigrade	Fan size after four days (in mm) A	B	Effect
-10	---	---	Death in 24 hours.
5	---	---	Produces encystment within a week.*
20	96	72	Good growth.
25	96	72	Good growth.
30	60	42	Slows growth.
32	6	36	Slows growth.
35	18	21	Slows growth.
37	0	0	Encystment within 24 hours.*
45	0	0	Death within 24 hours.

*Macroscopically encystments produced at 37°C are more tightly massed and more yellow than those formed at 5°C. Microscopically they appear the same.

TABLE V

The Effect of the Hydrogen Ion Concentration (M/75Phosphate Buffer) on the Growth of P. pusillum with Autoclaved Yeast Cells as Nutrient

pH	Temperature	Fan size after four days (in mm) A	B	Average age
5.3	20	27	18	23
	25	45	33	39
6.1	20	30	34	32
	25	18	22	20
6.4	20	6	6	6
	25	29	63	46
6.6	20	31	36	34
	25	21	34	28
6.8	20	33	37	35
	25	63	42	53
7.1	20	60	54	57
	25	96	43	70
7.4	20	42	45	44
	25	48	69	59
7.5	20	31	48	40
	25	39	40	40
Control	20	39	39	39
	25	72	48	60

TABLE VI

The Effect of the Phosphate Buffer Concentration on the Growth of *P. pusillum* with Autoclaved Yeast Cells as Nutrient

pH	MPO_4	Fan size after four days (in mm) A	B	Average
6.0	M/75	30	34	32
7.0		60	54	57
6.0	M/50	Encysted*	45	
7.0		45	46	46
6.0	M/30	dead	dead	—
7.0		3**	3**	3
6.0	M/15			
7.0		Death within 24 hours all cultures		

* Plastic structures formed as described at 5°C.

** Dead by the fifth day.

TABLE VII

The Effect of Continuous Light on the Growth of P. pusillum with Autoclaved Yeast Cells as Nutrient

Lighting conditions	Fan size after four days (in mm)		Average
	Plate A	Plate B	
Continuous light	12	18	15
Continuous darkness	42	48	45

TABLE VIII

The Effect of Prolonged Refrigeration on the Encysted Stage of P. pusillum*

Length of Refrigeration period in days	Number of cultures removed to room temperature	Number of cultures reconstituting plasmodium	Average time at room temperature before streaming resumed
7	5	5	Less than 24 hours
21	5	5	Less than 24 hours
35	5	4	About 4 days
56	5	5	Two days
84	5	5	About 3 days
112	1**	1	Two days

* Petri dish cultures.

** Others remain for further study.

TABLE IX

The Effect of 37°C Incubation Temperatures on the Encysted Stage of P. pusillum

Length of incubation period in days	Number of cultures removed to room temperature	Number of cultures reconstituting plasmodium
3	2	2
6	2	2
9	2	0

TABLE X

The Effect of Temperatures of $-10^{o}C$ on the Encysted Stage of P. pusillum

Length of freezing period in days	Temperature of thawing	Number of cultures removed from freezer	Number of cultures reconstituting plasmodium
1	Room temperature	3	1
1	$5^{o}C$	3	3
25	$5^{o}C$	1	1
41	$5^{o}C$	1	1

TABLE XI

The Effect of the Opposite Encystment Temperatures on the Encysted Stage of P. pusillum

Temperature of cyst formation	Temperature of test	Number of days of test	Number of cultures employed	Number reconstituting plasmodium
5°C	37°C	2	2	2
37°C	5°C	7	2	2

TABLE XII

The Effect of Refrigerator Storage on the Encysted Stage of P. pusillum*

Number of days refrigerated	Number of tubes removed to room temperature	Number of tubes reconstituting plasmodium	Time required for reconstitution
10	2	2	24 hours
30	4	4	About 2 days
60	4	4	About 4 days

*In screw cap tubes.

Plate I. The growth of P. pusillum on autoclaved yeast cells (isolate # 1).

