J.D.E

GENETIC ANALYSIS OF A MYXOMYCETE.

The work was undertaken because genetic analysis of Myxomycetes may eventually yield information about the action of genes in morphogenesis. The Myxomycetes are among the simplest organisms in which morphogenesis occurs and in which the effects of genes in different cellular environments may be studied. No previous work on genetics of Myxomycetes has been reported.

The species used was Physarum polycephalum, which had previously been described but had never been cultured through its life-cycle under controlled conditions. The plasmodium, an amorphous syncytium, grows vegetatively or ceases growth and produces spores. The spores release uninucleate "amoebae" which divide repeatedly. An unknown number of amoebae fuses to form a plasmodium. In the present work, techniques were developed by which each one of these four phases of the life-cycle could be produced when required and in the presence of only one other organism as food, a strain of Pseudomonas fluorescens.

Some of the processes underlying the life-cycle were elucidated. Experiments showed that amoebae cultured from a single spore would not form a plasmodium, that plasmodia were formed only by certain combinations of amoebae from different single-spore cultures, and that these combinations indicated

the existence of two mating-types among the cultures investigated.

Photometric measurements of desoxyribonucleic acid (DNA) content of nuclei in plasmodia and amoebae were done to determine the relative ploidy of the nuclei in these two stages, but the results were inconclusive.

A plating method for amoebae (or spores) was developed to provide a means of accurate quantitative estimation of growth, the isolation of rare mutants and the isolation of large numbers of progeny from crosses.

One clone of amoebae resistant to emetine hydrochloride was isolated. A plasmodium synthesised from this mutant clone and a sensitive clone of opposite mating-type was found to be sensitive to emetine. Three hundred clones of amoebae, each obtained from single spores produced by this "hybrid" plasmodium, were tested for emetine-resistance and mating-type and, with respect to these factors, both parental and both recombinant types were found. The frequencies of the four types indicated that emetine-resistance and mating-type are determined by pairs of allel at two unlinked genetic loci. These results show that genetic recombination occurs during the life-cycle of this Myxomycete and that genetic analysis of the organism is therefore possible.

Genetic Analysis of a Myxomycete.

A thesis submitted for the degree of Doctor of Philosophy to the University of Glasgow, December, 1960.

Jennifer Dee.

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CONTENTS.

INTRODUCTION.

The Myxomycetes, or true slime-moulds, have a unique life-cycle in which they pass from one stage to another through several startling transformations. The stage most easily seen in natural conditions is the fruiting body, which bears, on a stiff, erect stalk, numerous microscopic spores, so hard and resistant that they will exist for many years in dry conditions without losing their capacity to develop. In moist conditions, the spores break open to release uninucleate motile cells, which show amoeboid or flagellar movement depending on the amount of water present. These cells may lead an independent life for long periods of time, feeding on bacteria and increasing in number by mitotic divisions. Under certain conditions, however, by an unknown process, and in a remarkably short space of time, this population of independent, uninucleate cells gives origin to a multinucleate mass of protoplasm, which moves rapidly from place to place by vigorous protoplasmic streaming. This new form is the plasmodium, a mass of delicate, intricate branches, sometimes brightly coloured, constantly changing in form, and capable, in suitable surroundings, of unceasing growth. In natural conditions, the

plasmodium is usually found on dead leaves or wood, and it probably feeds mostly on bacteria. The final transformation is from the moving mass of the plasmodium to the static, regular forms of the fruiting bodies. This change, which occurs when all available food is gone, is also accomplished in a short space of time, it involves a change throughout the entire mass, and is usually accompanied by a striking colour change; in a few hours fruiting bodies have been formed and no trace of plasmodium remains.

Because of this unusual life-cycle, the Myxomycetes cannot justifiably be grouped with any other known type of animal or plant. Zoologists and Botanists have still not agreed in which domain they most properly belong. With a few other small groups of organisms, such as the Acrasiales, which they resemble in some respects, they comprise a group distinct from both animals and plants.

The life-cycle of the Myxomycetes, in the form described above, has been known for about a hundred years, since the work of de Bary (1854) and Cienkowski (1863). Many workers have since studied the organisms, but most of their efforts have been aimed at morphological and cytological description of the stages of the life-cycle

and have not led to any deeper understanding of them. A few studies were made of the factors influencing the occurrence of the different stages when Myxomycetes were cultured in the laboratory, but these were never carried out under controlled conditions. In recent years, very little attention has been paid to the life-cycle, but the properties of the plasmodium have been studied by a number of workers interested in the structure and activity of protoplasm.

Although the Myxomycetes are a distinct group, they are by no means specialized. On the contrary, both the uninucleate and multinucleate stages of their life-cycle show extreme simplicity of structure, and because of this combination of simple structure with unique life-cycle, they are excellent subjects for several kinds of investigation into general properties of biological systems. The chief use that has been made of them so far is in biochemical studies on the properties of protoplasm, which have utilized the simple structure and unlimited capacity for growth of the plasmodium. The possibility that has not been explored is that of using the organism for studies on morphogenesis, by means of the techniques of genetic analysis.

The Myxomycetes are among the simplest organisms to show morphogenesis; that is, change of form as distinct from and unaccompanied by growth. In addition, the life-cycle involves a dramatic change in the cellular environment of the nuclei, and, unlike the gametic stage of higher organisms, the uninucleate stage carries out an independent existence so that it may be as easily studied as the plasmodium. Genetic analysis of the Myxomycetes should therefore make possible a study of the effects of genes on morphogenetic processes and of the effects of genes in different cellular environments, which is a part of the study of differentiation. The object of the present investigation was to make a start on the genetic analysis of one strain of Myxomycete.

The chief obstacle to this programme was the difficulty of culturing Myxomycetes through their whole life-cycle. Much time had to be spent on this part of the problem, but the result has been that most of the difficulties have been overcome. In the course of this work on culture conditions, it was found that the uninucleate stage of the life-cycle could be easily handled by standard microbiological techniques. It was therefore possible to look for and isolate mutant strains at this stage of the life-cycle. Crosses between differently-

marked strains were then made and analysis of the progeny revealed genetic recombination. Concurrently with these preparations for the study of morphogenesis various experimental techniques were also used in attempts to understand the nuclear cycle and the processes underlying the formation of the plasmodium. These led to some conclusions, though much is still uncertain.

The Myxomycetes are such fascinating creatures to watch, and their transformation from uninucleate to multinucleate organism is so dramatic, that many previous investigators have attempted to discover the truth about this mysterious process by observation alone. These attempts have led to numerous conflicting descriptions and, except for the most recent, the results of cytological investigations of the nuclear cycle have been equally unconvincing. The purely observational approach to these problems has therefore been strictly excluded from the present investigation, and experimental and genetical techniques have been developed to tackle them, in the hope that their answers may be more decisive.

Classification.

The Myxomycetes are now generally regarded as forming a distinct class not closely related to any other. They may be loosely grouped in the Myxothallophyta with

three isolated orders, Acrasiales, Labyrinthulales and Hydromyxales, all of which show a pseudoplasmodium which, unlike the true plasmodium of the Myxomycetes, is wholly or partially cellular in structure (Ainsworth and Bisby, 1954).

Within the Myxomycetes are two subclasses:

Subclass 1: Exosporeae.

Subclass 2: Myxogastres.

Subclass Exosporeae consists of only one genus, Ceratiomyxa. In this, the spores are produced externally on individual stalks.

Subclass Myxogastres consists of 44 genera in four orders. The spores are produced internally in fruiting bodies of characteristic form, supported by a framework which often contains lime.

Since results on many different Myxomycetes are discussed in this thesis, a complete list of genera is given in Figure 1. The classification is taken from Martin (1949).

The species used in the present investigation was Physarum polycephalum, obtained in the form of a sclerotium (dried plasmodium) from Dr. H.P. Rusch of Wisconsin University. The identification of the species has been confirmed by Dr. G.W. Martin of the University of Iowa.

Fig. 1. Classification of the Genera in the Class Myxomycetes.

SUBCLASS 1. EXOSPOREAE

One genus only: Ceratiomyxa.

SUBCLASS 2. MYXOGASTRES

Orders	Families	Genera
1. Liceales	a) Liceaceae	Licea, Tubifera.
	b) Reticulariaceae	Alwisia, Lycogala, Dictydiaethalium, Reticularia, Enteridium.
	c) Cribrariaceae	Lindbladia, Cribraria, Dictydium.
2. Trichiales	a) Dianemaceae	Margarita, Dianema, Prototrichia.
	b) Trichiaceae	Perichaena, Arcyodes, Arcyria, Oligonema, Trichia, Hemitrichia, Calonema.
3. Stemonitales	a) Echinosteliaceae	Echinostelium.
	b) Collodermataceae	Colloderma.
	c) Stemonitaceae	Amaurochaete, Brefeldia, Elaeomyxa, Diachea, Enerthenema, Stemonitis, Comatricha, Clastoderma, Lamproderma, Macbrideola.
4. Physarales	a) Physaraceae	Fuligo, Badhamia, Physarella, Cienkowskia, Craterium, Physarum, Leocarpus.
	b) Didymiaceae	Diderma, Mucilago, Didymium, Lepidoderma, Leptoderma.

Terminology.

"Amoebae". In this thesis, the organisms composing the uninucleate stage of the life-cycle have been referred to exclusively as "amoebae". In former works, a variety of terms has been used, and these have been reviewed by Cadman (1931). In general, "amoeba" or "myxamoeba" has been used to denote a stage when the uninucleate organism has no flagellum, and "swarm cell" or "zoospore" for a stage when it has a flagellum or is capable of developing one. Ross (1957) groups species of Myxomycetes into three types, according to the relative lengths of time occupied by "flagellate swarm cells" and "nonflagellate myxamoebae" in the life-cycle, and for P. polycephalum, he reports that the flagellate stage persists for not more than 130 hours after emergence from the spore. In the present work, these distinctions have been found invalid; clones of the uninucleate, amoeboid stage that have been cultured for as long as two years on solid medium have still been observed to develop flagella when placed in water, and have also been found still capable of fusing to form plasmodia. A single name was therefore used for the uninucleate stage, and the term "amoeba" was chosen because it was the simplest and because it may validly

be used as a purely descriptive term and thus cannot add confusion to any further dispute there may be about the characteristics of the uninucleate stage of Myxomycetes in general. On the solid medium used for all cultures in this work, the amoebae move by pseudopodia, do not possess visible flagella, and in fact look exactly like common "soil amoebae".

"Hatching". In all previous works on Myxomycetes, the term "germination" has been used to denote the emergence of the amoeba from the spore. In the present work, the term "hatching" has been used for this event. This change is probably indefensible, since it is not strictly necessary. It resulted from the inability of a student of Zoology to conceive of any process involving the emergence of an independent, motile organism from a shell as "germination". The change of terminology is also well supported by the Oxford Dictionary.

PART I.

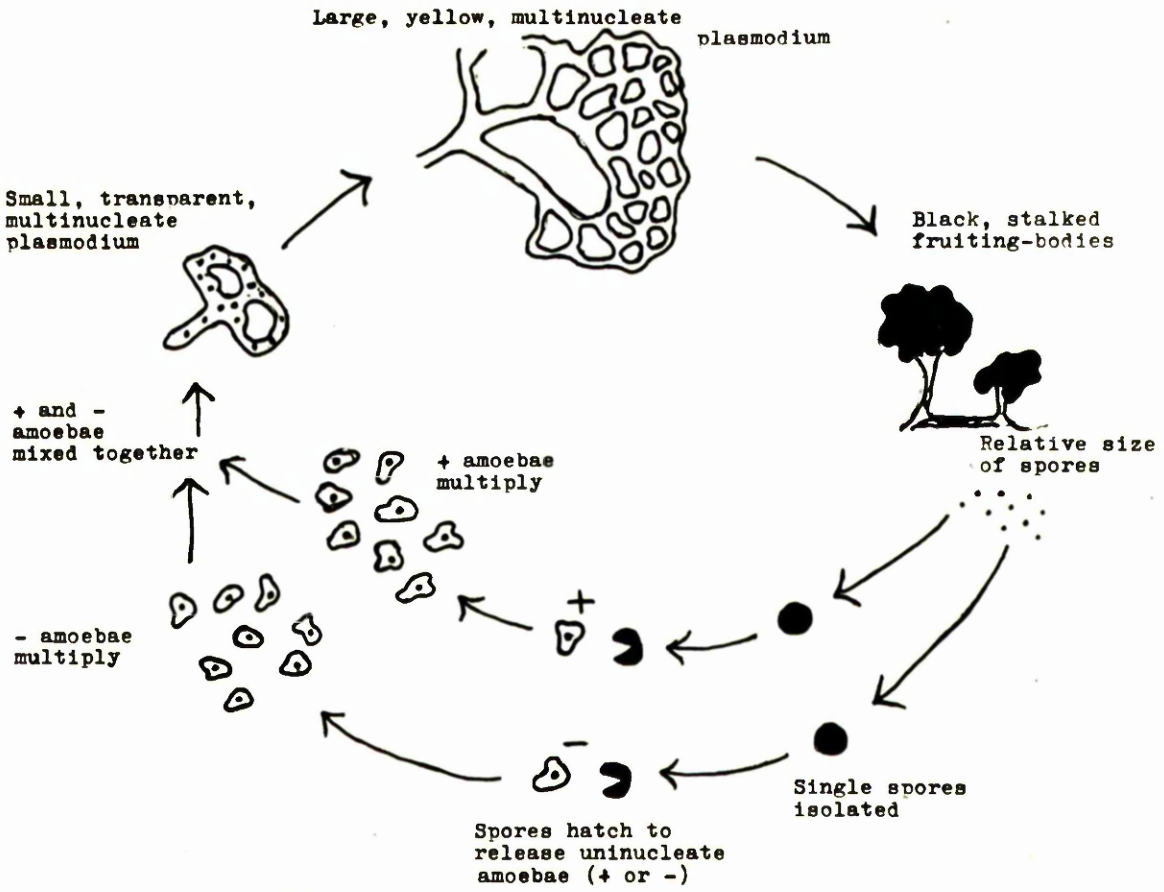
TECHNIQUES TO CONTROL THE LIFE-CYCLE.

Introduction.

The life-cycle of Physarum polycephalum follows the general description given above (see Fig. 2). The plasmodium, which is bright yellow in colour, grows indefinitely in a moist atmosphere, its size depending on the amount of food available. Under certain conditions it produces fruiting-bodies (sporangia) with a calcareous skeleton supporting large numbers of black (or dark purple) spores which can survive in dry conditions for several years. In moisture, a spore, which is about 10 μ in diameter, releases either one or two (both situations have been observed) transparent amoebae, slightly smaller in size. Under suitable conditions an unknown number of amoebae fuses to form a small, transparent plasmodium which immediately shows protoplasmic streaming, grows rapidly and soon develops yellow pigment. This whole life-cycle was observed in the laboratory by Howard (1931a).

In the present work, the object was not to describe the life-cycle, nor to investigate fully the factors influencing it, but simply to develop techniques that would allow the various stages to be produced at will.

Fig. 2. The life-cycle of *Physarum polycephalum*. The mating-type system indicated in the diagram (involving + and - strains of amoebae) was discovered during the present work.


Large, yellow, multinucleate plasmodium
Black, stalked fruiting-bodies
Relative size of spores
Single spores isolated
Spores hatch to release uninucleate amoebae (+ or -)
- amoebae multiply
+ and - amoebae mixed together
Small, transparent, multinucleate plasmodium
+ amoebae multiply

Culture conditions that were fully reproducible were therefore necessary; factors such as temperature, humidity and nutrition had to be controlled and all contaminating organisms eliminated. No previous workers had reported culturing the species under such conditions except a few who grew only the plasmodium (see below).

Throughout the present work, all cultures were incubated at a constant temperature of 25°C (except where it is stated otherwise) and the recognized precautions to maintain sterile conditions were used for all cultures.

1. Growth of the Plasmodium.

A number of authors have reported growth of P.polycephalum plasmodia in fairly well controlled conditions. Howard (1931a) grew the plasmodium on autoclaved oat agar, but did not take precautions to remove contaminating organisms so that it is not certain whether the plasmodium fed on the oats or on bacteria or yeasts present in the culture. Cohen (1939) purified the plasmodium carefully and found that it failed to grow on autoclaved oat agar unless a strain of yeast or bacteria was added. He did succeed in maintaining pure cultures of plasmodium on autoclaved yeast, as also did Hok (1954). Rusch (1959) reports the successful growth of plasmodium in pure culture in a liquid, "partially defined" medium, but the composition of this medium has not yet been published. He has also succeeded in growing the plasmodium in pure culture on a mixture of oats and water (personal communication).

Throughout the present investigation, the plasmodium has been grown on autoclaved oat agar, made by a method similar to that described by Howard (1931b).(see below). The plasmodium originally received was heavily contaminated. It was purified by the use of antibiotics and by migration

over non-nutrient medium. After purification, growth in oat agar was still vigorous, and a series of tests for the presence of contaminating organisms, made by placing samples from the cultures in tubes of broth and other nutrient media, showed that the plasmodium was probably feeding on the oats without any aid from other organisms. No further investigation of the nutrition of the plasmodium was done.

Preparation of Oat Agar.

i) A quantity (1-2 litres) of agar of concentration 1% (w/v) is made, sterilized and stored in flasks of 250 ml. capacity. (Throughout this work the agar used for all media was Oxoid No.3 and the water was Glasgow tap water).

ii) A 250 ml. flask containing 150 ml. 1% agar is heated until the agar is melted.

iii) 12 gs. "loose rolled oats" are added to the agar.

iv) The flask is heated in a water bath at 90°C for 10 minutes.

v) The cotton wool plug is removed and replaced by a fresh plug of cotton wool that is pressed in as tightly as possible and held in place with an elastic band. If this is not done, the plug is usually blown out during autoclaving.

vi) The flask is autoclaved at 15 lbs. pressure for 15 minutes.

vii) The flask is removed from the autoclave and the oat agar is immediately poured into Petri dishes (9 cms. diameter).

viii) Subculturing to the fresh oat agar is usually done soon after the medium has solidified, but the dishes of oat agar may be stored at room temperature for about one week without deterioration.

Subculturing of Plasmodium.

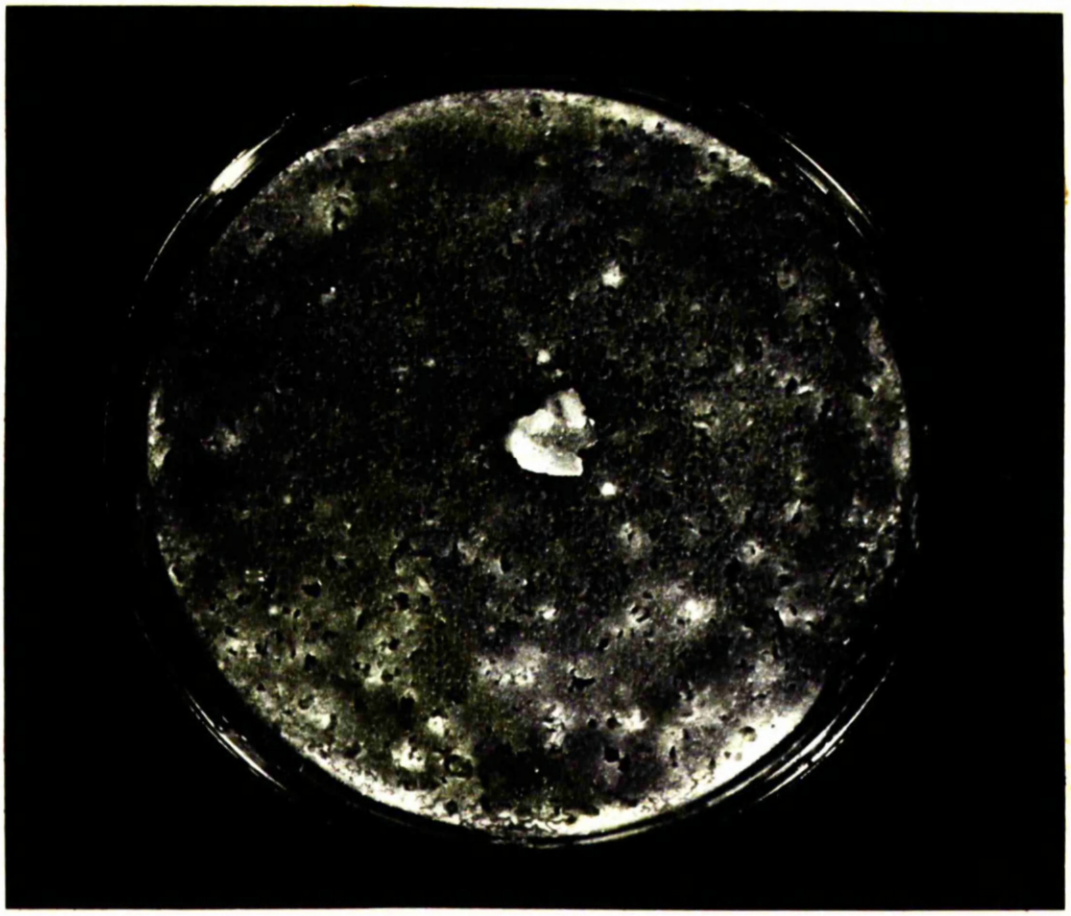
i) A piece of plasmodium about 1 sq. cm. in area and the medium underlying it is cut out from an oat agar culture in a Petri dish and transferred to the surface of fresh medium of the same kind.

ii) The dish is inverted and incubated in a moist chamber at 25°C for three days in the dark. In this time, the plasmodium usually spreads to cover the entire surface of the medium (see Figure 3).

iii) The dish is removed from incubation and stored at room temperature in the dark for about four days before subculturing.

Fig. 3. Plasmodium on an oat agar plate 10 days after inoculation. (Natural size).

In the centre is the lump of medium on which the inoculum of plasmodium was introduced. The plate has been incubated for three days and kept at room temperature for seven days. The plasmodium has spread over the entire surface of the dish and its thick "veins" may be seen in most areas. The white spots show where oats are embedded in the agar and the dark holes where oats have been eaten away by the plasmodium.



2. Formation of Spores by the Plasmodium.

a) Results of Previous Investigations. Several investigations have been made of the factors influencing formation of spores (fruiting) in Myxomycetes, Physarum polycephalum having been used for most of the work. With this species, Camp (1937) found that the plasmodium would not form spores in the presence of food, and Howard (1931a) found that spore formation would occur in the presence of food only if the food was "fouled" by bacteria. Gray (1938) reported that spore formation could occur in the presence of food but that it took place much more rapidly if food was absent.

Gray (1938) found that P. polycephalum and three other species with yellow plasmodia rarely formed spores when kept in the dark, and in experiments with cultures of P. polycephalum exposed to artificial light he found that in either the presence or absence of food, fruiting occurred more rapidly the greater the intensity of the light. When cultures were treated with alternating short periods of light and darkness, the total period of exposure to light necessary for spore formation was less than when exposure was continuous. In tests with different wavelengths, he found that the shortest were probably most satisfactory in producing spore formation. Ten species with white

plasmodia formed spores equally well in light or darkness.

In another series of experiments on the fruiting of P. polycephalum plasmodia deprived of food and exposed continuously to light, Gray (1939) found that both temperature and pH influenced spore formation and that the effects of these factors were inter-related; the higher the temperature, the lower the optimum pH. Seifriz and Russell (1936), after a series of experiments with P. polycephalum, reported that fruiting was unpredictable and affected by none of the many factors they tested, including temperature, nutrition and light.