After 1 day

After 2 days

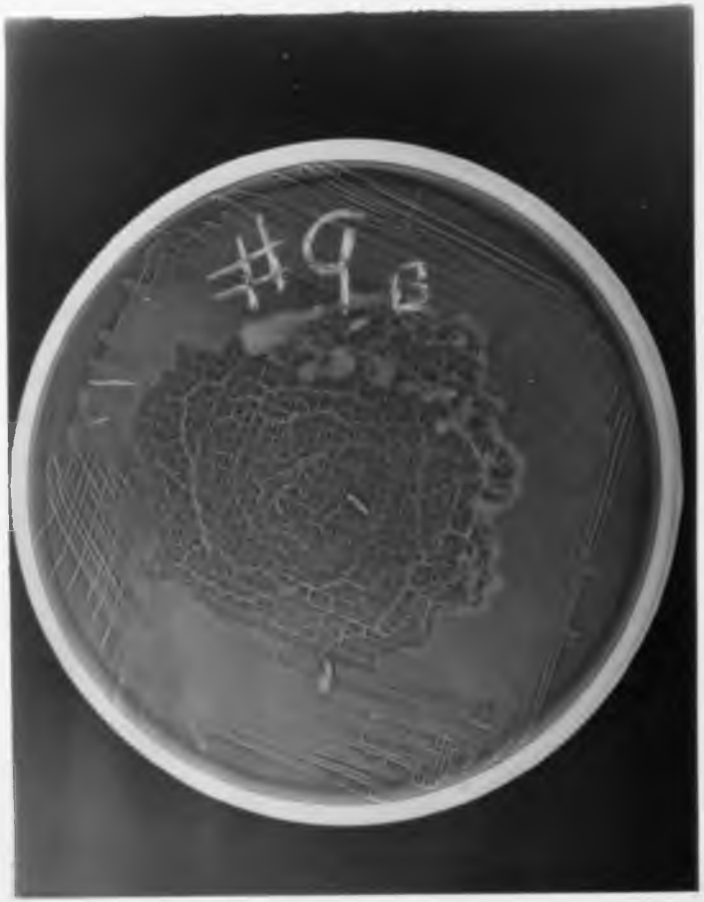
Plate # I (continued). The growth of P. pusillum on autoclaved yeast cells (isolate # 1).

After 3 days

After 4 days

Plate I (continued). The growth of P. pusillum on autoclaved yeast cells (isolate # 1).

After 5 days



After 6 days

Plate II. The growth and the chemotactic response of P. pusillum feeding on an autoclaved suspension of a pink mold-like yeast (isolate # 3).

After 1 day

After 2 days

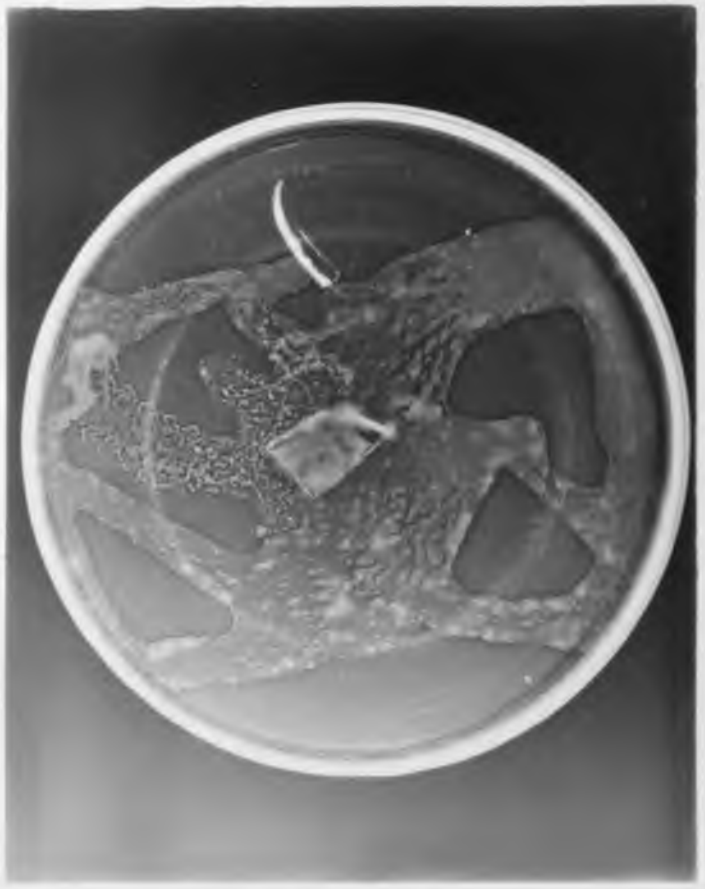
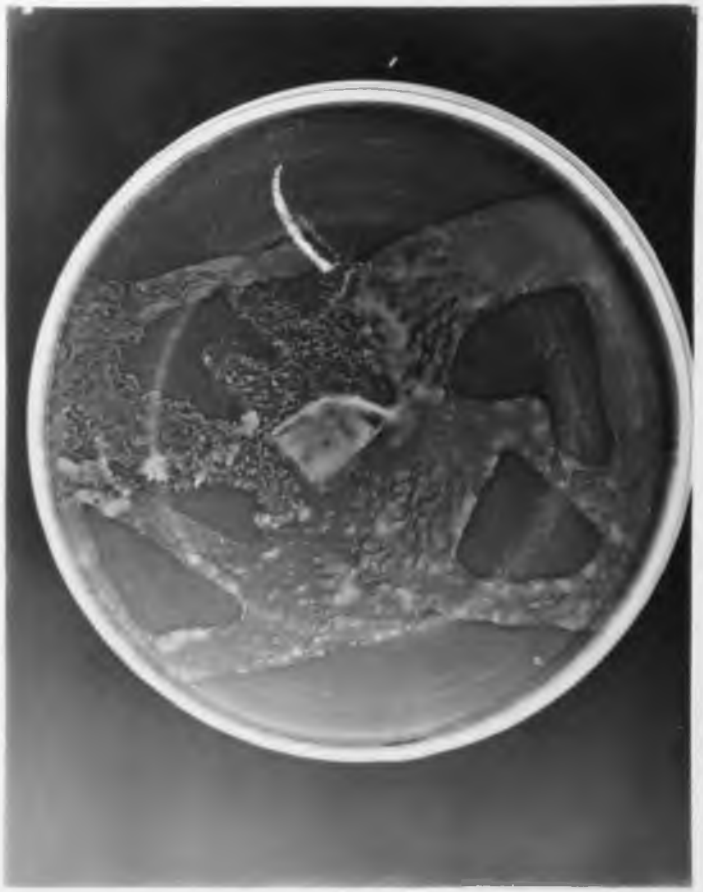


Plate II (continued). The growth and the chemotactic response of P. pusillum feeding on an autoclaved suspension of a pink mold-like yeast (isolate # 3).

After 3 days



After 4 days

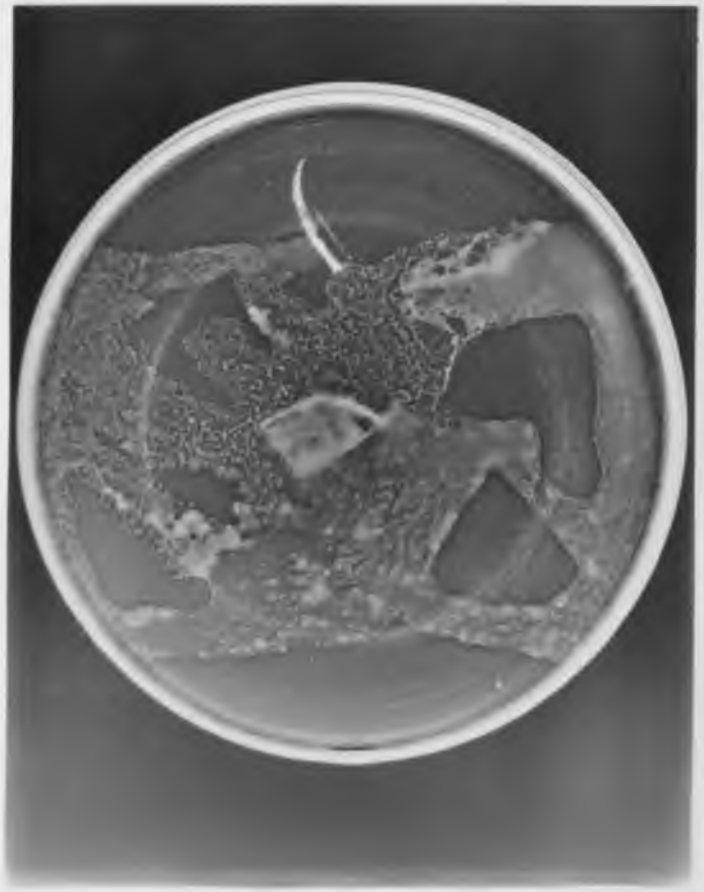
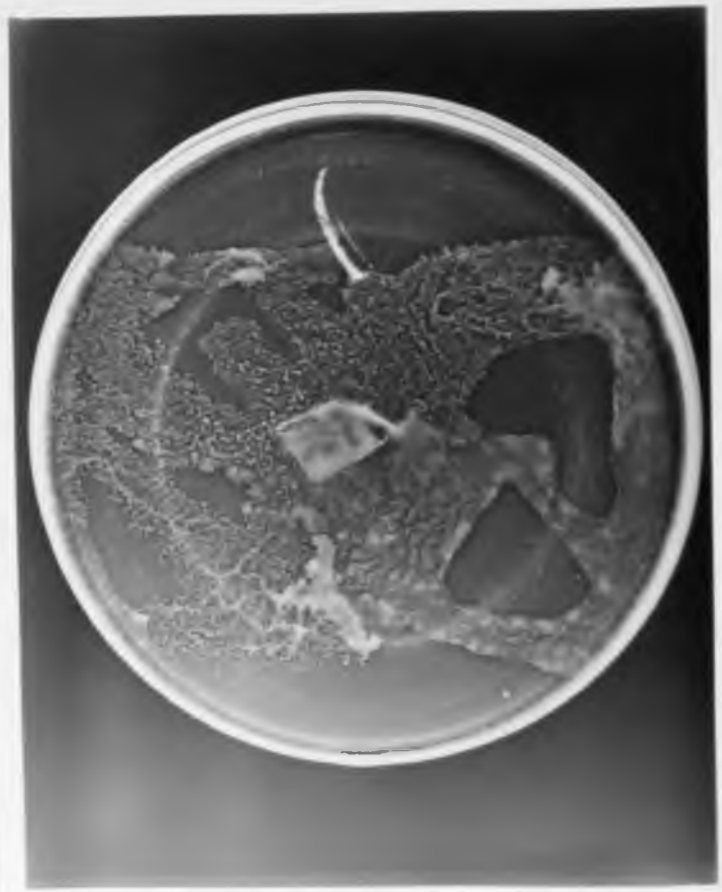