Sobels and van der Brugge (1950) described a method for obtaining spores from yellow plasmodia by growing them in flowerpots standing in water. They believed the method to be successful because the plasmodium could choose the most favourable position for fruiting on the gradient of humidity provided by the vertical side of the porous pot. The cultures were fed on oat agar in the presence of yeast and exposed to daylight. Spore formation in the absence of other organisms was not reported until Rusch (1959) described a reliable method for obtaining spores from P. polycephalum plasmodium grown in pure culture in a

"partially defined soluble medium", but unfortunately the necessary details of this method were not available until late in the course of the present investigation (see below).

b) Results of the Present Investigation. The results of previous workers had shown that some of the factors influencing spore formation in P. polycephalum were the amount of food present, light, temperature, pH and humidity, and had also suggested some possible methods of culturing the plasmodium in the most favourable conditions for spore formation. All these factors were taken into account in a series of tests to obtain spore formation in the present investigation, in which plasmodium grown in pure culture on oat agar was used. Of more than 100 cultures used in these tests, only three produced spores, and no conclusive results about the effects of the different factors were obtained.

The effect of adding bacteria to the cultures was then tested, using a pure strain of bacteria isolated from the original contaminated plasmodium. (The strain has since been identified as Pseudomonas sp.). The plasmodium was kept in flasks in the dark and allowed to feed on oat agar and bacteria until it migrated away from the food; the cultures were then exposed to daylight. In 41 cultures

of this kind to which bacteria were added to the oat agar before inoculation of the plasmodium, spores were formed in 30, while in 15 oat agar cultures to which no bacteria were added, no spores were formed. The successful method was therefore adopted as the standard method for inducing spore formation. (See Figs. 4 and 5).

Details of Method.

i) The culture vessels used are Pyrex conical glass flasks of capacity 150 ml., plugged with cotton wool. About 5 ml. oat agar (prepared as described above) is poured into each and allowed to solidify in a thin layer at the bottom of the flask.

ii) A suspension of the isolated strain of Pseudomonas, of concentration approximately 2×10^9 bacteria/ml. is made in saline and 0.5 ml. of this suspension is added to each flask and spread over the medium by agitation of the flask. (In practice, the concentrations of bacterial suspensions are standardized by culturing the bacteria on slopes of a Czapek-Dox-glucose medium of standard area for a standard length of time and washing all the growth off a slope into a known volume of saline).

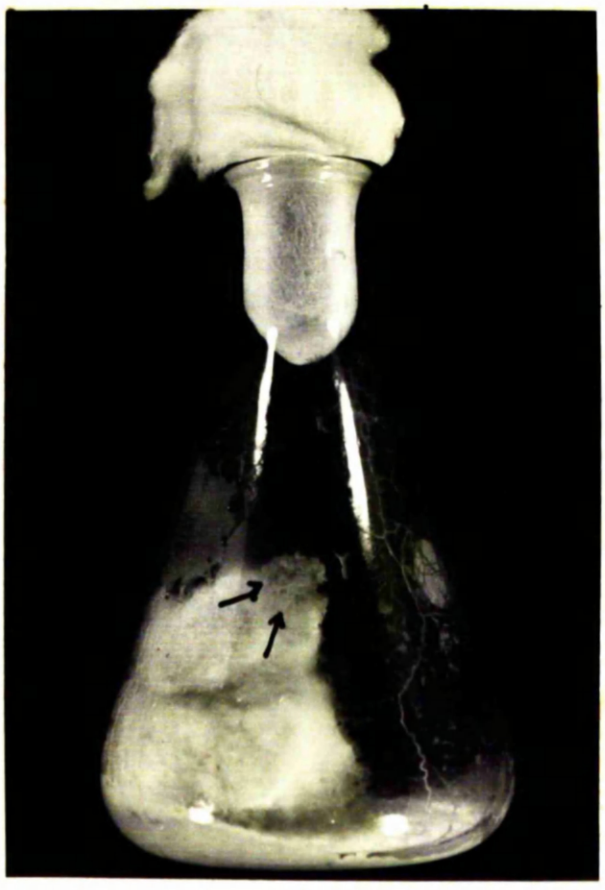
Fig. 4. The method for inducing spore-formation. (2/3 x natural size).

a. After five days incubation in the dark, The plasmodium has spread up the sides of the flask from the oat agar at the bottom and has condensed into thick veins. Cotton wool is added to the flask when all the plasmodium has left the oat agar.

b. Twenty-four hours after the cotton wool is added. The plasmodium has all left the sides of the flask and has formed a mass on top of the cotton wool (shown by the arrows). The marks on the sides of the flask are tracks of slime left by the plasmodium. The flask is now exposed to light.

c. Two days after the first exposure to light. A mass of black spores has formed on the cotton wool and no plasmodium remains. The flask is viewed from the side that was nearest to the light.

a

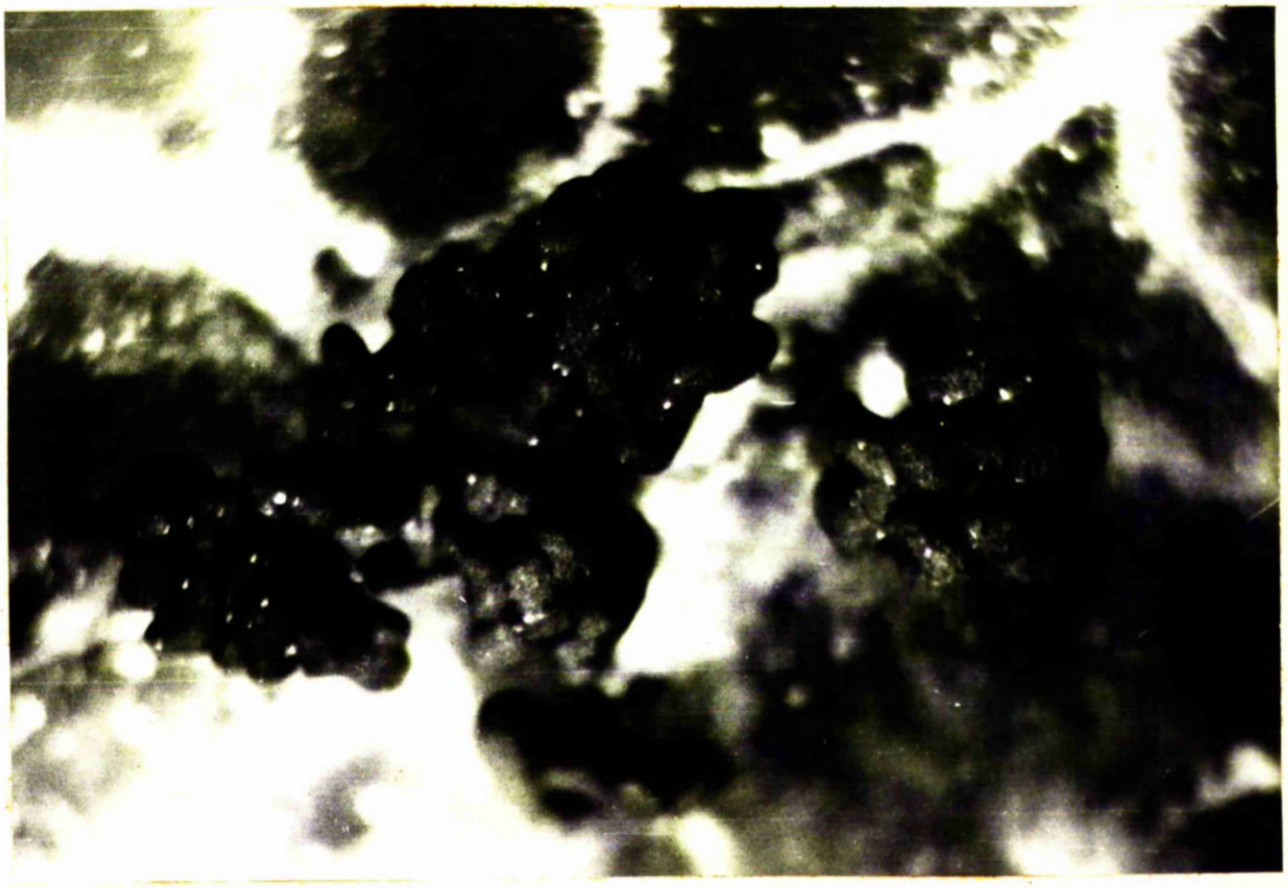


b

c

Fig. 5. Sporangia (fruiting-bodies). (x 36)

The sporangia consist of masses of black spores supported on a calcareous framework. The shape of the spore masses is characteristic of the species. Individual spores may just be distinguished on the surface of the mass at this magnification. In the background are branching tracks of slime left by the plasmodium. These sporangia were formed on a glass surface.



iii) The cultures are incubated at 25°C for 24 hours in the dark.

iv) A piece of plasmodium about 1 sq. cm. in area cut from an oat agar culture in a Petri dish is placed on the medium in each flask.

v) The cultures are incubated at 25°C in the dark until all the plasmodia has left the medium and has moved up the sides of the flask. (Usually about 7 days).

vi) A lump of sterile, non-absorbent cotton wool, big enough to cover about half the surface of the medium and about one third of the internal height of the flask is placed on the layer of medium in each flask, touching the glass at one side of the flask.

vii) The cultures are incubated at 25°C in the dark for 24 hours. At the end of this period, all the plasmodium is usually in a compact mass on the cotton wool.

viii) The flasks are placed about 15 cms. from two bars of Osram fluorescent, "Daylight" illumination in the 25°C incubator. The light is switched on and off by a time switch, giving alternate periods of 6 minutes light and 14 minutes darkness; with this timing, the cultures do not become over-heated.

Under these conditions, spores usually form in most of the cultures 1-2 days after they are first exposed to light. The spores may then be easily removed by pulling out the lump of cotton wool. The pieces of cotton wool to which spores are attached are separated from the rest of the mass, dried in Petri dishes covered with filter paper at 25°C, and stored in small tubes at room temperature. The entire process of spore formation takes about 10 days.

Alternative Method Derived from Rusch. Rusch (1959) reported that if a piece of plasmodium was deprived of food and incubated in the presence of nicotinic acid for several days in darkness, it would form spores soon after it was exposed to light. His method consists of incubating the plasmodium on moist filter paper supported on glass beads over a layer of medium containing only salts, calcium carbonate and nicotinic acid. The flasks are exposed to light for two hours after four days incubation in the dark. Spore formation usually occurs within 12 hours of illumination. The details of this method were not published until some time after the oat agar method had been developed, and it has so far been used only a few times. Spores were formed in some of the cultures, but the method is probably

less satisfactory than the oat agar method for plasmodia regularly grown on oat agar; Rusch's method was designed for use with plasmodia grown in liquid culture from which, unlike oat agar culture, large volumes of plasmodium may be easily harvested and washed free of growth medium.

c) Discussion. The work of Rusch (1959) has clarified the processes involved in spore formation. He found that a plasmodium formed spores if it was, in sequence,

(i) deprived of food,

(ii) incubated in the presence of nicotinic acid in the dark for four days,

(iii) exposed to light for two hours.

All these conditions are necessary: if nicotinic acid is supplied in the growth medium and not during the period of incubation in the absence of food, spores are not formed, and if the period of incubation with nicotinic acid in the absence of food is less than four days, spores are not formed. From his work, Rusch concluded that the period of incubation with nicotinic acid before illumination was "presumably required for the conversion of nicotinic acid to pyridine nucleotides, which in turn (presumably) re-orient metabolism towards sporulation by directly or indirectly sensitising the plasmodium to light".

He suggested that the minimum incubation period of four days represented the time required for this process to be completed. After this time, exposure to light initiated spore formation. He found that the process of re-orientation was reversible until the end of the period of exposure to light. If growth medium was added to the cultures before they were exposed to light, all plasmodia began to grow again and failed to form spores, but if it was added after two hours exposure to light, all cultures formed spores and failed to grow, even though none of the morphological changes associated with spore formation had been visible when the growth medium was added.

The Effect of Bacteria. In the present work, one of the conditions found to be necessary for spore formation was the presence of bacteria. If it is assumed that Rusch's findings apply equally well to the type of cultures used in the present work as to his own, it may be suggested that the bacteria help to supply one of the conditions that he found necessary. It is quite likely, in fact, that the bacteria produce nicotinic acid in the medium, which acts on the plasmodium after the nutritional content of the food has been exhausted. On the other hand, it is possible that the bacteria simply serve to cause migration

of the plasmodium away from the food. At the time when the plasmodium leaves the medium and moves up the sides of the flask, there are nearly always some unused oats left, but the surface of the medium is covered with bacterial growth. Bacterial waste products may cause the migration. In other words, the presence of food that is "fouled" by bacteria may be equivalent to "absence of food" for the purpose of inducing a plasmodium to cease growth and turn to spore formation. The effect of "fouling" by bacteria in producing spore formation was also found by Howard (1931a).

If the "absence of food" is the necessary requirement satisfied by the bacteria, it may be wondered why plasmodia grown on oat agar without bacteria do not eventually form spores also. It is possible that the reason for this is simply a matter of time; in the absence of bacteria growth on oat agar is slower and it is possible that by the time the food is exhausted other conditions in the cultures, such as humidity, have become unfavourable.

The Effect of Light. The results of Gray (1938) and Rusch (1959) showed conclusively that exposure to light is a necessary condition for fruiting. The present work

has supported the results of these authors in showing that artificial light is as satisfactory as daylight so long as it is of fairly short wave-length (white, not yellow), and that fractionation of the period of exposure does not inhibit spore formation. Since spores can be obtained from plasmodia of P. polycephalum exposed to light for short constant periods of time at constant temperature, as in this work and in Rusch's work, it appears that effects of daylength or seasonal change are not important in influencing spore formation in this species.

Humidity and Surface Texture. The results of the present work suggest that, as might be expected, there are other factors extremely important in influencing fruiting apart from those clearly implicated in the various investigations described above. Two of these factors appear to be the humidity of the cultures and the texture of the surfaces available in them. The evidence for this conclusion is derived chiefly from observation of a large number of unsuccessful cultures. It may be illustrated by two particular observations:

i) Before the details of Rusch's work were published, the information was available that nicotinic acid

induced spore formation (Rusch, personal communication). Early in the present work, a number of attempts were therefore made to test the effect of nicotinic acid, but these were so unsuccessful that they were eventually abandoned. But when the details of the method became known, it was used successfully. The chief difference between these tests was that in the unsuccessful ones, nicotinic acid had been supplied in a water agar medium, while in the successful ones, the plasmodium was placed on filter paper supported by glass beads over a liquid medium containing nicotinic acid.

ii) When the standard oat agar method of inducing spore formation was adopted, it was found that cultures were successful more often if cotton wool was added to them before they were exposed to light. The cotton wool clearly forms a very suitable surface for spore formation since, when it is present, the spores are almost invariably formed on it, and very few are ever formed on the glass or on the medium.

Both these observations suggest that, as also suggested by Sobels and van der Brugge (1950), a fibrous, porous surface is a favourable substrate for spore formation. Whether the effect is due to the fibrous structure itself or to the gradient of humidity that accompanies it is of

course uncertain, but it is likely that one or both of these factors is important.

3. Development of Amoebae from Spores.

a) Results of Previous Investigations. Previous workers with Myxomycetes seem to have had little difficulty in inducing the spores to hatch and the amoebae to multiply. Howard (1931a) found that the spores of P. polycephalum germinated satisfactorily in water. Smart (1937) found that the percentage of spores hatching in many Myxomycete species was raised by the presence of nutrient decoctions, but since in P. polycephalum he found that 80% of spores hatched in tapwater or distilled water, the effect of added nutrients was slight in this species. He also concluded that the optimum temperature for hatching was between 22°C and 30°C and that the speed of hatching, though not the percentage, could be increased by the presence of other hatching spores.

Elliott (1949) found that the use of bile salts, such as sodium taurocholate, as wetting agents increased the percentage of spores hatching. Gilbert (1929) found that the spores of some species gave a very low percentage of hatching until they were a few months old, while those of other species would hatch immediately after formation.

P. polycephalum was not included in this investigation. Cook and Holt (1928) and Smith (1929) found hatching of spores that were five years old and thirty years old respectively in many species, but P. polycephalum was not included in the investigation.

b) Results of the Present Investigation. In the present work also, it was found to be simple to induce hatching and multiplication of amoebae under conditions similar to those used by Howard and Smart. Heavy suspensions of spores obtained from the initial, contaminated plasmodium were inoculated on the surface of water agar in Petri dishes and the dishes incubated in moist chambers at 25°C. Many spores hatched and there was dense growth of amoebae on the surface of the agar. It was found that free water was not necessary to the amoebae; growth from spores to plasmodia took place in these cultures on the surface of the agar. Microscopic examination of these plates, inoculated with the contaminated collections of spores, revealed that the amoebae were multiplying most quickly in the areas where bacteria or other contaminants were growing on the surface of the agar. The greatest multiplication was found in translucent patches of bacterial growth. The strain of bacteria giving this

growth was therefore isolated from the plates, purified and established as a stock on slopes of a nutrient agar containing Czapek Dox and glucose. This strain of bacteria has been used throughout the present work for feeding amoebae and, in later parts of the work, has been used for feeding the plasmodium when spore formation is required (see I.2 above). The bacteria are gram-negative rods, motile and chain-forming. The strain has been identified by Dr. J. Norris as Pseudomonas sp., probably P. fluorescens. These bacteria give slight growth on water agar, forming translucent "pools" in which the amoebae feed and multiply. On richer medium, the bacterial growth is too heavy for successful growth of the amoebae.

As soon as the strain of bacteria had been purified, tests to induce hatching and multiplication of amoebae were done under controlled conditions. Spores formed by plasmodia that had been freed of contaminating organisms (as described in Section 1) were used and the spore suspensions were further cleaned by repeated washing by centrifugation before use. Spores treated in this way carried a negligible amount of bacteria with them. When placed on the surface of water agar, some of the spores hatched but the amoebae encysted without multiplying.

However, when a suspension of the isolated bacteria was inoculated on top of the encysted amoebae, they began to multiply. In all future tests, therefore, spores were freed of all contaminants before use, and the only food supplied for the amoebae was the isolated strain of bacteria growing slightly on the surface of water agar. (See Fig. 6).

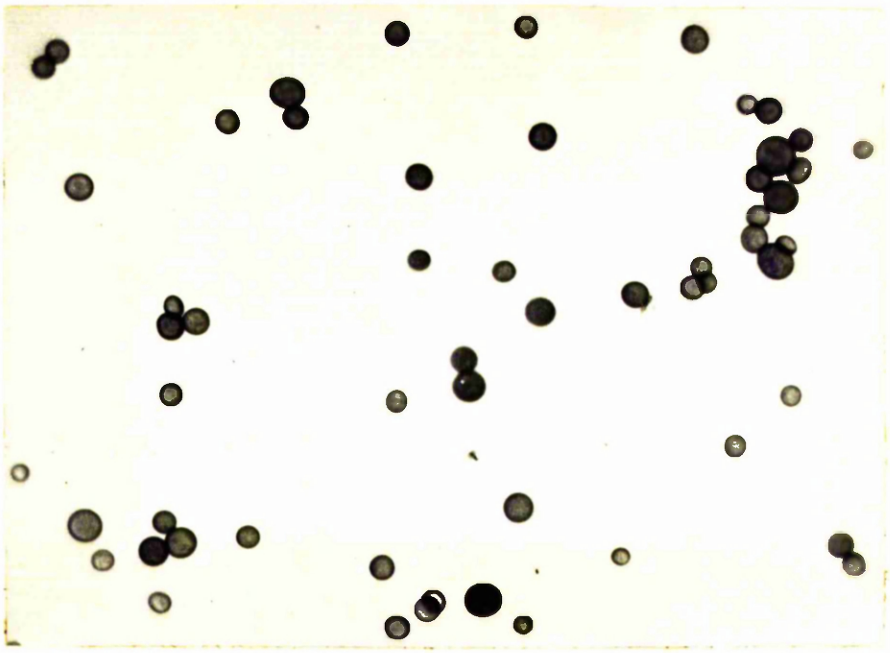
A series of tests was then done to induce the hatching and multiplication of amoebae from single, isolated spores. Spores were isolated by manipulation on the surface of agar with the aid of a dissecting microscope. Small squares of agar carrying single spores were cut out and transferred to plates of water agar, where a loopful of bacterial suspension was inoculated on top of each. If the spore hatched, the amoebae multiplied until they had eaten all of the small patch of bacteria and then encysted and remained viable until transferred to fresh medium. In the first series of spores treated in this way, from one set of sporangia (i.e. sporangia formed in one culture flask), 45 spores hatched of 221 isolated (20.4%). In another series, with spores from a different set of sporangia, only 7 hatched of 273 isolated (2.6%).

Fig. 6. a. Spores (x 310).

A suspension made by crushing sporangia in water. The photograph shows typical variation in size.

b. Amoebae and the strain of bacteria (Pseudomonas sp.) on which they feed (x 470).

A suspension made in water from a culture of amoebae and bacteria on water agar. Some of the amoebae are active, (irregular in shape) and some are encysted (round). The bacteria are chain-forming rods.



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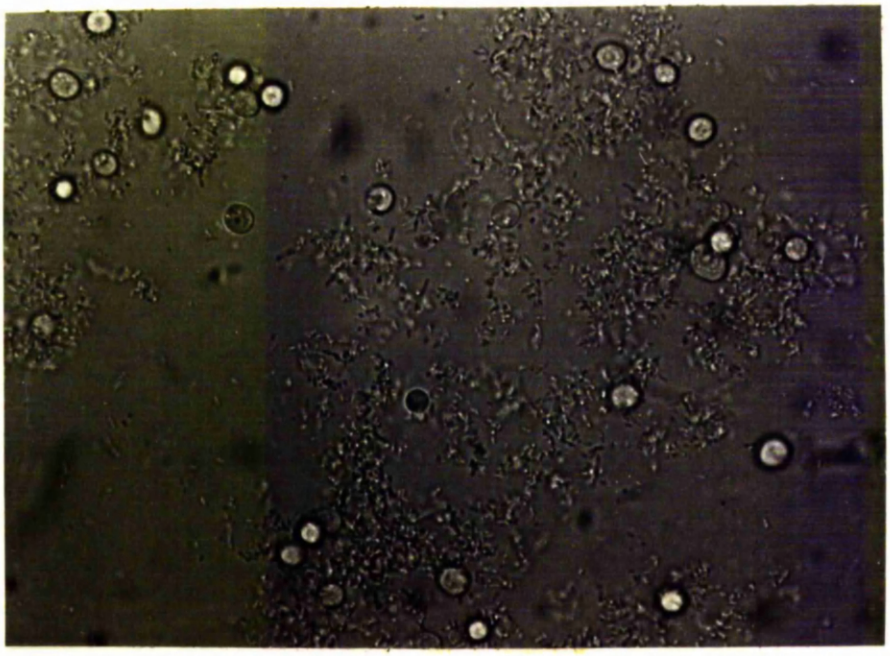


b

The Plating Method. When amoebae were growing under the conditions described above, by feeding on a layer of bacterial growth on water agar, it was noticed that the areas in which they had multiplied were clearly visible to the naked eye. As the amoebae multiplied and ate the bacteria, they caused a translucent area to appear in the more opaque background of the bacterial layer. It was noticed also that where a single amoeba had initiated such a patch of multiplication, the patch was always circular, this being presumably caused by the fact that each amoeba fed and divided without moving very far over the surface. The centre of such a patch consists of encysted amoebae which have finished the available food.

These observations suggested the possibility of a simple plating method for spores or amoebae. A similar method was developed independently by Kerr and Sussman (1958) in work with Didymium nigripes. The principles of the method used in the present work were derived from those commonly used for other micro-organisms. A method for successfully plating amoebae was easily worked out but plating spores was found to be more difficult. The details of the method used for amoebae are as follows:

i) The medium is water agar of concentration 2% (w/v) in 9 cm. diameter Petri dishes, poured about half an hour before plating.

ii) A suspension of amoebae is made in distilled water. The suspension is made homogeneous by pipetting with a Pasteur pipette.

iii) The density of the suspension is estimated by haemocytometer counts.

iv) The suspension is diluted to the required density by serial dilutions in distilled water. Dilutions are made so that 0.1 ml. or 0.2 ml. of the final suspension will contain the number of amoebae required on one plate (the most convenient number is 100).

v) The required volumes (0.1 ml. or 0.2 ml.) of the appropriate dilutions are plated on the surface of the water agar by spreading with a glass rod.

vi) The dishes are allowed to stand at room temperature for 1-5 hours to enable the water to soak into the agar.

vii) The dishes are spread with bacteria. The suspension of bacteria (the isolated strain of Pseudomonas) is made in saline from a two-day culture on Czapek-Dox-glucose medium. The concentration of the suspension

is approximately 1 x 10^{10} bacteria/ml. and 0.1 ml. is spread on each dish. Spreading is done with a glass rod. In practice, the concentration of the bacterial suspension is standardized by growing the bacteria on slopes of constant area and washing all the growth from a slope into a standard volume of saline.

viii) The dishes are allowed to stand at room temperature until the surface of the agar is dry (often they are left in this position overnight).

ix) The dishes are inverted and incubated at 25°C.

After four days incubation, growth is visible on the plates, a circular, clear area having been produced by each amoeba that has produced a viable colony. These circular, clear areas have been called "plaques" by the author (and also by Kerr and Sussman, 1958) because of their similarity to the "plaques" produced by bacteriophage (see Figs. 7 and 8). The number of plaques on each plate is counted after four days incubation and the counts are continued until the plates have been incubated for ten days. New plaques continue to appear during this time.

The efficiency of the plating method has been thoroughly tested by platings of amoebae. The viability of amoebae is

Fig. 7. Plaques on a Petri dish after six days incubation. (Natural Size).

A suspension of amoebae and a heavy suspension of bacteria were plated successively on water agar in the dish. The dark background is the layer of bacteria; the circular clear areas are the "plaques" where amoebae are multiplying.

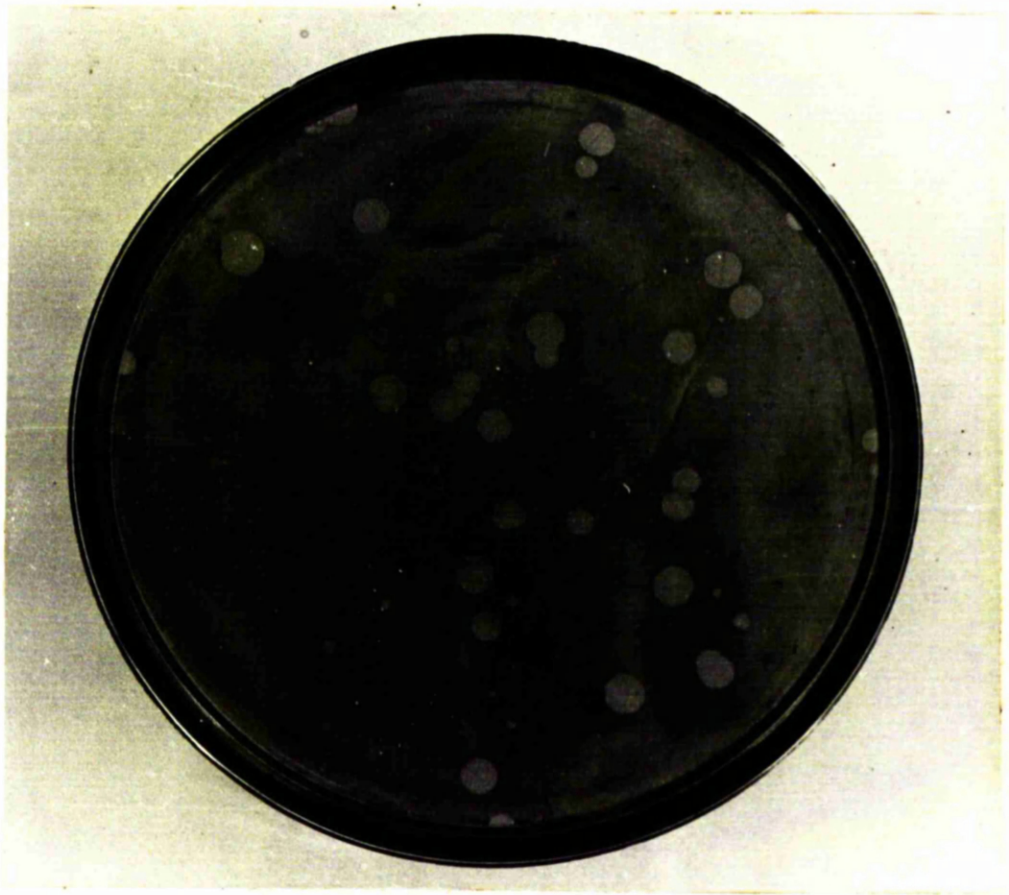
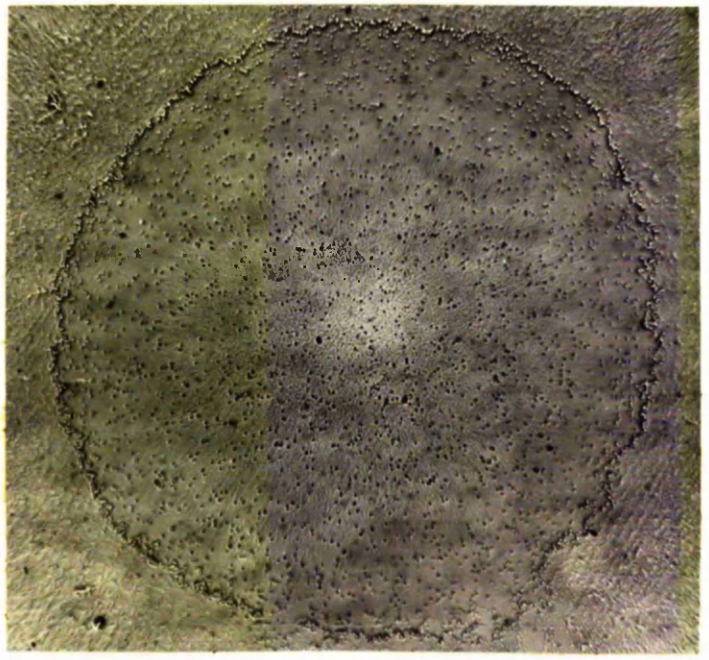


Fig. 8. a. A single plaque. (x 28).

The amoebae are densely crowded around the edge of the plaque, where they are feeding on the bacterial layer. In the centre, where the food has been finished, the amoebae have encysted.

b. The edge of a plaque (x 100).

The irregular shape of most of the amoebae shows that they are still actively moving around and feeding. A few have encysted and appear circular. At the edge, the amoebae are crowded close together but such crowding may be easily distinguished from the fusion involved in plasmodium formation (See Figs. 10-12).



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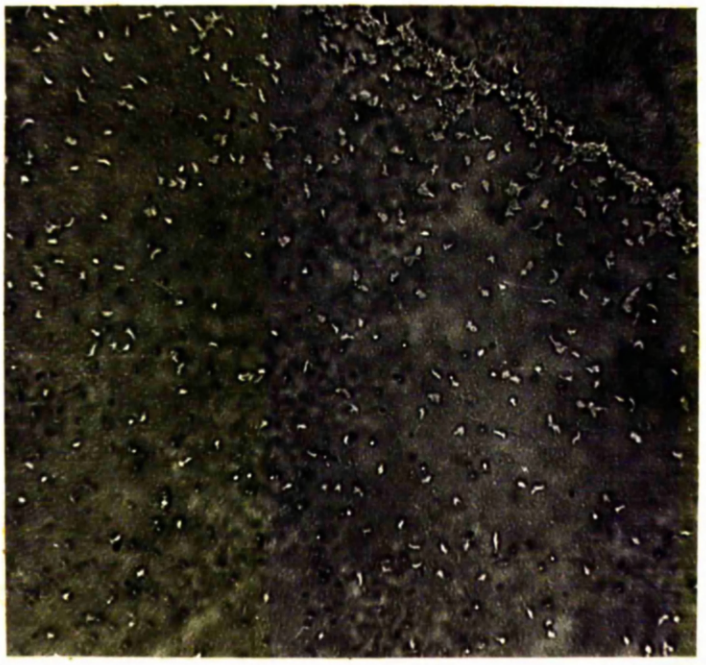


b

usually nearly 100%; that is, the number of plaques corresponds closely to the number expected on the basis of the haemocytometer counts. Also, the counts on different plates of a series show a variance not substantially greater than expected by chance.

It was concluded that the plating method could be used to estimate the viabilities of batches of spores. Since the 100% viability found in platings of amoebae indicated that each plaque arose normally from only a single amoeba, it was also concluded that platings of spores could be used as a quick method of isolating the products of single spores. Tests were done to discover the most suitable conditions for plating spores. In these, however, it was found that plating spores gave less regularly successful results than plating amoebae. Two series of platings with two different batches of spores were done before the conditions necessary for successful plating of spores were discovered.

Table 1. Results of the first series of spore platings. The spores used had been formed on 18.10.58. The "total viable count" for a plating represents the total number of plaques formed divided by the total number of spores plated and expressed as a percentage. Except where otherwise stated, the random variation among viable counts for plates of one plating was small.

Plating No.	Plating Date	Age of Spores	No. of Plates	Total No. of Spores	Result (Total viable count)	Treatment
1.	31.10.58	2 weeks	10	1,000	3.0%	"Calsolene" (lauryl sulphonate) used as wetting agent in spore suspension.
2.	3.3.59	4½ months	16	6,000	12.5%	a) Range of bacterial concentrations tested: 10^6 - 10^8 bact./dish. b) Treatments to increase humidity.
3.	24.7.59	9 months	20	10,000	2 plates covered with amoebae, 0.3% on the rest.	-
4.	3.8.59	9½ months	15	10,000	2 plates covered with amoebae, 0.5% on the rest.	Some plates incubated at 20°C; the rest (including the two good plates) at 25°C.
5.	4.8.59	9½ months	16	10,000	0.2%	a) Pretreatment of spores in 5% sucrose solution. b) Incubation at 20°C and 25°C.
6.	4.8.59	9½ months	16	10,000	0.5%	a) Pretreatment of spores with cold shock (-12°C). b) Incubation at 20°C and 25°C.
7.	8.10.59	11½ months	24	12,000	0.0%	-
8.	9.12.59	13½ months	24	9,000	0.01%	Bacterial suspensions made with cultures of different ages (2 - 42 days old).

Table 2. Results of the second series of spore platings. The spores used had been formed on 16.12.59. For explanation of terms see Table 1.

Plating No.	Plating Date	Age of Spores	No. of Plates	Total No. of Spores	Result (Total viable count)	Treatment
1.	6.1.60	3 weeks	20	70,000	1 plate partly covered with amoebae. 0.01% on the rest.	Bacterial suspensions made with cultures of different ages (2 - 25 days old)
2.	8.1.60	3 weeks	16	90,000	1 plate covered with amoebae. 0.03% on the rest.	Bacterial suspensions made with cultures of different ages (2 - 30 days old)
3.	18.1.60	5 weeks	a) 8	8,000	8 plates covered with amoebae.	Various treatments to increase humidity: dishes sealed with water or agar; surface of medium broken up; second layer of agar poured over spores.
			b) 10	10,000	1 plate partly covered with amoebae. 0.04% on the rest.	Nutrients added to the medium: vitamins; oat decoction.
4.	8.2.60	8 weeks	18	12,000	1 plate covered with amoebae 5 plates partly covered with amoebae. 0.03% on the rest.	Sodium taurocholate used as wetting agent in spore suspension.
5.	12.2.60	8 weeks	16	8,000	3 plates partly covered with amoebae. 0.00% on the rest.	Bacterial suspension mixed with agar and poured over spores after plating.
6.	18.2.60	9 weeks	a) 8	3,000	4 plates covered with amoebae. 0.00% on the rest.	Sodium taurocholate used as wetting agent. Agar poured over spores after plating.
			b) 8	3,000	2 plates partly covered with amoebae 0.00% on the rest.	- Bacteria plated on top of this second layer.
7.	24.2.60	10 weeks	21	7,000	6 plates covered with amoebae 0.00% on the rest.	Agar poured over spores after plating. Bacteria plated on top of this second

The results of the two series of platings are shown in Tables 1 and 2. Platings 1 and 2 in the first series (Table 1) gave quite good results, and the method used in Plating 2 was adopted as the basis for all the other platings. The method was similar to that described for amoebae, the chief differences being:

i) The medium was 1% agar instead of 2%.

ii) Approximately 1 x 10^8 bacteria were spread/dish instead of 1 x 10^9. (The results of Plating 2 were equally good on plates spread with 10^6 and 10^8 bacteria/dish).

iii) The dishes were not allowed to dry before they were incubated.

iv) The dishes were incubated in a moist chamber.

The various treatments applied in attempts to improve results with this method are shown in Tables 1 and 2. Some of the treatments were suggested by the results of other workers and some by a consideration of results in the present work. Comparison of the successful and unsuccessful platings in the present work suggested that high humidity was an important requirement for success. Much attention was paid to the conditions of humidity in the dishes and all factors that could affect humidity,

such as the concentration of the agar, the volume of spore and bacterial suspensions plated, the type of dish used, the conditions of incubation and the intervals of time between the different stages of the plating were as far as possible identical with those of the successful platings. However, none of the treatments used in the first series had any significant effect on the results.

In the second series, the use of sodium taurocholate as a wetting agent in the spore suspension (Elliott, 1949) perhaps gave a slight improvement in the results of Platings 4 and 6 (see Table 2), but soon after this a successful plating method was developed in which no wetting agent was used.

As shown in the Tables, Platings 3 and 4 in the first series and Platings 1 and 2 in the second series showed irregular results. In each of these platings, so many spores hatched on one or two plates that these plates were covered with amoebae and plaques could not be counted. A careful consideration was made of all possible differences that could exist between these successful plates and unsuccessful ones in the same plating. It was concluded that the two factors most likely to be causing the effect were:

i) Humidity. Variations in the conditions of humidity between different individual dishes all poured at the same time, plated with same volume of liquid and incubated under the same conditions could still be caused by differences in the depth of the agar, the temperature at which it was poured or the tightness of fit between the dish and its lid.

ii) Presence of nutrients. A few dishes that had been inadequately washed could contain traces of vitamins or other substances that might stimulate the hatching of spores.

Plating No.3 in the second series was done to test these two factors (Table 2). In eight plates, (a) several different treatments were applied to increase humidity in the dishes. In ten plates (b) traces of various vitamins were added to the medium in different amounts. The result was that every one of the first set of eight plates was covered with amoebae, while only one of the second set was covered and the total viability on the remaining nine plates was 0.04%.

It was concluded that the treatments used to increase humidity in this test were effective in increasing the percentage of hatching spores. The most convenient of these treatments consists of pouring a second thin layer

of well-cooled water agar over every dish several hours after it has been plated with spores and bacteria. The hatched amoebae multiply between the two layers of agar.

Before this method was finally adopted, two minor variations of it were tested in Platings 5 - 7:

i) In Plating 5, the bacterial suspension was mixed with the second layer of agar instead of being plated beneath it; and

ii) In Platings 6 and 7, the bacterial suspension was plated on top of the second layer of agar instead of beneath it.

Both these methods gave good results on some plates, but the original form of the two-layer method gave the highest percentage of growth and the most clearly-defined plaques. It was also found in these tests that the plaques were more clearly defined if the concentration of the bacterial suspension was higher than that used in most tests previously (10^9/dish instead of 10^8).

The standard method of plating spores is now as follows:

i) The medium is water agar of concentration 1% w/v in 9 cm. diameter Petri dishes, poured about half an hour before plating.

ii) Spores are suspended in distilled water, washed once by centrifugation, and then re-suspended and distributed homogeneously in the suspension by pipetting with a Pasteur pipette.

iii) The density of the suspension is estimated by haemocytometer count.

iv) The suspension is diluted to the required density by serial dilutions in distilled water.

v) The required volumes (0.1 ml. or 0.2 ml.) of the appropriate dilutions are plated in the surface of the water agar by spreading with a glass rod.

vi) The dishes are allowed to stand at room temperature for 1 - 4 hours to enable the water to soak into the agar.

vii) The dishes are spread with bacteria. The concentration of the suspension is approximately 1 x 10^{10} bacteria/ml. and 0.2 ml. is spread on each dish. Spreading is done with a glass rod. The bacterial suspension is made as described in the plating method for amoebae.

viii) The dishes are allowed to stand at room temperature for about 1 hour.

ix) The dishes are inverted and incubated at 25°C for 24 hours.

x) The dishes are removed from incubation and a second thin layer of 1% agar is poured on each. The agar is poured at a temperature of 45-50°C and about 4 ml. is poured on each dish. The thickness of the top layer is thus about 0.6 mm. The dishes are tilted and shaken about while the agar is still liquid so that it will set in a thin layer all over the surface.

xi) As soon as the top layer of agar has set, the dishes are returned to incubation at 25°C.

Six or seven days after incubation starts, plaques are counted and isolation of amoebae from plaques may be done. The plaques are formed between the two layers of agar and cannot easily be seen with the naked eye. They are counted with the aid of a dissecting microscope, and isolation of amoebae from the plaques is done under the same conditions.

This method was immediately used in four platings of spores obtained in the cross of doubly-marked strains and it enabled large numbers of progeny of this cross to be isolated (see Part III, 2). The results of these platings are given in Table 5 (p.101). The average viability ranged from 2.1% to 6.8% and in each plating all the plates showed similar numbers of clearly-defined

plaques. The irregular results found during the test platings described above have not recurred since the adoption of the two-layer method of plating.

c) Discussion. The work that has been described in this section did not have the object of discovering factors that promoted or inhibited hatching, but was aimed simply at developing a satisfactory method by which spores could be induced to hatch and their products isolated. Thus few of the factors that were tested were investigated exhaustively and no conclusions, apart from the few remarks that have been made above, can be drawn from this work about the effects of temperature, nutrients or wetting agents.

Neither have any tests been done on the effect of ageing on the viability of spores, a factor found to be of importance in some species by Gilbert (1929). In the series of platings described above, the smallest age tested at which spores hatched successfully was two weeks and the greatest was ten months. Since age may affect spore viability and since different batches of spores may have different viability, valid conclusions cannot be drawn from comparison of the results of different platings.

Results from which conclusions might validly be drawn, however, are those obtained in the platings in which many spores hatched on a few plates and very few spores hatched on the other plates, since in any one plating most of the conditions of treatment are the same for all plates. This effect was found in eight different platings.

As explained above, the two factors that seemed most likely to be causing this effect were humidity and the presence of nutrients. Plating 3 in the second series (Table 2) was designed to distinguish between these two alternatives and it showed that good results were obtained when any of a variety of treatments was applied to increase the humidity in the dish. However, most of the treatments that were applied to increase humidity in the dishes, in particular the addition of a second layer of agar, would also decrease the rate of diffusion of substances towards or away from the spores, and among the results of this would be a reduction in oxygen tension in the spore's surroundings. A change in oxygen tension, rather than an increase in humidity, may be the factor that induces a spore to hatch. Clearly, no conclusions about the particular factors that influence hatching can be drawn from these results.

An additional factor that must be considered is the effect upon unhatched spores of the presence of hatching spores. This was found by Smart (1937) to increase the speed though not the percentage of hatching. It is possible that this factor may have contributed to the high degree of success on the particular plates that were successful in the present investigation. If a sufficient number of spores hatch, they may stimulate others to hatch in environmental conditions that would otherwise be unfavourable. However, since the percentage of hatching on the other plates of the series in which the successful plates were found was always consistently low, it is extremely unlikely that sufficient hatching spores could occur on one plate by chance to cause such an effect. Other factors must first have caused a high degree of hatching, even if the stimulating effect of this hatching on other spores caused the plates to be so densely covered.

4. Formation of the Plasmodium by the Amoebae.

a) Results of Previous Investigations. The formation of plasmodia by amoebae has been achieved by many workers both in liquid and on solid medium. Many authors have

claimed, as a result of microscopic observation, that the formation of a plasmodium is initiated by the fusion of a single pair of morphologically similar amoebae. In Physarum polycephalum, this observation was made by Howard (1931a) and, most recently, by Ross (1957). Abe (1934) looked for physiological differences between the two members of a pair of fusing amoebae and concluded that they differed in electric charge and in oxidation-reduction potential, but Kambly (1939), who repeated Abe's experiments, found no such differences.

Only a few workers have performed precise experiments to discover whether or not plasmodia can be formed by the amoebae resulting from a single spore or single amoeba. Working on Didymium difforme, Skupienski (1926) found that although multispore cultures gave rise to plasmodia, single-spore cultures did not. When he mixed amoebae from two different single-spore cultures, he obtained plasmodia. However, in the same species, both Cayley (1929) and Schünemann (1930) found development of plasmodia in single-spore cultures.

With Didymium nigripes, Schünemann (1930) found development of plasmodia in single-spore cultures. Skupienski (1918) also obtained plasmodia from single-spore

cultures but found no plasmodia in cultures developed from single amoebae. He concluded that a spore contains nuclei of two different mating-types which segregate after the amoebae hatch. He does not record how many amoebae emerged from a single spore. However, Kerr and Sussman (1958) found development of plasmodia in cultures developed from single amoebae as well as in those from single spores. Von Stosch (1935) studied two varieties of D. nigripes and found that they gave different results. With D. nigripes var. xanthopus, he found development of plasmodia in single-spore cultures, but with D. nigripes var. cunigripes, he found no plasmodia in single-spore cultures. With this latter variety, he performed a series of tests, mixing samples of amoebae from pairs of single-spore cultures. From some of these mixtures, he obtained plasmodia, and he concluded from his results that a single pair of mating-type factors controlled plasmodium formation.

b) Results of the Present Investigation. Plasmodia were found frequently in the early cultures of amoebae grown from groups of contaminated spores on the surface of water agar. When controlled conditions for the culture of amoebae had been developed, some series of single-spore

and multispore cultures were grown. In one series of cultures, grown from spores taken from one batch of sporangia, plasmodia developed in ten of the twenty-five multispore cultures, but in none of forty-five single-spore cultures.