Plate II (continued). The growth and the chemotactic response of P. pusillum feeding on an autoclaved suspension of a pink, mold-like yeast (isolate # 3).

After 5 days



REFERENCES

1. Alexopoulos, C. J. 1959. The laboratory cultivation of Stemonitis. Am. J. Bot. 46:140-142.

2. Alexopoulos, C. J. 1960. Gross morphology of the plasmodium and its possible significance in the relationships among the Myxomycetes. Mycologia. 52:1-20.

3. Camp, W. G. 1936. A method of cultivating Myxomycete plasmodia. Bull. Torrey Bot. Cl. 63:205-210.

4. Cohen, A. L. 1939. Nutrition of the Myxomycetes. I. Pure culture and two-membered culture of Myxomycete plasmodia. Bot. Gaz. 101:243-274.

5. Cohen, A. L. 1941. Nutrition of the Myxomycetes. II. Relations between plasmodia, bacteria, and substrate in two-membered culture. Bot. Gaz. 103:205-223.

6. Cohen, A. L. 1942. The organization of protoplasm; a possible experimental approach. Growth 6:259-272.

7. Cohen, A.L. and J. C. Sobels. 1952. Mineral requirements of some Myxomycetes with possible bearing on their ecology. Bull. Georgia Acad. Science. 10:14-16.

8. Daniel, J. W. and H. P. Rusch. 1961. The pure culture of Physarum polycephalum on a partially defined soluble medium. J. Gen. Microbiol. 25:47-59.

9. DeBary, A. 1887. Comparative morphology and biology of the fungi, Mycetozoa, and bacteria. Clarendon Press. Oxford.

10. Elliott, E. W. 1949. The swarm cells of Myxomycetes. Mycologia. 41:141-170.

11. Evenson, A. E. Personal communication.

12. Gilbert, H. C. and G. W. Martin. 1933. Myxomycetes found on the bark of living trees. Iowa Studies Nat. Hist. 15:3-8.

13. Gray, W. D. 1938. The effect of light on the fruiting of Myxomycetes. Am. J. Bot. 25:511-522.

14. Harshburger, J. W. 1901. Observations on the plasmodium Fuligo septica. Bot. Gaz. 31:198-203.

15. Hok, K. A. 1954. Studies of the nutrition of Myxomycete plasmodia. Am. J. Bot. 41:792-799.

16. Howard, F. L. 1931. The life history of Physarum polycephalum. Am. J. Bot. 18:116-133.

17. Howard, F. L. 1931. Laboratory cultivation of Myxomycete plasmodia. Am. J. Bot. 18:624-628.

18. Jump, J. A. 1954. Studies on sclerotization in Physarum polycephalum. Am. J. Bot. 41:561-567.

19. Lister, A. 1888. Notes on the plasmodium of Badhamia utricularis and Brefeldia maxima. Ann. Bot. 2:1-24, cited from (16).

20. Martin, G. W. 1949. North American flora. The New York Botanical Garden. Part I.

21. Schweinitz, L. D. 1822. Synopsis fungorum. Naturforschende Gesellschaft in Leipzig. Supplement No. 382. p. 63, cited from (8).

22. Sobels, J. C. 1950. Nutrition de quelques Myxomycètes en cultures pures et associées et leur propriétés antibiotiques. 136 pp. Gouda, Netherlands.

23. Sobels, J. C. and A. L. Cohen. 1953. The isolation and culture of opsimorphic organisms II. Notes on isolation, purification, and maintenance of Myxomycete plasmodia. Ann. N. Y. Acad. Sciences 56:944-948.

24. Stokes, J. L. 1954. Studies on the filamentous sheathed iron bacterium Sphaerotilus natans. J. Bacteriol. 67:278-291.

25. Wickerham, L. J. 1951. Taxonomy of yeasts. U.S.D.A., Wash., Tech. Bull. No. 1029.