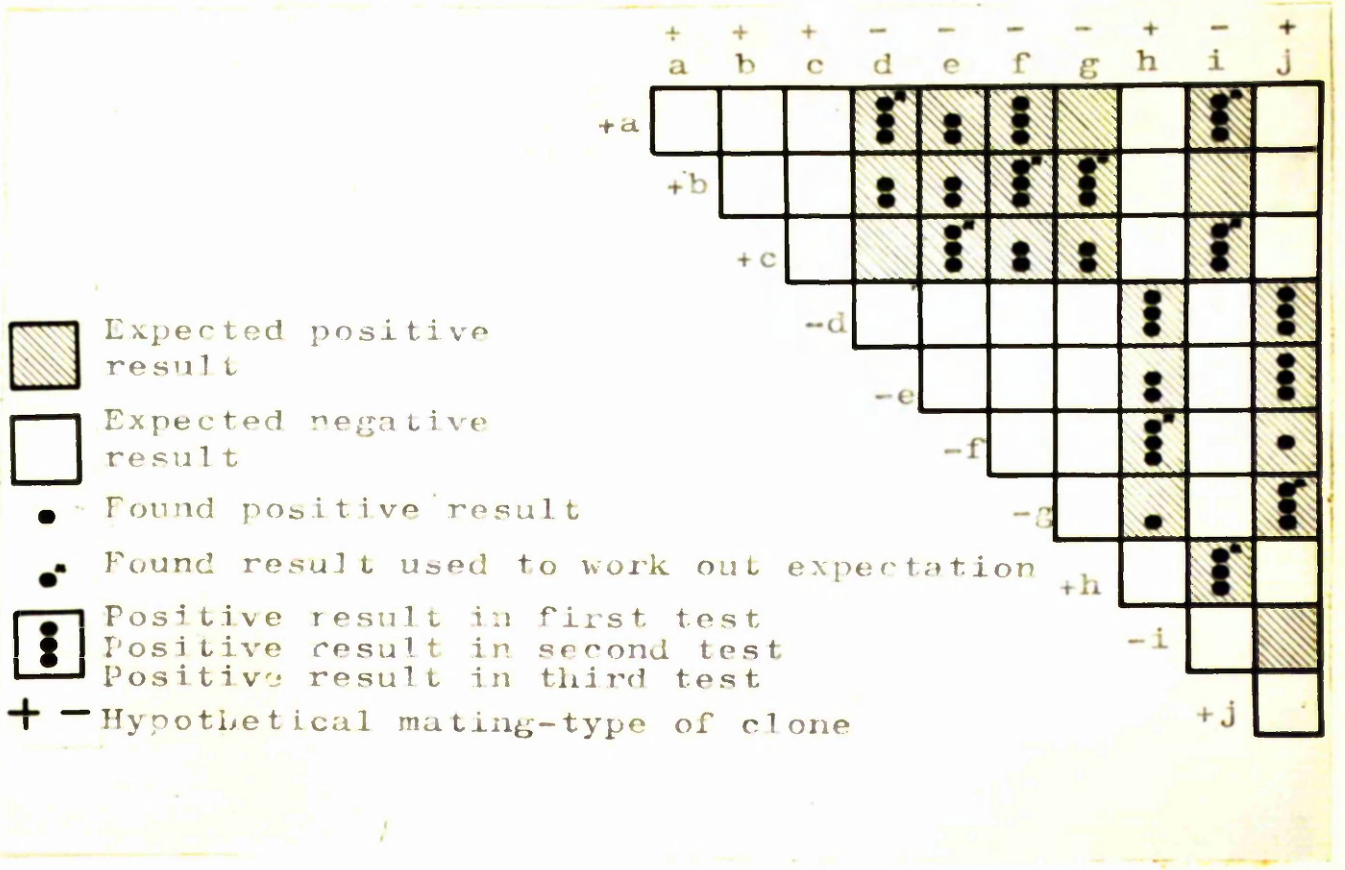
Ten groups of amoebae derived from ten different single-spore cultures were then mixed together systematically. They were used to set up fifty-five different cultures: ten of these cultures were started with amoebae of the ten groups taken separately, and forty-five were started with amoebae from all possible combinations of the ten groups taken two at a time. After several days incubation, plasmodia had developed in thirteen of these cultures. The incidence of plasmodium formation among the cultures was plotted on a chart, and the results indicated that a simple two-type (+/-) mating system was in operation. The test was repeated twice and on each occasion plasmodia developed in 20 of the 55 cultures. None of these results disagreed with the expectation derived from the results of the first test. In the combined results of all three experiments, plasmodium formation occurred in twenty-one of the twenty-five cultures in which it was expected on the basis of a +/- system, and it did not occur in any of

the combined-group cultures in which it was not expected or in any of the ten single-group cultures (see Figure 9).

The Effects of Environmental Conditions. The conditions used to induce amoebae to form plasmodia have been similar to those used for the culture of amoebae. Pools of a heavy bacterial suspension are inoculated on the surface of water agar, and loopsful of heavy suspensions of the amoebae are placed in them while they are still wet and mixed together. The plates are incubated in moist chambers at 25°C. The pouring and inoculation of each plate is done as rapidly as possible and the plate is incubated immediately. The surface of the plates is kept moist but is not so wet that the different pools on one plate oan run together and mix.

After four days incubation, the plates are examined with a dissecting microscope. The amoebae have eaten most of the bacteria by this time and plasmodia are usually found in some of the cultures. Plasmodia usually continue to appear in other cultures for several days, and the plates are incubated until a few days after plasmodia have ceased to appear. Plasmodia are at first little bigger than the amoebae and almost spherical, but they can easily be recognized even at this stage because

Fig. 9. Tests of the mating-type system. The ten clones were denoted by the letters a - j. Each square in the chart represents a culture. Forty-five cultures were started with amoebae of the ten clones taken in all possible combinations of two at a time, and ten control cultures were started with amoebae of the ten clones taken separately. The mating-type + or - was assigned to each clone on the basis of nine of the results of the first test. These nine results are marked as shown in the key.


+ + + − − − − + − +
a b c d e f g h i j
+a
+b
+c
−d
−e
−f
−g
+h
−i
+j
Expected positive result
Expected negative result
Found positive result
Found result used to work out expectation
Positive result in first test
Positive result in second test
Positive result in third test
+ − Hypothetical mating-type of clone

there is always an area around them from which the amoebae have disappeared. As the plasmodium grows, it assumes various forms and moves around in the culture by protoplasmic streaming. (See Figs. 10 - 12). Wherever it moves, the amoebae disappear. As soon as a streaming plasmodium is observed in a culture, the agar on which it is situated is cut out and removed from the dish so that the plasmodium cannot move to the other cultures. In some cultures, only one plasmodium is formed; in others, many are formed simultaneously (see Fig. 12).

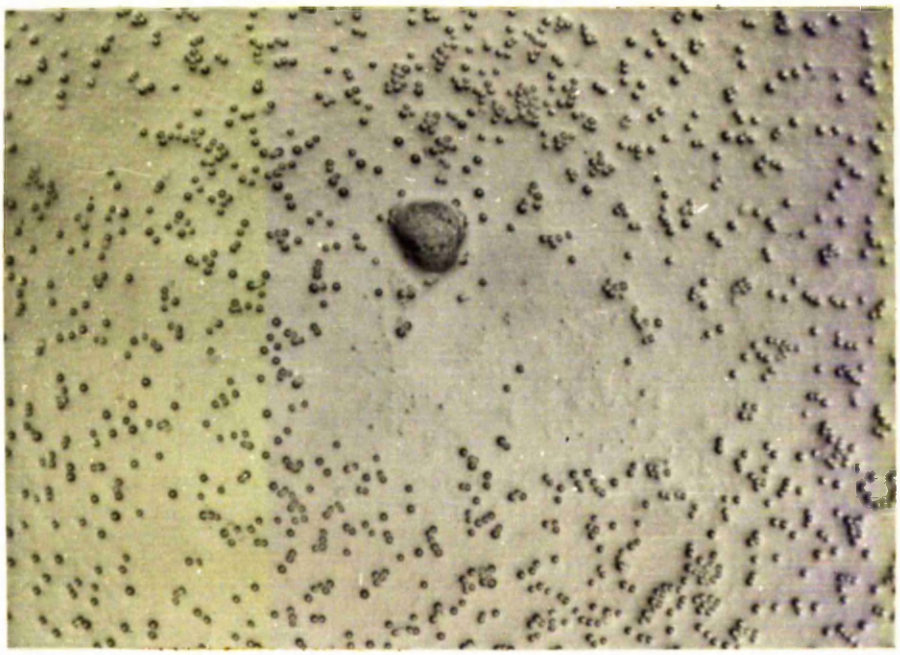
Under the conditions described above, plasmodium formation occurs in many cultures but it still does not occur in all of those in which it is expected. Slight variations in the treatment appear greatly to affect the success of the cultures. In the three tests described above, plasmodia were found in 20 cultures in each of the second and third tests, but in only 13 cultures in the first test. The treatment of the cultures in these tests was identical except that examination of the plates was done much more slowly in the first test than in the later ones. It was concluded from observation of these plates that drying of the medium may reduce the frequency of plasmodium formation, and much care was taken in the later

Fig. 10. Formation and growth of a plasmodium (cont. in Fig. 11) (x 130).

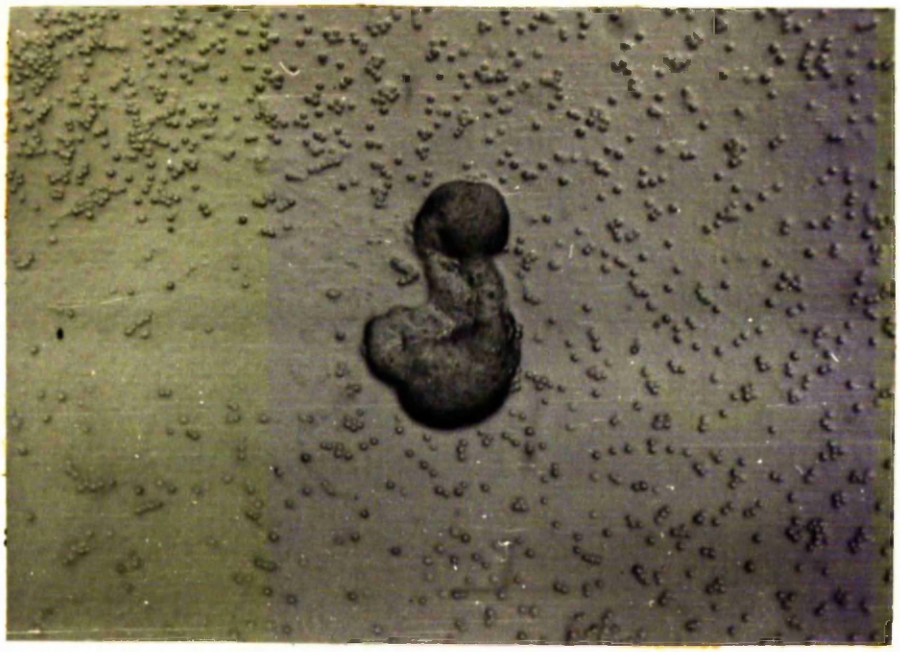
Amoebae of the two mating-types have been feeding together on bacteria on the surface of water agar for five days. Most amoebae are now encysted and they are rapidly disappearing as the plasmodia form and move around amongst them.

a. Typical appearance of a plasmodium when it is first detected in a culture. An area from which the amoebae have disappeared is always present around the plasmodium.

b. A typical stage in growth.



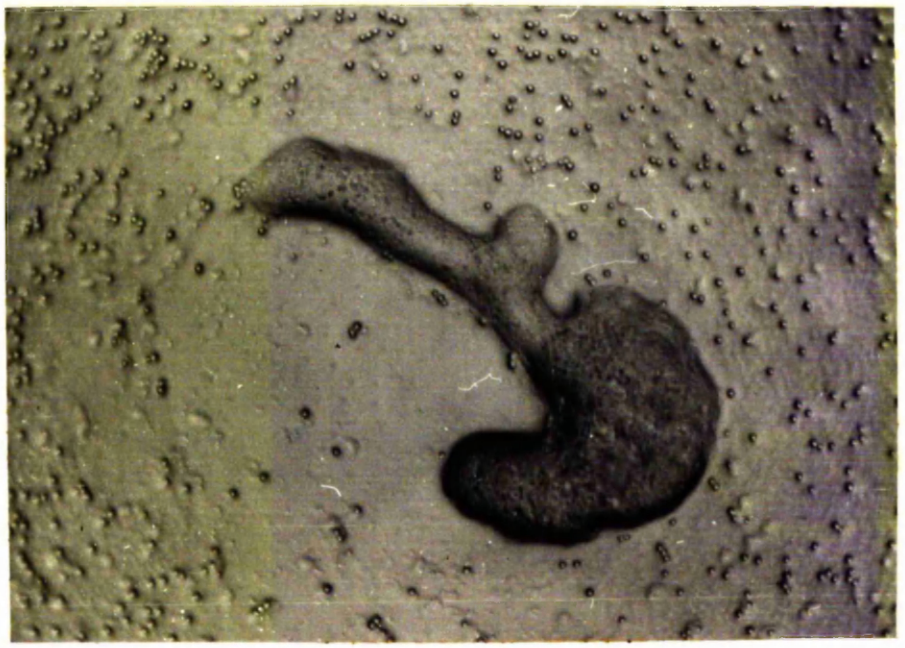
a



b

Fig. 11. Formation and growth of a plasmodium (cont.) (x 130) in (a) ; (x 110) in (b).

Typical stages in growth, which may be seen 1 - 2 days after a plasmodium is first detected. Some amoebae are apparently entering the substance of the plasmodium, but whether they are actively fusing with it or being engulfed by it is unknown.



a

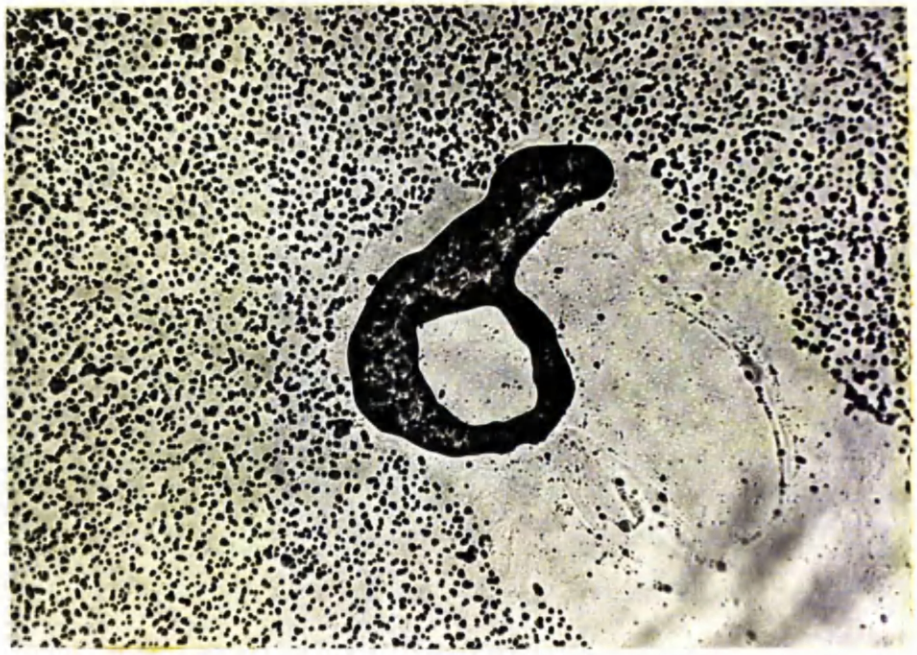
b

Fig. 12. Growth of plasmodia. (x 43).

The plasmodia have been formed in cultures of amoebae on water agar, as in Figs. 10-11.

a. A plasmodium moving among encysted amoebae; the amoebae have disappeared from the area over which it has moved.

b. Many plasmodia sometimes form in one culture of amoebae; six may be seen in this small area.



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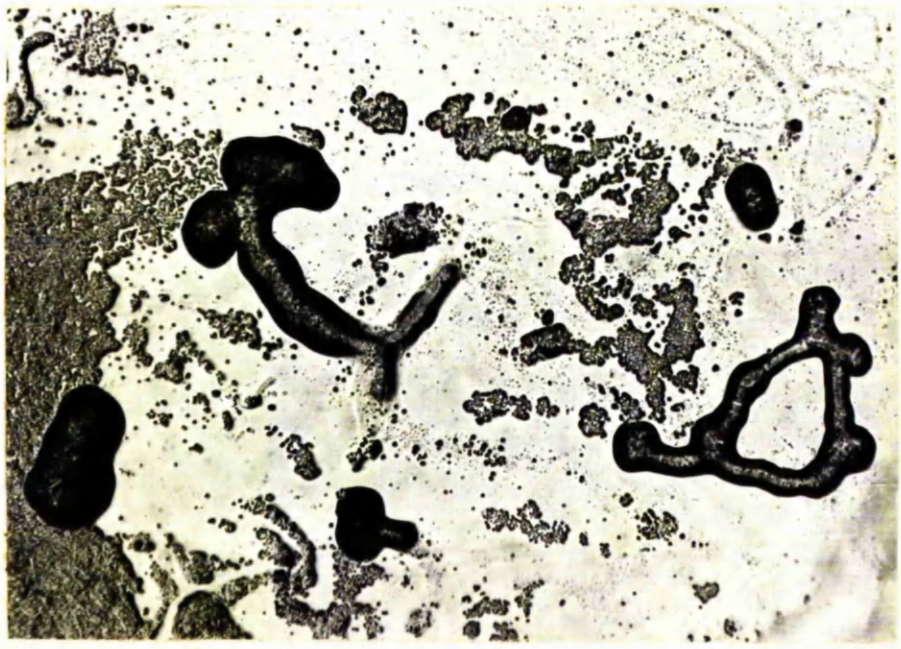


b

tests to ensure that the surface of the agar remained moist.

c) Discussion. The first hypothesis considered to explain the results obtained by mixing amoebae from single-spore cultures was that a +/- mating-type system was in operation. This is the simplest kind of mating system known. It implies that two types of amoebae, denoted by + and - respectively, exist, and that the fusion of amoebae of the two types is necessary to initiate plasmodium formation. Since plasmodia do not develop in single-spore cultures, it is assumed that all the amoebae developing from the hatching of one spore are of the same mating-type. Because of this, it is likely that all the amoebae in a single-spore culture are of the same genetic constitution, and the culture of amoebae derived from a single spore may therefore reasonably be called a "clone".

All the positive results obtained in the three tests, that is, all the observations of plasmodium formation, agreed with the expectation worked out on the basis of this hypothesis. A departure from perfect agreement with expectation was caused only by the absence of plasmodium formation in four of the cultures in which it was expected.

There are two reasons for believing that this departure from expectation is not caused by the existence of a more complex mating-type system.

i) The four cultures in which plasmodium formation was expected and did not occur involved amoebae of seven different clones. If the absence of plasmodium formation is to be explained by attributing mating-types more complex than simply + or - to the clones that failed to react, this treatment must be applied to all seven clones. When an expectation is worked out on this basis, using any of the known mating-type systems (Burnett, 1956), it is found that many of the positive results observed in the tests disagree with the expectation.

ii) In the first test, plasmodium formation did not occur in twelve cultures in which it was expected, but in the two repetitions of the same test it was absent in only four of these. Thus it is clear that environmental conditions affect the incidence of plasmodium formation, and it is likely that repetition of the test would eventually reveal plasmodium formation in the remainder of the cultures in which it was expected.

From the results reported in this section, it seems reasonable to conclude that, among the clones of amoebae investigated, only two mating-types occur and that there is a simple +/- mating-type system of the kind described. If other collections of plasmodia or spores of the same species were made, it is of course possible that different mating-type factors might be found in them; this has not been investigated.

The results found are similar to those obtained by Von Stosch (1935) from mixing single-spore cultures of D. nigripes var. unigripes. He also concluded that a simple +/- mating-type system was in operation. The results also agree with the less detailed observations of Skupienski (1926) with D. difforme and with the many recorded observations of amoebae fusing in pairs. However, in both D. nigripes and D. difforme, there has been disagreement among different investigators; some have recorded that plasmodium formation can occur in single-spore cultures and others that it cannot. It is possible that this disagreement results from the use of different strains of a species. Both types of mating system occur in other groups of organisms and different species or different strains of the same species are known to differ in some cases. For example, of two species of Rhizopus one is

homothallic and the other heterothallic (Burnett, 1956).

In strains in which plasmodium formation regularly occurs in single-spore cultures, there are three possible interpretations of the situation:

i) A mating-type system operates but a single spore produces amoebae of both mating types.

ii) A single spore produces amoebae of one genotype only but these fuse to initiate plasmodium formation.

iii) No fusion of amoebae is necessary to initiate plasmodium formation.

Results that provide any evidence to enable discrimination between these interpretations are available only for D. nigripes. Skupienski (1918) obtained plasmodia from single-spore cultures but not from single-amoeba cultures, and his interpretation was therefore the first one. Kerr and Sussman (1958) obtained plasmodia from both types of culture and thus provided evidence that the second or third interpretation is correct. Here again, it seems probable that different strains may differ in their behaviour.

The results reported in this section show that the formation of a plasmodium is initiated by the fusion of amoebae of two different types. They do not show (i) whether this initial fusion occurs between two individual

amoebae or between groups of amoebae, (ii) whether the amoebae that disappear from the culture as the plasmodium grows coalesce with the plasmodium or are eaten and digested by it, or (iii) whether nuclear fusion occurs when the plasmodium is formed. These questions will be discussed in Part IV.

Summary.

1. From a contaminated sclerotium of Physarum polycephalum received from Dr. Rusch, a plasmodium was cultured, purified and maintained on oat agar in the absence of contaminating organisms.
2. A strain of bacteria, Pseudomonas sp. (probably P. fluorescens), derived from the contaminated sclerotium, was isolated and maintained in pure culture.
3. A method for inducing the plasmodium to form spores was developed. The plasmodium is grown on oat agar in pure two-membered culture with Pseudomonas sp. and exposed to artificial light for fixed periods of time at constant temperature.
4. A method was developed for pure two-membered culture of amoebae on the surface of water agar. The surface is spread with Pseudomonas sp. and inoculated with spores or amoebae.
5. A simple plating method for amoebae was developed. A suspension of amoebae is spread on water agar together with a heavy suspension of Pseudomonas sp. Each amoeba multiplies to form a clear "plaque" in the bacterial layer. The plaque counts for a series of plates show low variance and correspond closely to the estimates made from haemocytometer counts of the suspension.

6. A modified plating method had to be developed for spores. Successful results were obtained when a second layer of agar was poured over each dish after plating with spores and bacteria. This provided a means of isolating the products of single spores.
7. Single spores were also isolated by hand and cultures of amoebae were established from them.
8. In these single-spore cultures, it was observed that plasmodia were never formed, though they appeared in a proportion of cultures from pairs of spores and in most cultures from larger numbers of spores.
9. Mixing of amoebae from ten single-spore cultures in all combinations of two at a time showed that only certain combinations formed plasmodia, and the incidence of plasmodium formation among the different combinations was that expected for a single-factor (+/-) mating-type system.
10. It was concluded from these results that plasmodia were formed only when amoebae of different mating-types were mixed, that a single spore produced amoebae of one mating-type only and that only two mating-types occurred in the spores investigated.

PART II.

ISOLATION OF MARKED STRAINS.

Introduction.

When techniques had been found for the control of the life-cycle, the second stage in the preparations for genetic analysis was the search for markers by which strains could be identified. The discovery of the mating-type system had provided one marker but for the detection of recombination at least one more had to be found. This search for markers was carried out only on the amoebae, since, for such work, they have several advantages over plasmodia:

i) The amoebae are uninucleate and probably haploid, so that any mutant arising should be immediately detectable. Even a small piece of plasmodium contains a very large number of nuclei.

ii) The amoebae can be handled in large numbers by the usual techniques of microbiology. The large size and the extreme mobility of the plasmodium make it very difficult to handle.

iii) A clear distinction between growth (i.e. multiplication) and absence of growth can be obtained in the amoebae. In the plasmodium, the form is so irregular that it is difficult to distinguish between growth and movement.

Techniques were developed for the efficient handling of amoebae for the purpose of isolating mutants. A search was then carried out for amoebae resistant to certain drugs that normally inhibit growth, and one clearly-defined resistant mutant was obtained.

1. General Techniques.

a) Conditions of growth. The conditions of growth used in this work have been described in detail in Part I,3. The amoebae feed on a pure strain of bacteria (Pseudomonas sp.) which grows slightly on the surface of 2% water agar. The stock of bacteria is maintained on slopes of a minimal medium. A few attempts were made to feed amoebae with the same strain of bacteria killed by heat or by streptomycin, but they gave little or no growth of amoebae.

b) Isolation of Clones. It was shown in Part I, 4 that amoebae in a single-spore culture will not give rise to plasmodia. A stock of amoebae may therefore be established from a single-spore culture and maintained for long periods. The stocks of amoebae used in the work described in this Part all belonged to two single-spore cultures and were called a and i in the mating-type tests described in Part I, 4. Both stocks were purified by plating the amoebae and picking from single plaques, so that it was certain that each purified stock was a clone; that is, that it consisted entirely of amoebae descended from one amoeba by repeated division. These stocks, a and i, have now been maintained for over two years. It is, of course, important to use clones for experiments in which reproducible results are required, since the majority of individuals

in a clone are genetically uniform and only individuals with the same genetic constitution may be expected to behave in a consistent way.

Clones are maintained on water agar slopes in test-tubes, which are of course inoculated with bacteria. When the amoebae have multiplied sufficiently to have eaten all the bacteria, they encyst and remain alive in this state for at least several months. Subcultures to fresh slopes are made at intervals of a few months.

c) Plating Method. The plating method has been described in detail in Part I, 3. It is an important technique because it allows accurate quantitative estimates of viability of different strains under the same conditions or of one strain under different conditions. It also provides a simple method for the isolation of clones, which is a necessary requirement for the genetic analysis of the results of crosses.

The efficiency of the method and the effects of a number of factors that might influence it were thoroughly tested by repeated platings of stock a, and these tests resulted in the choice of the standard method described above. In the course of these tests it was found that there was no significant difference in the viability of cultures of amoebae that were still active at the time of

plating and those that had been in the encysted state for various lengths of time.

d) Test Plates. To discover simply whether or not a given clone of amoebae will grow on a certain medium, the plating method is unnecessarily complicated. Instead of it, for this purpose, test plates are used. A small area of a plate of water agar is inoculated with a bacterial suspension and the amoebae to be tested are transferred to the area in a loopful of water. After a few days incubation, it is easy to see whether or not the amoebae are multiplying. Since the amoebae do not spread very far beyond the area of the bacterial inoculum, a number of different clones can be tested on the same plate. When a number of clones have to be tested on a series of different media, they are arranged in a corresponding pattern on all the dishes of a series so that each clone is easily identifiable on each dish. (See Fig. 13).

2. Isolation of Drug-Resistant Mutants.

The method of searching for drug-resistant mutants is very simple. Large numbers of amoebae are spread on medium containing a drug to which the amoebae are sensitive but which does not affect the bacteria. Amoebae resistant to the drug will then multiply and will form plaques,

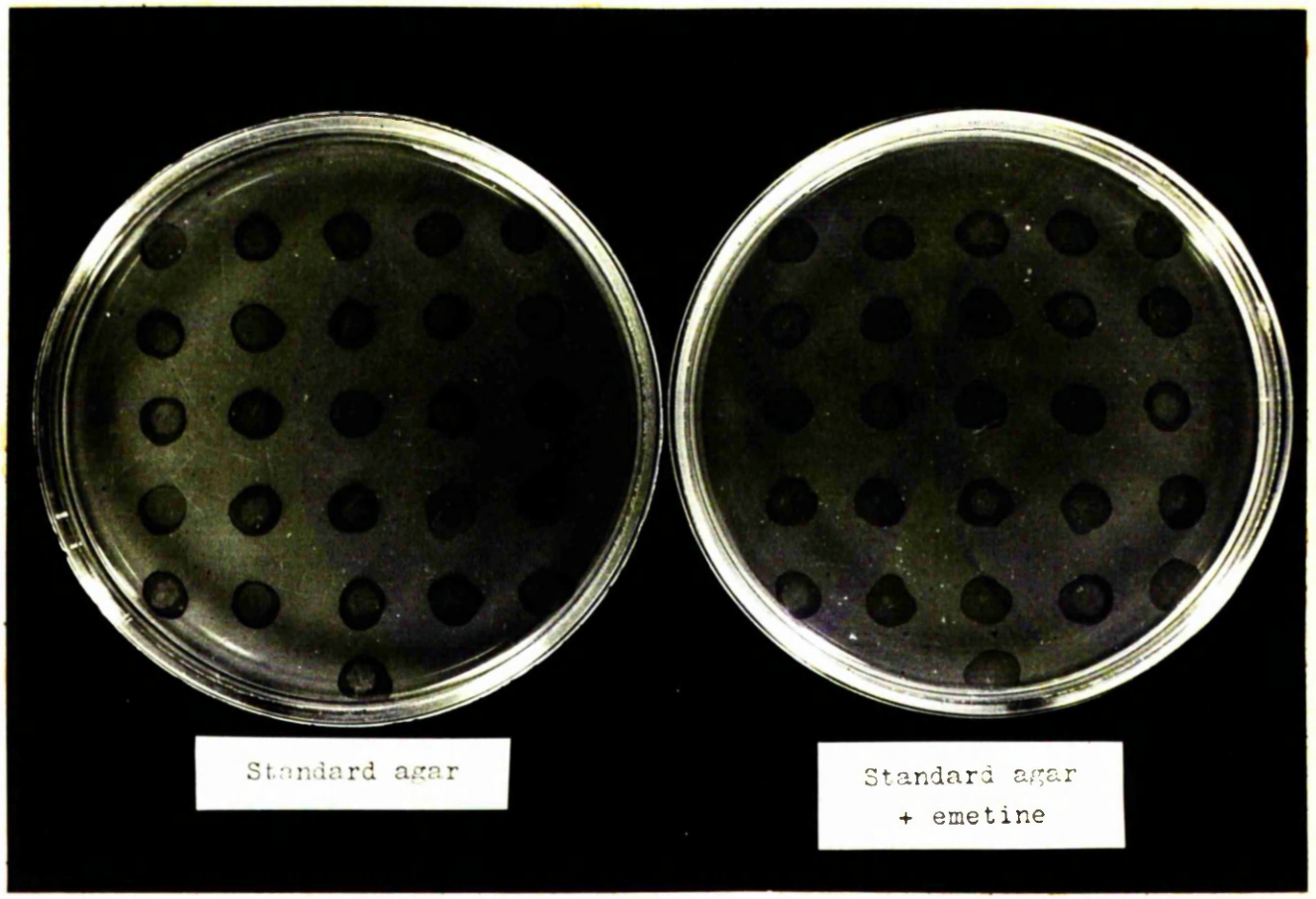
Fig. 13. Test plates. (3/4 x natural size).

Loopsful of bacterial suspension were placed at 26 points on each plate. A loopful of a suspension of amoebae was then inoculated in the bacterial patch at each point. The plates are seen after three days incubation. The bacterial patches appear dark, but those in which amoebae have multiplied have a clear area at the centre.

The following three strains of amoebae were inoculated in order from left to right at corresponding points on the two plates:

1) a; sensitive to emetine, mating type +
2) i; " " " , " " -
3) E19; resistant " " , " " -

On standard agar (2% water agar), all strains have grown; on standard agar containing emetine, only strain E19 has grown.


Standard agar
Standard agar
+ emetine

and thus can be easily detected and isolated.

a) Selection of Suitable Drugs. The suitability of a drug was tested as follows: two streaks of bacterial suspension were made across a plate of water agar, and a streak of amoeba suspension was made on top of one of these. A very heavy suspension or solution of the drug was then streaked across the plate at right-angles to the bacterial streaks so that bacteria and amoebae were exposed to a gradient of concentration of the drug. After incubation, the plates were examined for growth of bacteria and amoebae.

Of the eight drugs tested, four were unsuitable because they did not inhibit growth of amoebae: mycostatin, quinine, pamaquin, sulphadiazine. The other four drugs did inhibit growth of amoebae and were therefore used in further tests. These were:

i) Thiram, a sulphur fungicide (tetramethylthiuram disulphide).

ii) Venturicide, a mercury fungicide (2⅛% phenyl mercury chloride).

iii) Captan, a polyhalogeno fungicide (N-trichloromethyl-thiotetrahydrophthalimide).

iv) Emetine, an alkaloid amoebicide (emetine hydrochloride).

Captan and Venturicide also inhibited the growth of the bacteria but this effect appeared at concentrations higher than those necessary to inhibit the growth of amoebae.

b) Method for Plating Amoebae at High Density. In the search for drug-resistant mutants, amoebae were plated in most tests at densities of 1×10^6 to 3×10^6 per dish. If higher densities were used, it was difficult to decide whether or not multiplication of amoebae took place because of the background of encysted amoebae. In order that large numbers of amoebae would be ready for regular platings, a stock of amoebae of one clone was maintained and subcultured frequently. The amoebae were grown in Petri-dishes by the usual method, fed on a heavy suspension of bacteria. For each plating, a suspension was made by thoroughly washing the amoebae from the surface of about ten of these stock plates after they had been incubated for about five days. About 5×10^6 amoebae were usually obtained from each stock plate. A large volume of suspension was made, which was concentrated by centrifugation. The concentrated suspension was spread on the drug plates. For each plating, control plates of water agar alone were spread with about 100 amoebae/dish from a dilution of the concentrated suspension. From the number of plaques

observed on the control plates, the average number of viable amoebae per dish plated on the drug plates was estimated. All numbers quoted in the following account are those derived from such calculation and not those estimated from the haemocytometer counts.

c) Platings on the Selected Drugs.

i) Thiram. The stock of amoebae used was of clone a. Three platings were done in which plates containing Thiram at a series of different concentrations were spread with about 1×10^6 amoebae/dish. The results were found to be inconsistent; for example, growth sometimes occurred on plates in which the drug was ten times as concentrated as in other plates on which growth did not occur. No further tests were done with this drug.

ii) Venturicide. The stock of amoebae used was of clone a. Preliminary platings on a series of concentrations of Venturicide were done and the lowest concentration at which consistent inhibition of the amoebae occurred was selected. Two platings were then done on this concentration, the first with a total of 12×10^6 amoebae and the second with 56×10^6. Thirteen plaques were found on the first set of plates, but when the amoebae were transferred to medium of the

same concentration, they did not grow. Six plaques were found on the second set of plates, and the amoebae from these did grow when transferred to similar medium. However, they were found to be mixed with a contaminating strain of bacteria, and when this was removed, they would no longer grow on Venturicide. It therefore seemed likely that Venturicide was easily broken down by contaminating organisms, thus giving misleading results. No further tests were done with Venturicide.

iii) Captan. The stock of amoebae used was of clone a. Three preliminary platings on a series of concentrations of Captan were done, and the lowest concentration at which consistent inhibition of the amoebae occurred was selected. Nine platings were then done on medium of this concentration, and a total of 4×10^8 amoebae was plated. In only two platings was any growth found. In the first of these, amoebae from five plaques were isolated but they all failed to grow when transferred to medium of the same concentration. In the second, amoebae from one plaque were isolated and these did grow when transferred to medium of the same concentration, but after three further transfers on Captan, including

a purification by plating, they failed to grow on the medium. Thus, no permanently resistant mutants were found.

iv) Emetine. The stock of amoebae used was of clone i. Four preliminary platings on a series of concentrations of emetine were done and, of the concentrations tested, the lowest on which consistent inhibition of the amoebae occurred was found to be 0.031%. (i.e. 0.031 g. emetine hydrochloride in 100 ml. 2% water agar medium). The next concentration in the series tested was 0.015%, which allowed patches of growth on plates spread with 1×10^6 amoebae or more.

Five platings, involving a total of 0.7×10^8 amoebae, were done on medium containing 0.015% emetine, and amoebae were isolated from 24 patches of growth that appeared on these plates. All of these amoebae failed to grow when transferred to medium of the same concentration, and all were therefore discarded.

Eleven platings, involving a total of 2.6×10^8 amoebae were done on medium containing 0.031% emetine. Growth was found in only three of these platings - nos. 5, 6, 9.

Plating No.5. Amoebae from eighteen plaques were isolated and tested. The clones were called E1 - E18. All failed to grow when transferred to 0.031% emetine, but four of them (E2, E13, E16, E18) grew well on 0.015% emetine. These four strains were subcultured and tested further. After five successive transfers on water agar, they were tested five times on a range of concentrations of emetine. Each time, they gave growth slightly better than the stock strain but not good enough for them to be clearly distinguished from it. During the tests, all four strains were purified by two successive selections from single plaques. These strains have been maintained, but have not yet been used for any further tests.

Plating No.6. Amoebae from two plaques were transferred to medium of the same concentration but failed to grow and were discarded.

Plating No.9. Amoebae from one plaque were isolated and tested. They failed to grow on 0.031% emetine but showed very good growth on 0.015% emetine. The clone was called E19. After three successive transfers on water agar, the strain was tested on concentrations of emetine up to 0.019% and showed good growth on all. After two successive transfers on 0.019% medium, it was then plated at low

density on concentrations up to 0.031% and on all concentrations it showed approximately the same average growth (number of plaques) as on the water agar controls.

The strain was then taken from 0.019% emetine medium and purified by selection from single plaques in two successive platings on 0.019%. The purified strain was maintained on slopes in two parallel series, one on water agar and the other on 0.019% emetine.

Thus one strain resistant to emetine has been isolated after 3.3×10^8 amoebae have been tested on this drug.

Summary.

1. Two stocks of amoebae (a and i) were established from two single-spore cultures and purified by repeated cloning on water agar (plating and picking from single plaques).
2. With these stocks, the efficiency of the plating method was thoroughly tested and further minor techniques were developed for the convenient handling of large numbers of amoebae.
3. Four drugs were selected that inhibited multiplication of amoebae without noticeably affecting the bacteria provided as food.
4. On each of these four drugs, incorporated in water agar at appropriate concentration, dense platings of a or i amoebae were done. All plaques that appeared were tested for the presence of amoebae resistant to the drug.
5. One strain of amoebae resistant to emetine was isolated from stock i after 3×10^8 amoebae had been plated on this drug.
6. This emetine-resistant strain (E19) was purified by successive cloning on water agar containing emetine and maintained in culture on water agar.

PART III.

CROSSES OF MARKED STRAINS.

Introduction.

The work described in Parts I and II provided two characteristics by which strains of amoebae could be classified into distinct groups. These characteristics, mating-type and resistance to emetine, could therefore be used as genetic markers. Crosses between strains carrying different forms of each marker were now made. The first cross analysed was between strains of amoebae differing in mating-type only (Part III, 1). When preliminary tests had shown that emetine resistance was suitable as a marker, a cross between the resistant strain and a sensitive strain was made, these strains of course being of different mating-types. The preliminary tests and the cross are reported and discussed in Part III,2.

1. Singly-Marked Strains.

a) First-Generation Progeny of the Cross a x i. A plasmodium was obtained by mixing amoebae of the two clones, a (mating-type +) and i (mating-type -), which had been originally isolated for the mating-type tests described in Part I, 4. Spores formed by this plasmodium were plated and fifty clones of amoebae were isolated. These first-generation progeny were tested for mating-type by backcrossing to the two parent clones; that is, amoebae from each clone were mixed with amoebae of clone a and, separately, with amoebae of clone i, and the incidence of plasmodium formation in the cultures was recorded.

This test was repeated seven times because so many of the cultures failed to produce plasmodia. In the combined results of all seven tests, fifteen of the fifty clones produced plasmodia in combination with a only and ten with i only; the remaining twenty-five failed to produce plasmodia with either parent. The full results of the seven tests are given in Table 3. Slight changes were made in the conditions under which these tests were performed, in an attempt to increase the incidence of plasmodium formation. These will be mentioned in the discussion.

Table 3. Results of seven tests in which fifty clones of the first generation from the cross a x i were tested for mating-type by backcrossing with the parent clones, a (+) and i (-).

* = plasmodium formation in any one test.

** = plasmodium formation in any two tests, etc.

Clone	Reaction with a	Reaction with i	Clone	Reaction with a	Reaction with i
1			26		
2		*	27		
3			28		
4	****		29		
5	*		30	****	
6			31		
7	***		32		*
8			33		
9			34	**	
10			35	*****	
11			36	****	
12		*	37		
13			38		
14			39		*
15	*****		40		****
16	**		41		
17		**	42		*
18			43		
19			44		***
20	*****		45		***
21		**	46	**	
22			47		
23	******		48		
24	*		49	*****	
25			50	***	

Ten of the fifty first-generation clones (those numbered 27-36) were also tested for their reactions with one another. This test was identical in plan with the original test for mating-types performed with the ten clones a - j: all possible pairing combinations of the ten clones were made. The test was done twice. The results of both repetitions are given in Figures 14 and 15, in which two different theoretical interpretations of the results are represented.

b) Discussion. Three alternative hypotheses to explain the results are considered below:

i) Single-Factor Mating-Type System: +/- at One Locus. This is the mating-type system that was deduced to be in operation from the results of the original test for mating-types. It is assumed that a single pair of factors at one locus controls the mating-type: a single amoeba carries either + or - at this locus. None of the positive results recorded in this section disagrees with the result expected if this is the system in operation. When the fifty first-generation clones were tested by backcrossing, each reacted with only one of the two parents, and the ratio of the numbers that were classified as + and - from this test was approximately 1:1. When ten of these clones

were crossed with one another, five of them had already been classified as + or - from the backcross tests and the other five, which had failed to react with either parent, could all be classified as + or - from the results of this second test. Assuming a +/- system, the full expectation of the results in this test could then be worked out. None of the positive results obtained disagreed with this expectation (see Fig. 14).

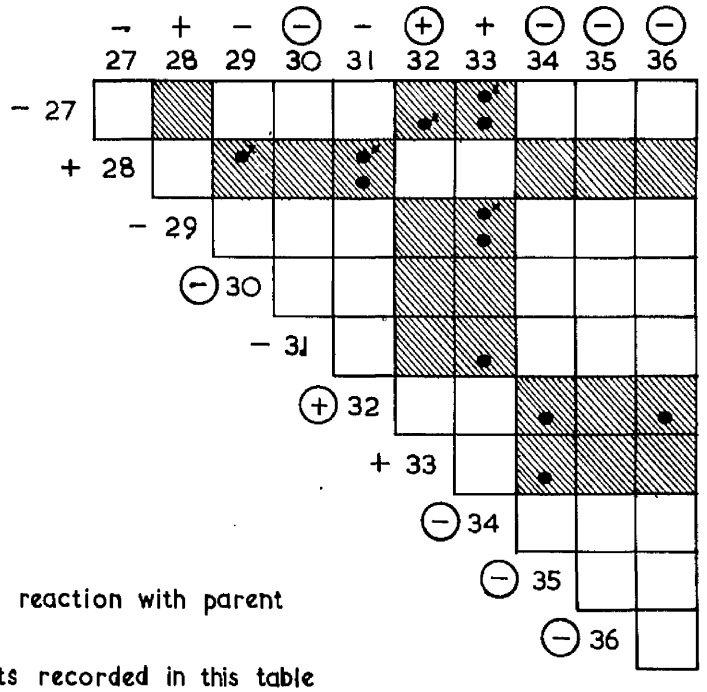
However, in both the backcrossing and intercrossing tests, many of the combinations of clones that were expected to produce plasmodia failed to do so. In the backcross tests, fifty clones were expected to produce plasmodia in combination with one or other of the parents, but, in the combined results of all seven tests, only twenty-five did so. In the intercross tests of ten of the clones, twenty-one combinations were expected to produce plasmodia but, in the combined results of two tests, only nine did so.

The high proportion of failures among cultures that are expected to succeed in producing plasmodia suggests that a more complex mating-type system may be operating, and this possibility is discussed below. On the other hand, it is still possible to defend the hypothesis of the single-factor system when the evidence concerning failure of crosses in all the mating-type tests is considered.

Fig. 14. Tests of the mating-type system using first-generation progeny of the cross a x i.

The results of crossing clones nos. 27-36 in all combinations of two at a time.

The expectation indicated in the chart is based on the assumption that a single-factor (+/-) mating-type system operates. (For further explanation of the form of the chart, see Fig. 9).



Expected positive result

Expected negative result

• Found positive result

•* Found result used to work out expectation

•
• Positive result in first test
Positive result in second test

⊕ or ⊖ Mating-type deduced from positive reaction with parent in backcross tests

\+ or − Mating-type deduced from the results recorded in this table

Backcross Tests of First Generation (Table 3): Although 25 of the 50 clones crossed successfully with one parent when the results of all seven tests were combined, the total number that produced plasmodia in each of the seven tests was 6, 0, 1, 18, 23, 12, 11 respectively. There were only slight differences in technique between these tests and the only known factor that the more successful tests had in common was that harder agar was used as the medium.

Intercross Tests of First Generation (Fig. 14): Five crosses that failed when this test was first done were successful when it was repeated.

Original Mating-Type Tests of a - j (Fig. 9, p.56): Seven crosses that failed when this test was first done were successful both times it was repeated.

Multispore Cultures. In the early tests when multispore cultures were compared with single-spore cultures (I, 4), plasmodia developed in only ten of twenty-five cultures started from very large groups of spores. It is extremely improbable that these cultures did not all contain amoebae of both mating-types.

All these results show that mixtures of amoebae in combinations which are known to be capable of producing

plasmodia sometimes fail to give this result. The reason for these failures is presumably that the conditions of the environment are not suitable for plasmodium formation. There is a little evidence that slight changes in technique tend to alter the number of failures. The environmental factors involved have not been identified. In the original mating-type tests, there was evidence that drying of the medium inhibited plasmodium formation. In the backcross tests, on the other hand, there was evidence that harder agar (containing less water and therefore drier) encouraged plasmodium formation. It is possible that the desirable environment involves both high humidity and good bacterial growth, which is encouraged by a harder, drier medium.

If many cultures that are known to be capable of producing plasmodia frequently fail because of unfavourable environmental conditions, it is reasonable to suppose that the cultures that have been expected to produce plasmodia and have never done so have failed for the same reason.

However, the failure of crosses among those expected to succeed does not appear to be completely random. This is most clearly seen in the results of the backcross

tests (see Table 3). Although statistical analysis of these results would be difficult, it appears from inspection that when conditions are not suitable for all crosses to succeed, some are more likely to succeed than others. In other words, factors inherent in the clones other than the mating-type factors, + and -, may determine whether a cross is likely to succeed or fail.

It is possible that this effect involves interactions between the factors carried by the two clones in a cross. In the original mating-type test of a - j (Fig. 9), it was seen that the same four crosses failed in three successive tests and that there were seven different clones involved in these four crosses. This suggested that failure of a cross was determined not by characteristics of a particular clone but by interactions of the characteristics of a particular pair of clones. Similarly, some of the clones that failed in the backcross tests crossed successfully with clones of the same generation.

Conclusion: When the results of the backcross and intercross tests are compared with those expected on the basis of a single-factor mating-type system, the formation of plasmodia is found in about half of the

crosses in which it is expected and in none of the crosses in which it is not expected. Many of the crosses which were expected to produce plasmodia have failed to do so. There is evidence that crosses frequently fail as a result of unfavourable environmental conditions, but there is evidence also that such random factors may not be sufficient to account for all the failures. The others must be assumed to be caused by inherent characteristics of the clones other than mating-type or by interactions of such characteristics.

ii) Two-Factor Mating-Type System: +/- at Each of Two Loci. On this hypothesis, it is assumed that two pairs of factors at two unlinked loci control mating-type. Each clone of amoebae is of one of the following four types: ++, --, +-, -+. When pairs of clones are crossed, only those clones that carry different factors at both loci produce plasmodia. Thus only two successful combinations are possible: ++ with --, and +- with -+.

This system was postulated to explain the results of the backcross tests. It is assumed that the parents, a and i, are ++ and -- respectively. Since it is assumed that the loci are unlinked, all four types are expected in equal proportions among the first-generation progeny.

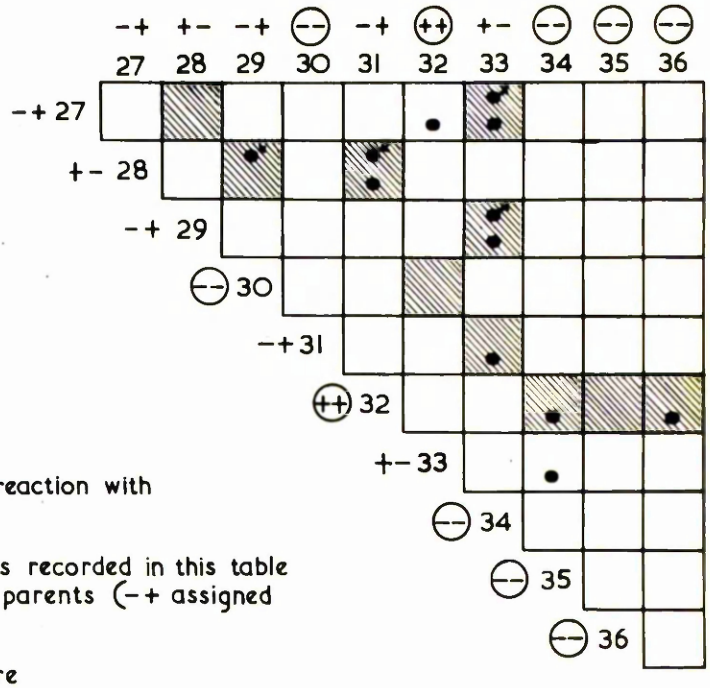
Approximately 25% of the progeny should be ++ and should cross successfully with parent i, 25% should be -- and should cross with parent a, and the remaining 50% should be +- or -+ and should not cross with either parent. This corresponds very closely with the results obtained: of the 50 clones tested, 10 crossed with i, 15 crossed with a and 25 crossed with neither.

When ten of the clones were crossed with one another, five of them could already be classified as ++ or -- from the results of the backcross tests. The remaining five could be assumed to be +- or -+ because they had failed to cross with either parent. One of these clones, number 27, was classified arbitrarily as -+. (It makes no difference to the results whether this is -+ or +-). The others could then all be classified as +- or -+ by the results of the tests. The full expectation of the results on the basis of this system could then be worked out (see Fig. 15). When this was done, it was found that plasmodia had been formed in all but three of the ten cultures in which they were expected, but also in two cultures in which they were not expected (Fig. 15). This latter kind of disagreement with expectation has not been found in any previous tests. The unexpected positive results can be explained by

Fig. 15. Tests of the mating-type system using first-generation progeny of the cross a x i.

The results of crossing clones Nos.27-36 in all combinations of two at a time.

The results recorded are the same as those in Fig. 14, but the expectation indicated in this chart is based on the assumption that a two-factor (+/- at each of two loci) mating-type system operates.



⊕⊕ or ⊖⊖ Mating-type deduced from positive reaction with parent in backcross tests

+− or −+ Mating-type deduced from the results recorded in this table for clones that did not react with parents (−+ assigned arbitrarily to no. 27)

Other symbols as in previous figure

assuming a breakdown of the mating-type system in these crosses, or by assuming chance contamination of the cultures with amoebae of a different mating-type, or emergence of amoebae of a different mating-type by mutation in the clones.

If the mating-type system is assumed to be a two-factor one of this kind, it must be concluded, from the results recorded in Fig. 9, that the original ten clones, a - j, were all of type ++ or --. Since all four types (++, --, +-, -+) are expected in equal proportions from any cross, this is unlikely; in fact, the segregation obtained among these ten clones disagrees significantly with the expectation on the basis of a two-factor system.

Conclusion: When the results of testing first-generation progeny are compared with those expected on the basis of a two-factor mating-type system, the formation of plasmodia is found in all but three of the ten crosses in which it is expected. However, plasmodium formation is also found in two crosses in which it is not expected and there is no evidence that helps to explain these unexpected results. The results of the original tests for mating-type disagree with the expectation on the basis of this system.

iii) Multifactorial Systems. The systems that have been considered are the simplest known systems that could be used to explain the results (Burnett, 1956). The only other mating-type system well known among micro-organisms is that in which many alleles exist at each of one or two loci. This system is not relevant to the present results, since they concern the progeny of only a single pair of clones and can thus involve only two factors at each locus.

The above considerations show that there is at present insufficient evidence to decide whether a single-factor or a two-factor system controls mating-type and plasmodium formation, but that it is very likely that the system is one of these two. In order to use mating-type as a genetic marker, it is not important to know which of these two systems is the true one, since in either case an equal segregation of mating-types is expected when the progeny are backcrossed to the parents, and the progeny could not easily be scored for a second mating-type marker. Further tests of mating-type will be done, however, in the hope that the system may be identified precisely.

2. Doubly-Marked Strains.

a) Preliminary Tests of Strains. The doubly-marked strains selected for this cross were:

E19: resistant to emetine, mating-type -

a: sensitive to emetine, mating-type +

Both strains were purified by picking from single plaques in two successive platings immediately before the following tests were done.

i) Comparison of Growth on Various Concentrations of Emetine.

Suspensions of amoebae were made from the following three cultures: strain a, growing on SA (= Standard Agar = 2% water agar inoculated with Pseudomonas sp.)

strain E19, growing on SA after two successive transfers on SA.

strain E19, growing on SA + 0.019% emetine (i.e. 0.019 g%. emetine hydrochloride in 100 ml. 2% water agar, inoculated with Pseudomonas sp.).

Each of these three suspensions was plated at a density of about 100 amoebae/plate on the following series of plates:

Plates 1 - 3	SA
" 4 - 6	SA + 0.019% emetine
" 7 - 9	SA + 0.025% emetine
" 10 - 12	SA + 0.031% emetine.

The results are shown in Table 4.

It is clear from these results that on concentrations of emetine from 0.019% to 0.031%, the resistant strain, E19, may be easily distinguished from the sensitive strain, a. The resistant strain shows the same viability on concentrations of emetine up to 0.031% as it does on SA, while the sensitive strain shows no growth on any of the concentrations (see also Fig. 13, p.73).

It is also clear that the culture of strain E19 that had been grown in the absence of emetine for two sub-cultures did not show lower resistance to the drug than the culture that had been kept on emetine. This was evidence that resistance to emetine was a genetic characteristic caused by a change selected by the drug, rather than a characteristic induced by the drug. Further tests supported this view.

ii) Tests for Adaptation of E19. Two lines of the strain E19 were grown in parallel: one on slopes of SA and the other on slopes of SA + 0.019% emetine. Five subcultures were made at intervals of about one week and both lines were tested for growth on SA and on emetine at the times when the fourth and fifth subcultures were made. Strain a was used as a control in both tests.

In the first test, the strains were tested on 0.019% emetine; in the second on both 0,019% and 0.031%. In

Table 4. Results of plating the resistant and sensitive strains, E19 and a, on standard agar and on standard agar containing different concentrations of emetine.

		Strain a amoebae plated/dish = 120			Strain E19 from culture on SA amoebae plated/dish = 96			Strain E19 from culture on SA + 0.019% em. amoebae plated/dish = 100		
Medium	Plate	No. of plaques	Average No.	Viability	No. of plaques	Average No.	Viability	No. of plaques	Average No.	Viability
SA	1	85	92	77%	70	84	88%	81	83	83%
	2	94			85			85		
	3	98			98			84		
SA + 0.019% emetine	4	0	0	0%	83	98	102%	86	94	94%
	5	0			86			93		
	6	0			126			103		
SA + 0.025% emetine	7	0	0	0%	83	91	95%	87	93	93%
	8	0			85			96		
	9	0			104			96		
SA + 0.031% emetine	10	0	0	0%	77	89	93%	73	79	79%
	11	0			88			87		
	12	0			103			78		

both tests, the two lines of E19 grew equally well on all the emetine plates, while the control strain, a, grew on none of them. It was concluded that the resistance to emetine of strain E19 was not a result of adaptation. The line of E19 growing on SA was used in most of the subsequent work.

b) Plasmodium (a + E19) and its Reaction to Emetine. Amoebae from the two strains, a (sensitive, +) and E19 (resistant, -) were streaked together on SA and many plasmodia were formed. From these, a single stock of plasmodium (a + E19) was established on oat agar. The growth of (a + E19) on a series of concentrations of emetine was compared with the growth of (a + i) on the same media. Since strain E19 was derived from strain i, the strains i and E19 and consequently the plasmodia (a + i) and (a + E19) presumably differ only by the factor that causes emetine resistance. Any difference in behaviour between (a + i) and (a + E19) may therefore be attributed to this factor.

The plasmodia were tested on emetine incorporated both in SA and in oat agar. No clear difference in reaction was detected between (a + i) and (a + E19) on any of the concentrations of emetine. Both strains appeared to be inhibited by emetine at concentrations from 0.031% upwards. On 0.006% emetine, growth was normal, and on concentrations

between 0.006% and 0.031%, intermediate degrees of growth occurred, which were difficult to define quantitatively.

c) First-Generation Progeny of the Cross a x E19.

Plasmodium (a + E19) was induced to form spores. Samples of spores were plated on SA and the resulting clones of amoebae isolated and tested for resistance to emetine and for mating-type.

i) Spore Platings. The results of six of the first seven platings of spores from (a + E19) are shown in Table 5. Plating 5 is omitted because the total number of plaques was so low that no isolates were taken. The spores used for all the platings were formed by the same stock of plasmodium, which was used to inoculate series of flasks for spore formation on several different dates. As indicated in the table, spores from only three different flasks were used: Platings 3 and 4 were done with spores formed in the same flask and Platings 2, 6 and 7 with spores formed in another single flask.

Each sample of spores was plated on a series of plates. The plaques were counted and amoebae isolated from them as soon as possible, usually after about 7 days incubation. The percentage viable count in each plating and the number of isolations made are shown in Table 5. Since Set 3 was so small, it was discarded without testing when Set 4 was isolated from the same spores.

Table 5. Results of plating spores formed by plasmodium (a + B19).

Plating No.	Plating Date	Details of Spores: Date formed	Flask No.	Age when plated	Spores plated/dish	No. of dishes	Av. no. of plaques	%age viable count	Isolates: Total No.	Set
1	3.3.60	25.2.60	14	7 days	230	19	14.6	6.3%	104	1
2	30.3.60	26.2.60	7	5 weeks	150	8	6.0	4.0%	104	2
	"	"	"	"	300	8	20.3	6.8%		
3	21.4.60	11.2.60	6	10 weeks	70	8	2.1	3.0%	37 (discarded)	3
	"	"	"	"	140	8	3.0	2.1%		
4	4.5.60	11.2.60	6	12 weeks	400	16	13.7	3.4%	100	4
6	23.9.60	26.2.60	7	30 weeks	400	16	2.0	0.5%		
7	6.10.60	26.2.60	7	32 weeks	500	8	3.0	0.6%	54	5
	"	"	"	"	1000	8	3.9	0.4%		

Samples of amoebae from the plaques were isolated on plates of SA by means of a small wire loop, manipulated under observation through a low power stereoscopic microscope. Care was taken not to sample more than one plaque at a time and plaques that had grown close together were avoided. Apart from this precaution, no selection was applied; all suitable plaques on a plate were sampled, whether small or large.

ii) Tests for Emetine Resistance and Mating-Type and Analysis of Results. After growth on SA, the clones of amoebae isolated from the spore platings were transferred to plates of SA + emetine and control plates of SA. The first set of isolates was tested on 0.019% emetine; the second and fourth on 0.031%; the fifth on 0.016%. Strains a and E19 were used as controls in all tests, to ensure that the emetine medium used would discriminate between resistant and sensitive strains. Segregation for resistance was found in each set. The distinction between ability and inability to grow on emetine was clear for most clones, since, where growth occurred, many amoebae on a spot would start to multiply at once, giving a

large patch of growth (Fig. 13), but a few clones gave only slight multiplication and these were classified as "doubtful".

All clones were also tested for mating-type by backcrossing with the strains a and 1 (Sets 1 and 2) or a and E19 (Sets 4 and 5). As in previous tests for segregation of mating-type, a large proportion of the clones could not be classified for this marker by the backcross tests. However, those that could be classified for mating-type were sufficient to show that recombination had occurred between mating-type and resistance to emetine. The numbers in each class are shown in Tables 6 - 8. The total samples shown in this table are slightly smaller than the numbers originally isolated because in each set of isolates a few failed to grow or became contaminated and were discarded.

Analysis of the results is shown in Tables 9 - 11. The ratio emetine resistant: emetine sensitive (Table 9) deviates significantly from 1:1 in three of the four sets and the results for the four sets are heterogeneous. The ratio mating-type +: mating-type - (Table 10) does not deviate significantly from 1:1 in any of the four sets and the results for the four sets are homogeneous. The ratio recombinants:

Tables 6 - 8. Results of Testing First-Generation Progeny of the Cross a x E19.

Table 6. Segregation for Emetine Resistance.

	Set 1	Set 2	Set 4	Set 5
Resistant	76	61	62	21
Sensitive	20	34	33	25
Doubtful	6	3	2	3
Total	102	98	97	49

Table 7. Segregation for Mating-Type.

	Set 1	Set 2	Set 4	Set 5
Mating-Type +	14	11	17	11
Mating-Type -	11	12	25	6
No Result	77	75	55	32
Total	102	98	97	49

Table 8(a). Full Classification for Emetine Resistance and Mating-Type.

Classification for emetine resistance.	Classification for mating-type.	Set 1	Set 2	Set 4	Set 5
Resistant	+	13	5	10	4
Resistant	-	9	9	14	3
Resistant	No Result	54	47	38	14
Sensitive	+	1	5	6	7
Sensitive	-	2	3	11	3
Sensitive	No Result	17	26	16	15
Doubtful	+	0	1	1	0
Doubtful	-	0	0	0	0
Doubtful	No Result	6	2	1	3
Total		102	98	97	49

(b). Classification omitting "doubtfuls" and Those that gave no result in the Mating-Type Test.

		Set 1	Set 2	Set 4	Set 5
Parental Types	Res -	9	9	14	3
	Sens +	1	5	6	7
Recombinant Types	Res +	13	5	10	4
	Sens -	2	3	11	3
Recombinants:parentals		15:10	8:14	21:20	7:10

Tables 9-11. Analysis of the Results Shown in Tables 6-8.

Table 9. Segregation for Emetine Resistance.

Resistant	Sensitive	Total	χ^2 (for deviation from 1:1 ratio)	D.F.	P
76	20	96	32.67	1	< 0.001
61	34	95	7.67	1	0.01-0.001
62	33	95	8.85	1	0.01-0.001
21	25	46	0.35	1	0.7-0.5
			49.54	4	
220	112	332	35.13	1	< 0.001
		Heterogeneity χ^2	14.41		
		Corrected heterogeneity χ^2 (allowance for deviation from 1:1).	16.12	3	0.01-0.001

Table 10. Segregation for Mating-Type.

+	-	Total	χ^2 (for deviation from 1:1 ratio)	D.F.	P
14	11	25	0.36	1	0.7-0.5
11	12	23	0.04	1	0.9-0.8
17	25	42	1.52	1	0.3-0.2
11	6	17	1.47	1	0.3-0.2
			3.39	4	
53	54	107	0.01	1	0.95-0.90
		Heterogeneity χ^2	3.38	3	0.5-0.3

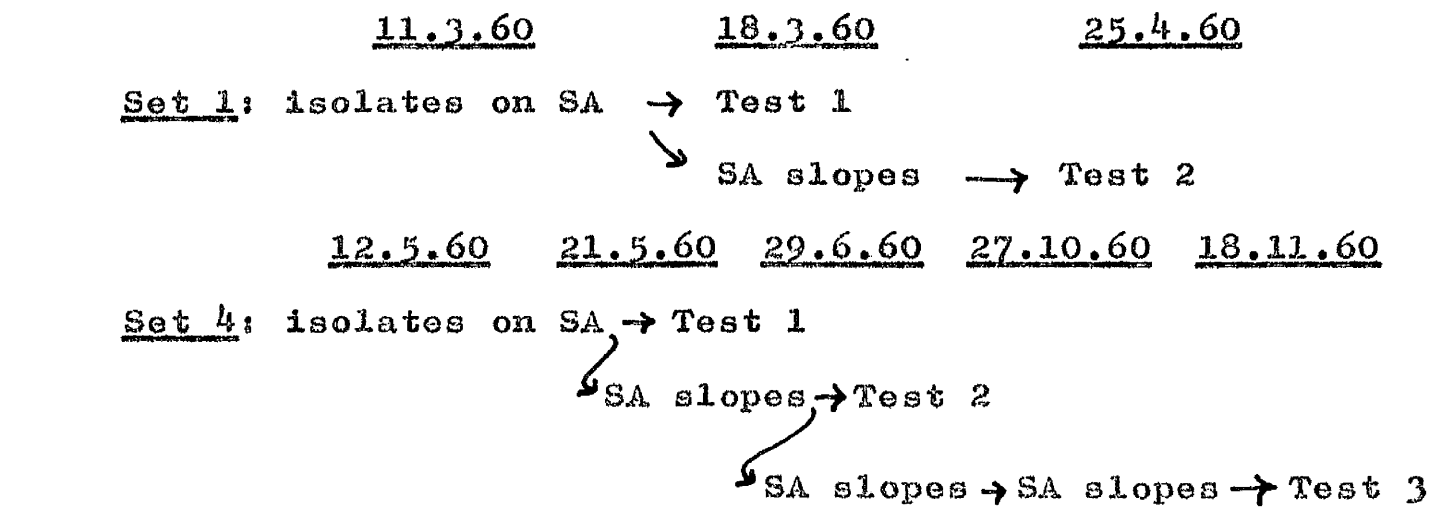
Table 11. Recombination Data for Fully-Classified Progeny (omitting "doubtfuls" and those that gave no result in the mating-type test.)

Recombinants	Parentals	Total	χ^2 (for deviation from 1:1 ratio)	D.F.	P
15	10	25	1.00	1	0.5-0.3
8	14	22	1.64	1	0.3-0.2
21	20	41	0.02	1	0.9-0.8
7	10	17	0.53	1	0.5-0.3
			3.19	4	
51	54	105	0.09	1	0.8-0.7
		Heterogeneity χ^2	3.10	3	0.5-0.3

parentals (Table 11) also agrees with 1:1 and the results for the four sets are homogeneous.

The results agree with the hypothesis that mating-type is determined by a single pair of alleles and that the factors determining mating-type and emetine resistance recombine freely. Emetine resistance may also be determined by a single pair of alleles, but if it is, some factor has disturbed the segregation of the alleles in three of the four samples tested. If it is assumed that mating-type and emetine resistance are determined by single pairs of alleles at two unlinked loci, then whether the cause of disturbed segregation for emetine resistance is viability or misclassification or both, the ratio recombinants : parentals is still expected to be 1:1. Thus, if a disturbing cause is assumed to be present, the results are consistent with the hypothesis that mating-type and emetine resistance are determined by single pairs of alleles at two unlinked loci. Results that give further evidence of disturbed segregation for emetine resistance are given below.

iii) Results of Re-Testing Sets 1 and 4 on Emetine. The first and fourth sets of isolates were re-tested on emetine after one or more subcultures on slopes of SA. The timing of the tests may be summarized as follows:



For both sets, it was found that the classification of some of the isolates was different in the different tests. The changes in the total numbers classified as resistant, sensitive and doubtful are shown in Table 12, and the different ways in which the classification changed in Table 13. In Set 1, 88 of the 101 isolates were classified in the same way in both tests, and in Set 4, 77 of the 97 isolates were classified in the same way in all three tests.

Table 12. Results of Re-testing Sets 1 and 4 on Emetine. One isolate of Set 1, resistant in the first test, was discarded in the second test because it produced plasmodia.

	Set 1		Set 4		
	Test 1	Test 2	Test 1	Test 2	Test 3
Resistant	76	68	62	51	64
Sensitive	20	31	33	38	30
Doubtful	6	2	2	8	3
Total	102	101	97	97	97

Table 13. Changes in Classification of Sets 1 and 4 When They Were Re-tested on Emetine. + = resistant; - = sensitive; (+) = doubtful.

(a) Set 1.

Test 1	Test 2		
+	+	68	
-	-	19	
(+)	(+)	1	
		88	= Total classified the same way in Tests 1 and 2.
+	-	7	
-	(+)	1	
(+)	-	5	
		13	= Total classified differently in Tests 1 and 2.
		101	= Total

Table 13 (continued)

(b) Set 4.

Test 1	Test 2	Test 3		
+	+	+	50	
-	-	-	27	
(+)	(+)	(+)	0	
			77	= Total classified the same way in Tests 1,2,3.
+	-	+	6	
+	(+)	+	4	
-	(+)	-	3	
			13	= Total classified the same way in Tests 1 and 3 only.
-	-	+	2	
-	-	(+)	1	
+	+	(+)	1	
			4	= Total classified the same way in Tests 1 and 2 only.
+	(+)	(+)	1	
			1	= Total classified the same way in Tests 2 and 3 only.
(+)	-	+	2	
			2	= Total classified differently in Tests 1, 2, 3.
			97	= Total

There was little variation in technique between the tests. Several different concentrations of emetine were used but the results of a test were recorded only if there was a clear difference between the reactions of the resistant and sensitive parents, E19 and a, on the medium used. There was slight variation in the age of the cultures of amoebae from which samples were taken for the tests. The variations in technique are summarized in Table 14, from which it may be seen that there is no obvious correlation between these conditions and the results.

iv) Results of Re-testing Set 5 on Emetine after Cloning.

It was suspected that some of the changes in classification recorded above might be caused by the presence of isolates that were not true clones. To test this, the fifth set of isolates was re-tested after cloning on SA. Cloning was done by streaking out each original isolate on a plate of SA and picking amoebae from a single plaque.

There was very little change in classification between tests made before and after cloning. The results are shown in Table 15. Forty-five of the forty-nine isolates were classified in the same way

Table 14. Comparison of Conditions in Tests of Sets 1-5 on Emetine.

	Set 1		Set 2	Set 4			Set 5
	Test 1	Test 2		Test 1	Test 2	Test 3	
Concentration of emetine	0.019%	0.031%	0.031%	0.031%	0.031%	0.016%	0.016%
Age of culture of amoebae	1 week	5 weeks	1 week	1 week	6 weeks	3 weeks	1 week
Result (Res:Sens)	76:20	68:31	61:34	62:33	51:38	64:30	21:25

Table 15. Results of Testing Set 5 Before and After Cloning.

(a) Totals in Each Class.

	Original Isolates	After Cloning
Resistant	21	21
Sensitive	25	26
Doubtful	3	2
Total	49	49

(b) Changes in Classification.

Original Isolates	After Cloning		
+	+	19	
-	-	25	
(+)	(+)	1	
		45	= Total Classified the same way in both tests.
+	-	1	
+	(+)	1	
(+)	+	2	
		4	= Total classified differently in the two tests.
		49	= Total

in both tests. Set 5 was also tested for mating-type before and after cloning and the only difference found between tests was that four of the isolates could be classified for mating-type in one test but not in the other. Seventeen were classified in the same way for mating-type in both tests, although among these were three of the four isolates that were classified differently for emetine resistance.

These results give no clear evidence of the presence of isolates that contain mixtures of amoebae of different types. Evidence that such mixtures were sometimes present was found, however, among the original isolates in Sets 1 and 5. In Set 1, two out of 104 isolates and, in Set 5, three out of 54 isolates had to be discarded before testing because they had produced plasmodia. These isolates presumably contained mixtures of amoebae of different mating-type.

d) Discussion. From the results of the cross described above, it may certainly be concluded that recombination of hereditary determinants has occurred during one life-cycle of the Myxomycete. The two characteristics, mating-type and resistance to emetine, were inherited consistently through many divisions of the amoebae in the parent strains. When these strains were mixed,

a plasmodium was formed which carried the factors determining the characteristics although it did not manifest them, and when this plasmodium formed spores, they produced amoebae that showed the two characteristics in the two possible new combinations as well as in the parental ones. Thus at some stage in the life-cycle, recombination of the factors determining mating-type and emetine resistance occurred.

The nature of the determining factors and the mechanism of their recombination are, however, not certain. The simplest hypothesis to explain the facts, and the one most in accordance with results in other organisms, is that the two characteristics are determined by chromosomal genes which recombine during meiosis. In order to discuss the results expected on this hypothesis, some assumptions must be made about the nuclear cycle.

Since the amoebae are of two different mating-types which fuse to form the plasmodium, it is reasonable to assume that the amoebae correspond to the gametes of other organisms and are consequently haploid. This is supported by the observation of Ross (in press) that meiosis occurs in P. polycephalum just before spore formation. The plasmodium may be heterokaryotic or diploid, but it must be assumed that fusion of nuclei in pairs and, subsequently, meiosis occur at some time before spore formation.

If this life-cycle is assumed and if it is furthermore assumed that only nuclei of opposite mating-type can fuse, then when a plasmodium is formed by two strains of amoebae carrying different alleles at one genetic locus, 50% of the amoebae of the next generation are expected to carry one of the alleles and 50% the other. If the parent amoebae carry different alleles at two unlinked loci, 50% of the next generation of amoebae will carry alleles at the two loci in the same combinations as the parents, and 50% will carry the two other possible combinations.

The analysis shown in Tables 9 - 11 tested for disagreement of the results with this hypothetical situation. The segregation of mating-type in the cross agrees with the hypothesis; the two mating-types, + and -, carried by the parents, segregate consistently in the ratio 1:1 in every sample of spores from the hybrid plasmodium. Mating-type may therefore be assumed to be determined by two alleles of a chromosomal gene. The inheritance of mating-type has already been discussed in Part III, 1, and it was shown that the system may involve + and - alleles at either one or two loci. However, as was also pointed out in that discussion, when mating-type is tested only by ability to cross with one parent or the other, the segregation of the two types expected is 1:1 whether

the system involves one or two pairs of alleles. The results in the present section support the hypothesis that the factors determining mating-type are alleles of chromosomal genes and they are consistent with a system involving either one or two pairs of alleles.

The analysis of recombination data in Table 11 shows that mating-type and emetine resistance recombine freely. The parental and recombinant classes agree consistently with the ratio 1:1 in all samples of spores from the hybrid plasmodium. Thus whatever the factors determining inheritance of emetine resistance, they segregate independently of the factors determining mating-type.

The analysis of segregation of emetine resistance in Table 9 shows that nearly all the results disagree with the hypothesis that this characteristic is determined by alleles of a chromosomal gene. Except in Set 5, the segregation deviates significantly from 1:1. The segregation in Set 5 agrees with 1:1 but this sample is inconsistent with another sample (Set 2) which was taken from the same batch of spores. If emetine resistance is assumed to be determined by alleles of a chromosomal gene, explanations must be found for the deviation from 1:1 and for the heterogeneity of the samples.

The factors that are most commonly found to cause deviation of allele ratios from 1:1 are selfing, viability difference and misclassification.

i) Selfing. "Selfing" is said to occur when pairs of nuclei fusing before meiosis consist sometimes of nuclei derived from the same parent strain. In some organisms, selfing never occurs; only fusions of nuclei of different parental origin result in meiosis and the formation of the next generation. If selfing of one parental nuclear type occurs more often than selfing of the other, the alleles carried by that parent will appear in greater frequency among the next generation and the allele ratios will deviate from 1:1. However, the segregation of parental:recombinant types must also deviate from 1:1 since there will be an excess of parental types. In the results of the present cross, the segregation of parentals:recombinants agrees with 1:1 and thus it may be concluded that selfing does not occur.

In connection with the investigations of the nuclear cycle (see Part IV), it is interesting to notice that this conclusion means that nuclear fusion occurs normally only between nuclei of different mating-type. If nuclear fusion occurs when a pair of amoebae fuse to

initiate plasmodium formation (as suggested by Ross, 1957), this is not surprising because the amoebae that fuse are presumably of different mating-type, but if it occurs just before spore formation it suggests that the mating-type factors function at two stages of the life-cycle, controlling both cell fusion and nuclear fusion.

ii) Viability Difference. If spores or amoebae carrying the allele for emetine resistance are in any way more viable than those carrying the allele for sensitivity, a deviation of the allele ratio from 1:1 is expected, accompanied by no deviation of the ratio of recombinants to parentals. The resistant spores may survive longer, mature more quickly or hatch more quickly, or the amoebae may multiply more quickly after hatching. Any of these differences would result in more isolations of resistant than of sensitive clones when amoebae are taken from the plaques formed in the spore platings. There is no great difference in viability between the amoebae of the parent strains, a and E19 (see Table 4), but it is possible that a difference between resistant and sensitive strains may be manifested during formation, maturation or hatching of spores even if it does not show in the amoebae.

The heterogeneity of Sets 2 and 5 might also be explained by such a viability difference between the strains. If, for example, spores carrying the resistant allele matured more quickly than those carrying the allele for sensitivity, platings done with young spores would give an excess of resistant clones while platings with older spores would not. The spores that gave rise to Set 5 were seven months old when they were plated, whereas those used for Sets 1, 2 and 4 were one week, five weeks and three months old respectively (see Table 5). Consistent with this hypothesis also is the fact that Set 1, which contained the greatest excess of resistant isolates (Table 6), was derived from spores plated at the smallest age.

The difference between Sets 2 and 5 could also have been caused partly by selective death of resistant spores. The viability of the spores fell during the time between the isolations of Set 2 and Set 5 from 5% to 0.5%. With this drop in viability, the proportion of resistant clones would fall from 60% in Set 2 to 50% in Set 5 if the number of deaths among resistant spores was greater than the number among sensitive spores by only 4%.

iii) Misclassification. An excess of resistant isolates among the progeny would also be found if misclassification occurred and some genetically sensitive clones were classified as resistant. As with a viability difference, misclassification is not expected to cause deviation of the ratio of recombinants:parentals.

A number of results recorded in section (c) suggest that misclassification has been a source of error in the cross. Principal of these is of course the fact that so many changes in classification occurred. If these changes had all occurred in one direction, for example if many isolates originally classified as resistant had later been found to be sensitive, the changes in classification might be taken as evidence of real changes in the constitution of the amoebae. But in Set 4 (Table 13), which was tested three times, many of the isolates that had apparently lost their resistance in the second test were found to have gained it again in the third.

The behaviour of the "doubtful" isolates is also evidence that misclassification occurred. Since the initial tests of all the Sets (Table 6) showed a small proportion of isolates, varying between 2% and 6%, that appeared to be intermediate in their reaction to

emetine between the clearly-defined "resistant" and "sensitive" strains, it might have been suspected that more than two classes existed and, consequently, that the control of emetine resistance was multifactorial.

In Sets 1, 4 and 5, however, in which 23 isolates out of a total of 247 were classified as "doubtful" at one time or another, only two were consistently classified as "doubtful" in all tests (see Tables 13 and 15). The total number of isolates classified consistently as resistant or sensitive in all tests of these sets was 208. The fact that nearly all the "doubtful" isolates were involved in changes of classification and that their changes showed no consistent trend (as, for example, resistant → doubtful → sensitive, which seems a logical trend but which in fact never occurred) suggests that they are not a distinct class carrying a genetic constitution intermediate between that of the resistant and sensitive classes, but merely a symptom of uncertain classification.

The two most likely causes of this uncertainty in classification are unnoticed variations in technique and the presence of isolates containing more than one clone. Variations in technique would lead to misclassification and deviation of the allele ratio from 1:1 in the observed direction if amoebae carrying the

allele for sensitivity were under some conditions able to grow on emetine.

The known variations in technique were shown in Table 14, and it was concluded from this table that there is no correlation between these variations and the segregation for resistance found in the tests. However, one tentative suggestion of a correlation may be made. If only Sets 1 and 4 are considered, the nearest approximation of the allele ratios to 1:1 is found in the tests in which the oldest cultures of amoebae were used. It might be assumed that when young cultures of amoebae are tested, the allele ratio apparently deviates from 1:1 because some genetically sensitive clones are able to adapt to growth on emetine. When older cultures are tested, fewer succeed in adapting. If this is so, however, this ability to adapt to growth on emetine must itself be an inherited property of the isolates, since in the tests of Set 4 (Table 13) the majority (13 out of 20) of the isolates that varied in their reaction behaved identically in Tests 1 and 3. If a random sample of sensitive isolates succeeded in adapting in any one test, those that adapted in Test 1 would be expected to be different from those that adapted in Test 3. In other words, fewer + - + changes would be expected in Table 13 and more + - - and - - +.

The deviation of the allele ratio and the changes in classification may also be partially accounted for if some of the isolates consist of mixtures of amoebae of more than one clone. If the ratio of resistant to sensitive clones is 1:1, isolates containing mixtures of two clones will consist of one quarter that contain two resistant clones, one quarter that contain two sensitive clones and one half that contain a mixture of resistant and sensitive clones. The mixtures of resistant and sensitive amoebae will show growth and will therefore be classified as "resistant". If there are very few resistant amoebae in a sample of the mixture, only slight growth may occur and the classification will be "doubtful". When a mixed isolate is subcultured to a slope of SA, allowed to multiply and then sampled again, as in the re-tests of Sets 1 and 4, differences in classification may result from local differences in the proportions of the two types of amoebae in the culture.

As shown in Section (c), there is positive evidence that some of the isolates contained more than one clone, since plasmodia were found in them. In Set 1, for example, 2 out of 104 isolates gave plasmodia. From this figure, a rough estimate may be made of the total number of mixed isolates present (considering mixtures of two clones

only). This involves first calculating the total number of mixtures containing amoebae of compatible mating-types, since not all such mixtures give rise to plasmodia. For this purpose, the data of Table 7 may be used. This table shows the number of isolates in each set that failed to give plasmodia when crossed to both parents ("No result") and the numbers that gave plasmodia with one parent or the other (classified as "+" or "-"). All the isolates were crossed with both parents. If the mating-type system is a single-factor one, every isolate is expected to produce plasmodia with one or the other of the parents; if it is a two-factor system, only 50% are expected to produce plasmodia. In fact, the proportion of isolates that gave plasmodia with one or the other of the parents ranged in the four sets from 40% to 20%. Thus assuming a single-factor system, the proportion of compatible mixtures expected to give plasmodia is in the range 40% to 20%; assuming a two-factor system, it is in the range 80% to 40%.

Thus, assuming a single-factor mating-type system, if the proportion of isolates giving plasmodia is 2%, the total proportion containing compatible mating-types will be 5% to 10%. The total proportion of isolates containing mixtures of two clones, regardless of mating-type, will be twice as great as this, i.e. 10% to 20%.

Assuming a two-factor mating-type system, if the proportion of isolates giving plasmodia is 2%, the total proportion containing compatible mating-types will be 2.5% to 5%. The total proportion of isolates containing mixtures of two clones, regardless of mating-type, will be four times as great as this, i.e. 10% to 20%.

Thus, for either mating-type system, the estimate of the total proportion of mixed isolates in the sample is in the range of 10% to 20%. If the allele ratio for emetine resistance is 1:1, half of the mixed isolates may be assumed to be mixtures of resistant and sensitive clones, that is, 5% to 10% of isolates. This proportion will be misclassified as resistant and may be variable in their reaction when re-tested. If 10% of isolates are misclassified as resistant, the ratio of resistant to sensitive expected from a sample of 100 isolates is 55 to 45 instead of 50 to 50. The ratio found in Set 1 is 76 to 20. Thus the proportion of mixtures found in this sample is not sufficient to account for the deviation from 1:1 although it is sufficient to account for the proportion for which classification was variable.

Set 5 was isolated and tested before and after cloning, with the purpose of estimating the error caused by the presence of mixed isolates. Out of 49 isolates, two that were originally classified as resistant were classified

differently after cloning. If these two isolates are assumed to have been mixtures of resistant and sensitive clones, the proportion of such mixtures may be estimated as 4%, with confidence limits of 1% and 14%. This is a minimal estimate, since not all mixtures would be expected to change their classification after cloning. However, if even 14% from a sample of 100 are misclassified as resistant, because they are mixtures, the ratio of resistant to sensitive expected is 57 to 43 instead of 50 to 50. This is almost sufficient to account for the excess of resistant isolates in the other samples and it is sufficient to account for the proportion for which classification was variable in Sets 1 and 4. However, since no excess was found in Set 5 and the samples are heterogeneous, it is difficult to draw any general conclusions from these results.

It may be concluded from this discussion that some misclassification has certainly occurred in the tests and that a considerable proportion of isolates containing mixtures of two or more clones has been present. The proportion of mixtures present is great enough to account for the variations in classification that occurred on re-testing and almost enough to account for the deviation from 1:1 in the ratio of resistant to sensitive isolates.

It is of course surprising that with spores of such low viability as 0.5% (as in Set 5), isolates containing mixtures of clones should arise at all. Nevertheless, in Set 5, three of the 54 isolates were discarded because they gave rise to plasmodia. It is very unlikely that these mixtures result from the chance proximity of two hatching spores and the haemocytometer counts before plating showed that clumps of spores were rare. It is possible, however, that the spores in the few clumps that are present hatch more readily than single spores because of some effect of one hatching spore on another. If this is so, the proportion of mixtures is expected to be high. Alternatively, the mixtures may result from single spores containing amoebae of more than one nuclear type, although such spores were never found among those isolated by hand.

It may be concluded that although there is insufficient evidence that any one of the factors considered has caused the deviation of the emetine resistance allele ratio from 1:1, there is evidence that several factors capable of causing the deviation have been operating in this cross.

Summary.

1. The strains a (mating-type +) and i (mating-type -) were crossed; spores obtained from the plasmodium were plated, and isolates of amoebae were made from 50 plaques.
2. These 50 isolates were tested for mating-type by back-crossing to the two parent strains and it was found that some formed plasmodia with one or the other of the parent strains only and some with neither. The results were consistent with the original hypothesis of a single-factor mating-type system but were also consistent with the hypothesis of a two-factor system.
3. Intercrossing of ten of the 50 isolates in all combinations of two at a time gave results that agreed better with a single-factor than with a two-factor system.
4. The strain E19 (emetine resistant, mating-type -) was found to maintain its resistance to emetine through successive subcultures in the absence of the drug.
5. Platings of amoebae of strains E19 and a (emetine sensitive, mating-type +) on medium containing emetine showed that there was a clear difference in the viability of the two strains on the drug.
6. The strains a and E19 were crossed. The plasmodium formed, (a + E19), was found to be as sensitive to emetine as the plasmodium (a + i).

7. Spores obtained from plasmodium (a + E19) were plated and four sets of isolates of amoebae were made from a total number of 346 plaques. The four sets of isolates were separately tested for mating-type and emetine resistance.

8. In all four sets, the ratio mating-type + : mating-type -, tested by backcrossing the isolates to the two parent strains, agreed with 1:1 and the results for the four sets were homogeneous. The results agree with the hypothesis that mating-type is determined by a pair of alleles at one chromosomal locus or by pairs of alleles at two unlinked chromosomal loci.

9. In all four sets, the ratio recombinant types : parental types agreed with 1:1 and the results for the four sets were homogeneous. Emetine resistance and mating-type therefore recombine freely. The results are consistent with the hypothesis that these characteristics are determined by pairs of alleles at two unlinked chromosomal loci.

10. In three of the four sets, the ratio emetine resistant: emetine sensitive deviated significantly from 1:1 because of an excess of the resistant type. The results for the four sets were heterogeneous. The results are not

consistent with the hypothesis that emetine resistance is determined by alleles of a chromosomal gene unless factors can be demonstrated that may cause this deviation.

11. Consideration of the factors that may cause deviation of allele ratios shows that selfing has not occurred in the cross but that misclassification for emetine resistance has occurred and that a viability difference may exist between emetine resistant and emetine sensitive spores.

PART IV.

CYTOLOGICAL OBSERVATIONS.

Introduction.

A very large proportion of the work that has been devoted to Myxomycetes in the past has involved attempts to understand the nuclear cycle by means of cytological observation. These studies have resulted in so much confusion that the simply observational method of approach has not been used at all in the present investigation. One quantitative method has been used, which will be described after a discussion of the work of previous investigations.

Three major problems about the nuclear cycle of the Myxomycetes have long been in doubt and have been the subject of numerous investigations:

i) The stage at which meiosis occurs,

ii) The stage at which nuclear fusion occurs,

iii) The number of cells that contribute active nuclei to the plasmodium.

If all three problems were solved, the ploidy of the different stages would also be known with certainty. Conversely, determination of the ploidy would assist in solving the problems and this is the basis of the quantitative approach used in the present investigation.

1. Results of Previous Investigations.

i) The Stage at which Meiosis Occurs. A decisive answer to this question has been given, for some species at least, by the recent work of Wilson and Ross (1955). They observed meiosis during sporangium formation, prior to final cleavage, in nine species of Myxogastres, representing three of the four orders. Subsequently (personal communication), they have also observed meiosis at the same stage in other species, including a member of the fourth order, Physarum polycephalum, the species used in the present investigation. In Ceratiomyxa, the only representative of the Exosporeae, they observed meiosis in the maturing spores after cleavage. Their photographs are the first convincing illustrations of meiosis in Myxomycetes that have been published.

Several previous workers, however, claimed to have seen two meiotic divisions preceding cleavage in developing sporangia in the Myxogastres: Wilson and Cadman (1928) in Reticularia lycoperdon, Schinnemann (1930) in Didymium nigripes and Cadman (1931) in D. nigripes. Other authors failed to see more than one division in the developing sporangia: among others,

Howard (1931a) in Physarum polycephalum and Von Stosch (1935) in D. nigripes. Von Stosch recorded that both meiotic divisions occurred in the spore and that three of the four resulting nuclei degenerated, leaving a uninucleate spore. Two other workers, Jahn (1933) with Badhamia utricularis, and Schure (1949) with Mucilago spongiosa, recorded that the first meiotic division occurred before final cleavage, the second afterwards in the maturing spore.

In Ceratiomyxa, two authors, Olive (1907a,b) and Gilbert (1935) recorded the same result as Wilson and Ross (1955); that meiosis occurs in the spore giving the mature spore four nuclei. Jahn (1908, 1936) disagreed, stating that meiosis occurred at the time of cleavage.

It is clear now that in Ceratiomyxa meiosis occurs in the maturing spore and that in many species of the Myxogastres meiosis occurs in the developing sporangia prior to cleavage. Wilson and Ross have not yet examined all the species in which conflicting observations have been made by past workers, so that it is possible that in some species of the Myxogastres, meiosis does occur partly or wholly in the spore. However, there is little evidence of this at present; none of the

illustrations given by previous workers have been convincing, and since Wilson and Ross (1955) noted that all the nuclei in any one sporangium completed meiosis in a very short period of time, it seems very probable that this rapidity alone has led to the failure of some workers to observe meiosis.

A few investigators have concluded from indirect observations that meiosis must take place in the spore. Skupienski (1918), with D. nigripes, obtained plasmodia from single-spore cultures but not from single-amoeba cultures and concluded that the amoebae from a single spore must segregate for mating-type. There is no answer to this observation at present (see Part I, 4). Some of the other workers who have obtained plasmodia from single-spore cultures and have seen fusion of pairs of amoebae in the cultures, for example Cayley (1929) with D. difforme, have used this result to conclude that amoebae from a single spore carry different mating-types and that therefore meiosis occurs in the spore. When single-amoeba cultures have not been tested, this conclusion is not valid, since there is no reason to suppose that fusion cannot occur between amoebae of the same genotype. Indeed, Kerr and Sussman (1958) have shown that in their strain of D. nigripes, single-amoeba cultures usually produce

plasmodia. Similarly, the finding in other species that amoebae from a single-spore culture are always of one mating-type only cannot be used to conclude that meiosis occurs before spore formation, since it is quite reasonable to suppose that three products of meiosis in the spore might degenerate, as reported by Von Stosch (1935) and as commonly found in the eggs of higher animals.

ii) The Stage at which Nuclear Fusion Occurs. The fusion of nuclei in pairs has been reported at two different stages of the life-cycle of Myxomycetes; at the time when the amoebae fuse in pairs, and at a time just prior to spore formation in the plasmodium.

Investigators who have reported nuclear fusion at the time of cell fusion are Jahn (1911) in Physarum didermoides, Skupienski (1917) and Cadman (1931) in Didymium nigripes, Wilson and Cadman (1928) in Reticularia lycoperdon, Gilbert (1935) in Ceratiomyxa and Ross (1957) in nineteen species of Myxogastres representing all four orders and including P. polycephalum. Investigators who have reported nuclear fusion in the plasmodium just prior to spore formation are Olive (1907a,b) and Jahn (1908) in Ceratiomyxa, Skupienski (1927, 1928) in Didymium difforme and Schünemann (1930)

in D. nigripes.

It is not possible at present to decide which of these investigators are likely to be correct, since pictures of nuclear fusion are rarely convincing. It is a process more difficult to demonstrate cytologically than meiosis and most of the illustrations meant to demonstrate it could be taken simply as showing the chance adjacence of two nuclei, or as the final stage of nuclear division. The most thorough investigation was that of Ross (1957) who observed both living and stained cells in cultures in which amoebae were fusing and forming plasmodia, and the series of photographs that he published give good evidence of nuclear fusion at this stage in some species at least.

iii) The Number of Cells that Contribute Active Nuclei to the Plasmodium. There is no doubt that as a plasmodium increases in size in a culture, the number of amoebae around it decreases; those that disappear are certainly adding to the bulk of the plasmodium. What is not certain is whether the nuclei of those amoebae that disappear are being broken down and digested by the plasmodium or whether they are continuing to exist as active nuclei in this new acellular environment. This question has not yet been answered.

Only a few workers have recorded that more than two amoebae may fuse to form a plasmodium or that amoebae may fuse with a plasmodium without being digested by it: these are Cienkowski (1863), in several species, Skupienski (1926, 1927, 1928) in Didymium difforme, Schünemann (1930) in D. nigripes and Von Stosch (1935) in D. nigripes var. xanthopus, D. squamulosum and Physarum cinereum, the three species in which he found that plasmodia could be formed in single-spore cultures. Among these four authors, Cienkowski and Skupienski recorded the fusion of pairs of amoebae, and Skupienski the digestion of amoebae by the plasmodium, in addition to the other processes.

Fusion of pairs of amoebae and digestion of amoebae by the plasmodium have been recorded also by Jahn (1911) in Physarum didermoides, Skupienski (1917) and Cadman (1931) in Didymium nigripes, Wilson and Cadman (1928) in Reticularia lycoperdon, and Ross (1957) in nineteen species of Myxogastres representing all four orders and including Physarum polycephalum. Among these authors, Skupienski, Cadman, Wilson and Cadman, and Ross recorded also the fusion of small plasmodia to produce bigger ones. These authors believed that plasmodium formation was initiated by the fusion of one pair of amoebae only, that this was followed by fusion of the two haploid nuclei, and that all

the nuclei in the plasmodium were then produced by division of the diploid nucleus or were diploid nuclei acquired by fusion with other plasmodia. They believed that whenever haploid amoebae entered the plasmodium, by fusion or by ingestion, their nuclei were broken down and digested.

It may be noted that the recorded observations of fusion of pairs or of groups of amoebae do not depend on whether the observations were made on multispore or single-spore cultures. Von Stosch (1935) recorded fusion of groups of amoebae in the species in which plasmodia could be obtained from single-spore cultures and fusion of pairs in the species in which they could not, while Skupienski recorded fusion of groups in the species in which plasmodia could not be obtained from single-spore cultures and fusion of pairs in the species in which they could. Cayley (1929) also observed the fusion of pairs of amoebae in single-spore cultures of D. difforme.

It is not possible at present to decide which of the many recorded observations are correct. It is reasonable to suppose that all the suggested processes may sometimes occur, but that not all of them occur in every species or under every type of culture condition.

2. Results of the Present Investigation.

The cytological observations made in the present investigation consisted of an attempt to determine the relative ploidy of amoebae and plasmodium by means of photometric measurements. The method is based on the fact that, when a sample of nuclei is stained by the Feulgen method, the density of stain in any nucleus of the sample is directly proportional to its content of desoxyribose nucleic acid (DNA), although the ratio of stain to DNA is not constant for nuclei stained under different conditions (Walker and Richards, 1957). The method depends also on the fact that accurate measurements of the density of stain in individual nuclei may now be made by the use of an integrating microdensitometer of the type first designed by Deeley (1955).

a) Details of Method. (For instruction in these methods, the author is grateful to Mrs Sylvia Wilson). Two smears were made on each slide, one of plasmodium and the other of amoebae. Samples of plasmodium were taken from two-day cultures on oat agar plates, on which the plasmodium was growing vigorously. Samples of amoebae were taken from water agar plates in which some of the amoebae were still actively feeding and dividing and some were encysted.

The smears were immediately frozen in isopentane and fixed in butanol and later stained by the Feulgen method. The hydrolysis time used was 12 minutes, since this was found most satisfactory by preliminary tests. After staining, the smears were mounted in glycerine and covered with cellophane. With these methods of fixing and mounting, the nuclei remain soft and flexible, so that when each is brought under pressure from the crushing condenser on the microscope, it spreads to form a layer of more even density, which is more accurately measured by the densitometer.

The microdensitometer consists of a microscope connected with a scanning device and a photomultiplier. Each smear is examined systematically with the oil-immersion objective of the microscope. Whenever a single nucleus is found sufficiently isolated from its neighbours, it is brought in to the centre of the field and crushed gently until it is of fairly even density; the field around it is then reduced as far as possible by means of a diaphragm in the tube of the microscope. The scanning device is then set into operation, so that the total density of the field is measured. The measurement is read on a dial which is marked with arbitrary values.

Three readings are taken for the same field and then

the nucleus is moved from the field and three readings of background density are taken. The difference between the averages of the two groups of readings is the arbitrary measurement of density, and thus of DNA content, that is recorded for the nucleus.

A consideration of the possible sources of error in such readings was made by Deeley (1955), who concluded that the random error was not more than 2% and that error from other causes was equally small.

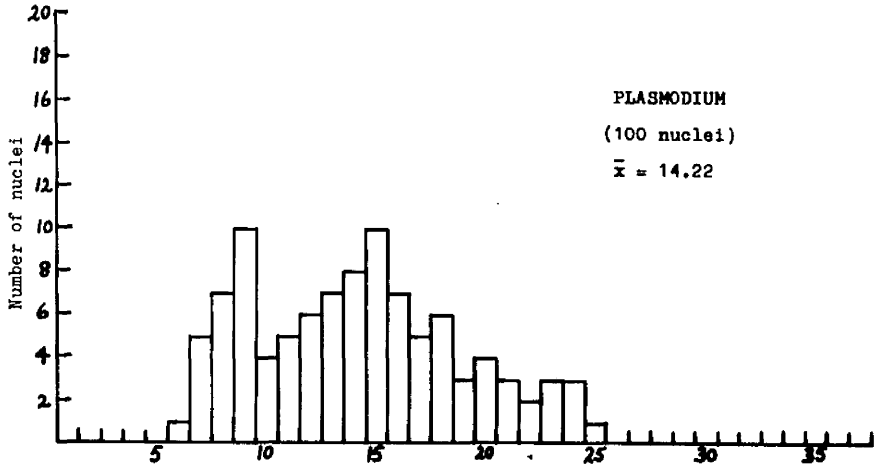
b) Results. The results of two sets of measurements are shown in Figures 16 and 17. Each set shows the distribution of values for nuclei from one slide only, since only measurements for nuclei stained under identical conditions are strictly comparable. All the nuclei measured were in interphase; no dividing nuclei were seen during the investigation.

In the first set, 100 nuclei of a plasmodium and 100 nuclei of amoebae were measured; in the second, 150 nuclei of a plasmodium and 129 of amoebae. Neither of the distributions for the first set (Fig. 16) deviates significantly at the 5% level from a normal distribution when tested by the χ^2 method. The distributions for the second set (Fig. 17) were not tested for deviation from normality. In both sets, the mean value for nuclei of amoebae is significantly different from and slightly higher than the

Fig. 16. Results of the first set of photometric measurements of DNA content in interphase nuclei of plasmodium and amoebae.

The values on the abscissa represent the photometric values of individual, Feulgen-stained nuclei, measured in arbitrary units. For nuclei of one set, these values are directly proportional to the DNA content of the nuclei.

$\bar{x}$ represents the mean photometric value in arbitrary units.


PLASMODIUM
(100 nuclei)
$\bar{x}$ = 14.22
Number of nuclei
Arbitrary units representing DNA content of single nuclei

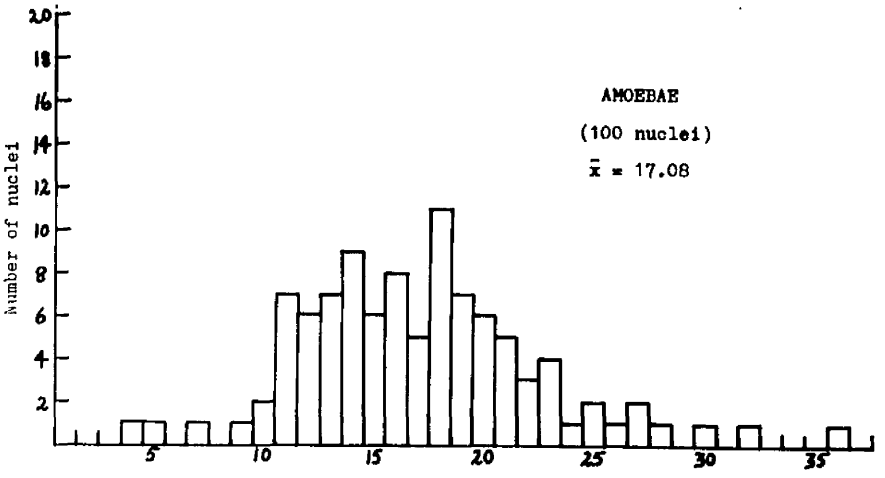
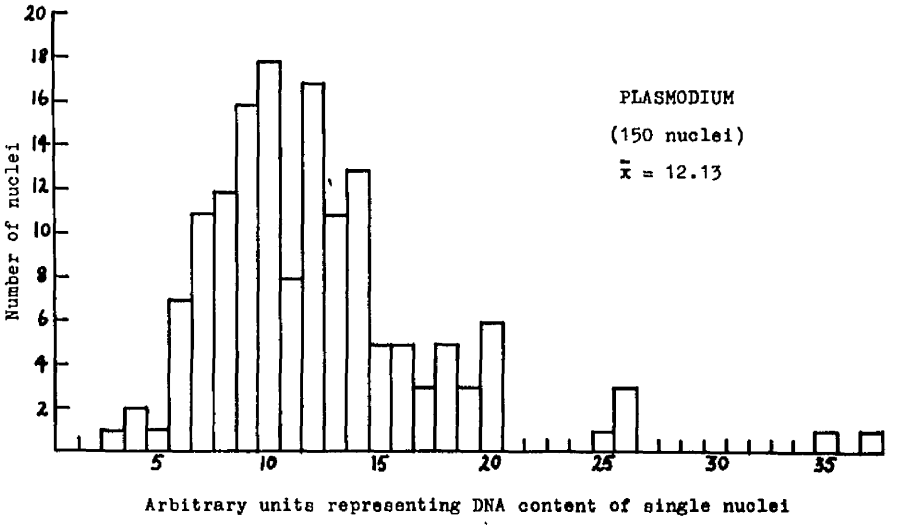
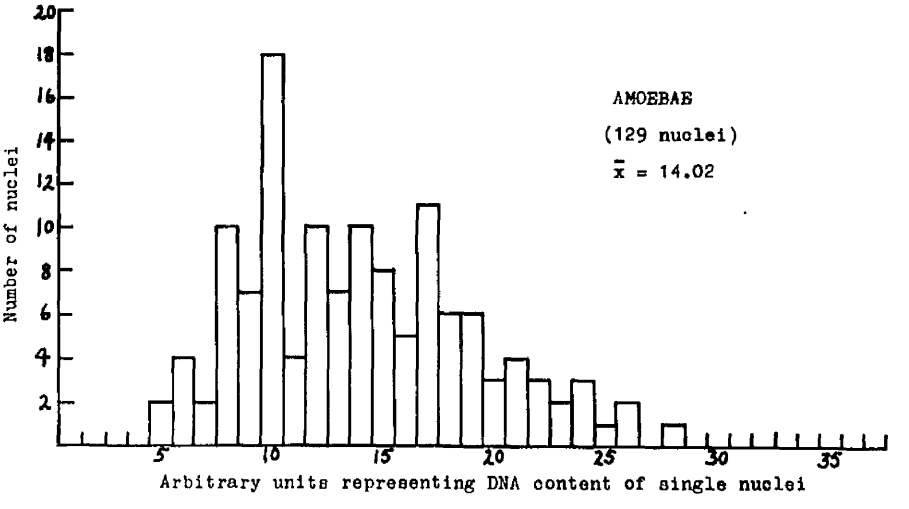

AMOEBAE
(100 nuclei)
$\bar{x}$ = 17.08
Number of nuclei
Arbitrary units representing DNA content of single nuclei

Fig. 17. Results of the second set of photometric measurements of DNA content in interphase nuclei of plasmodium and amoebae.

For explanation, see Fig. 16.

(Note: The absolute photometric values obtained in the two sets of measurements may not validly be compared; photometric values are proportional to DNA content only for nuclei stained on the same slide i.e. measured in the same 'set').


PLASMODIUM
(150 nuclei)
$\bar{x}$ = 12.13
Number of nuclei
Arbitrary units representing DNA content of single nuclei


AMOEBAE
(129 nuclei)
$\bar{x}$ = 14.02
Number of nuclei
Arbitrary units representing DNA content of single nuclei

mean value for plasmodial nuclei.

For the first set of measurements, more detailed analysis was carried out by Dr. S.D. Silvey and Mr J. Aitchison. For each of the two observed distributions, an attempt was made to fit to the data a mixture of two normal distributions of which the mean of one was twice the mean of the other. No such mixture was found that fitted the distribution for nuclei of amoebae better than a single normal distribution, but for the nuclei of plasmodium, a mixture was found that fitted the data better, though not significantly better, than a single normal distribution. This mixture was of two normal distributions with means 8.5 and 17.0 and variances 6.25 and 13.5 respectively, one third of the observations belonging to the distribution with the smaller mean. The larger mean is twice as great as the smaller and almost exactly equal to the mean of 17.1 found for the amoebae.

3. Discussion.

It was shown in Part IV, 1 that although the position of meiosis in the life-cycle of Physarum polycephalum is almost certainly just before spore formation, there is very little evidence on which to decide the position of nuclear fusion in the life-cycle or the nuclear constitution of the plasmodium. If nuclear fusion occurs immediately after fusion of a pair of amoebae and if subsequent growth

of the plasmodium is accompanied by no further fusions with amoebae, as believed by Ross (1957), the plasmodial nuclei must all be diploid and the amoebae haploid. If nuclear fusion takes place later, prior to spore formation, and if no fusions of the plasmodium with amoebae occur, the plasmodium must contain a mixture of haploid nuclei of two kinds, carrying the two mating-types; in other words, it must be a heterokaryon. If fusion of the plasmodium with amoebae occurs, then if nuclear fusion occurs early, the plasmodium will carry a mixture of diploid and haploid nuclei; if it occurs late, the plasmodium may carry haploid nuclei of more than two kinds.

It was hoped that the measurements of the DNA content of nuclei would show clearly the relative ploidy of amoebae and plasmodium. The results have, however, been unexpectedly difficult to interpret. The chief difficulty is that the values obtained for individual nuclei are so small that it is not possible to decide whether or not the distributions obtained are bimodal. The densitometer was designed chiefly for use with the much larger nuclei of mammalian tissues, with which the mean value obtained for any sample is usually at least 100 units on the arbitrary scale. The largest mean value obtained in the present investigation was 17 units. Since the closest marked

divisions on the scale are 10 units apart, the random error in such readings is so great that extremely large numbers of nuclei would have to be measured to make certain of the true nature of the distributions.

The failure to find a clearly bimodal distribution in any of the samples has led to difficulty in interpretation. In previous studies with microdensitometers, measurements of interphase nuclei, in both animals and plants, have usually formed a clearly bimodal distribution, the values of the two means being in the ratio 1:2 and there being few intermediate values (Deeley et al., 1954; McLeish, 1959). The explanation of the bimodal distribution is presumably that DNA synthesis takes place during interphase, and that it takes place rapidly, so that nuclei carrying an intermediate amount of DNA are rarely found.

It may be that the distributions found in the present investigation are bimodal; with such small values it is impossible to know for certain. However, there is some evidence that the distributions should not be expected to be bimodal. Several investigators (Howard, 1932; Nygaard, Guttes and Rusch, 1960; Ross, personal communication) have reported that mitoses of the nuclei in a plasmodium of Physarum polycephalum are essentially synchronous, even in large plasmodia, and both in liquid culture and on

solid medium. Furthermore, studies to discover the time of DNA synthesis in the plasmodium of P. polycephalum (Nygaard, Guttes and Rusch, 1960) showed that it occurred almost directly after mitosis and occupied a length of time that represented only about 10% of the total length of interphase.

If both these observations apply to the plasmodium used in the present investigation, then for any one sample of interphase nuclei there is a 90% chance that all the nuclei will contain a doubled amount of DNA, and a single normal distribution may be expected, with a mean corresponding to the diploid value if the plasmodium is haploid, and to the tetraploid value if it is diploid. In the remaining 10% of samples, a single normal distribution is also expected, but its mean may correspond to any point between the haploid and diploid or diploid and tetraploid values.

No similar studies have been reported on nuclear division or DNA synthesis in the amoebae. It would be surprising if mitoses were synchronous in a population of amoebae in which some are feeding and some are encysted, and a bimodal distribution should therefore be expected. If mitosis is infrequent at the time of sampling and if DNA synthesis takes place either just before or just after mitosis, it is possible that a distribution approximating

to a single normal may be found for a sample of interphase nuclei. In this case, the mean may correspond to the haploid or diploid value, depending on the time at which DNA synthesis occurs. Because of the mating-type characteristics of the amoebae and the probable position of meiosis in the life-cycle, we may discard the possibility that the amoebae are diploid.

In the first set of measurements (Fig. 16), if both distributions are considered as single normal distributions, the means for the nuclei of amoebae and of plasmodium are 17.1 and 14.2 respectively. Since it has been shown above that the majority of nuclei in the plasmodium are likely to carry a doubled amount of DNA, the value 14.2 may be taken as representing this doubled amount. The value of 17.1 for the amoebae, though significantly different from the value for plasmodium, is certainly not less than it. Since the amoebae must be assumed from other evidence to be haploid, it may be concluded that the nuclei of the plasmodium are also haploid; in other words, contrary to the observations of Ross (1957), nuclear fusion does not occur when the amoebae fuse to form the plasmodium. If this is true, the nuclei of the amoebae in this sample also carry a doubled amount of DNA.

If the distribution for plasmodial nuclei in the first set of measurements is taken to be bimodal, though this is difficult to reconcile with the observations on the synchrony of mitoses, the conclusion about the ploidy of the plasmodium is not changed. The two mean values for the nuclei of plasmodium are 8.5 and 17.0 and the mean value for the nuclei of amoebae is 17.1. These values agree closely with the relative values that would be expected if the amoebae contained haploid nuclei with the doubled amount of DNA and the plasmodium contained haploid nuclei in only some of which the DNA was doubled. However, the results could also be explained by assuming that the plasmodium contained a mixture of diploid and haploid nuclei in none of which the DNA had doubled. This interpretation is not easy to reconcile with the observations on the timing of DNA synthesis. Thus all the possible interpretations of the first set of results disagree with the results of some previous investigator.

The second set of results (Fig. 17) is not more satisfactory. Both distributions appear to be single normal distributions and the mean values for amoebae and plasmodium are 14.0 and 12.1 respectively. This relationship is similar to that found for the first set when both

distributions were considered to be single normal distributions, and the same arguments apply. Since it may be assumed that amoebae are haploid, the conclusion must be either that the plasmodium also contains haploid nuclei, which disagrees with Ross's results (1957), or that it contains diploid nuclei in none of which the DNA has doubled, which disagrees with the expectation derived from the results of Nygaard, Guttes and Rusch (1960).

It is obvious that the results of the measurements of DNA content are very unsatisfactory, but it would probably not be worthwhile to make further measurements except with a densitometer capable of measuring small nuclei more accurately. It may still be hoped that clear, quantitative results could be obtained by this method. The method is particularly desirable since chromosome counts on this species are extremely difficult, the chromosomes being very small and numerous. A count of approximately 90 has been made for the plasmodium by Ross (personal communication).

One final point that may be relevant is the possibility that many of the nuclei in a plasmodium are abnormal. Wilson and Ross found numerous chromosome bridges during meiosis in five species (1955) and in P. polycephalum (Ross, personal communication) and they conclude that these may be caused by crossing-over in translocations, complicated

by polyploidy, and, consequently, that many of the nuclei are abnormal. If inversions or translocations are common in the population, there is no need to propose polyploidy also to explain the occurrence of bridges, and the genetical results found in the present investigation make it appear unlikely that P. polycephalum, at least, is a polyploid. However, it is possible that the nuclei is a plasmodium may vary more than in a cellular organism. Since the nuclei are very densely distributed in the plasmodium and are not separated by any cell walls, it is possible that some carrying abnormal or deficient chromosome complements may survive for some time, being supported by the activity of the rest. Such variations would complicate the results obtained from measurements of DNA content.

Summary.

1. A review of past literature on the nuclear cytology of Myxomycetes is given. It is shown that although meiosis probably occurs just before spore formation in some Myxogastres, including P. polycephalum, and in the maturing spore in Ceratiomyxa, the position of nuclear fusion in the cycle and the nuclear processes involved in plasmodium formation have not yet been determined with certainty in any Myxomycete.
2. Two sets of photometric measurements were made on samples of Feulgen-stained, interphase nuclei from amoebae and plasmodium of P. polycephalum. In each set, the photometric values obtained may be assumed to be directly proportional to the DNA content of the nuclei.
3. The results of these measurements are difficult to interpret. The distributions do not demonstrate conclusively the relative ploidy of nuclei in plasmodium and amoebae and each of the possible interpretations of them disagrees with the results of some previous author using a different method of investigation.

References.

Abe, S. (1934). On the syngamy of some Myxomycetes. Sci. Rep. Tokyo Bunrika Daig., B.1: 193-202.

Ainsworth, G.C. and Bisby, G.R. (1954). A Dictionary of the Fungi. Commonwealth Mycological Inst., 4th edit.

Bary, A. de. (1854). Euglenaartige Gebilde aus Sporen von Trichia rubiformis. Flora, 12: 648.

Burnett, J.H. (1956). The mating systems of fungi, I. New Phytol., 55: 50-90.

Cadman, E.J. (1931). The life history and cytology of Didymium nigripes Fries. Trans. Roy. Soc. Edinb., 57, 93-142.

Camp, W.G. (1937). The fruiting of Physarum polycephalum in relation to nutrition. Amer. J. Bot., 24; 300-303.

Cayley, D.M. (1929). Some observations on Mycetozoa of the genus Didymium. Trans. Brit. Mycol. Soc., 14: 227-248.

Cienkowski, L. (1863). Das Plasmodium. Jahrb. Wiss. Bot., 3: 400-441.

Cohen, A.L. (1939). Nutrition of the Myxomycetes 1. Pure culture and two-membered culture of Myxomycete plasmodia. Bot. Gaz., 101: 243-275.

Cook, W.R.I. and Holt, E.M. (1928). Some observations on the germination of the spores of some species of Mycetozoa. Mycologia, 20: 340-352.

Deeley, E.M. (1955). An integrating microdensitometer for biological cells. J. Sci. Instrum., 32: 263-267.

Deeley, E.M., Richards, B.M., Walker, P.M.B. and Davies, H.G. (1954). Measurements of Feulgen stain during the cell cycle with a new photo-electric scanning device. Exp. Cell. Res., 6: 569-572.

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

Gilbert, F.A. (1929). Factors influencing the germination of myxomycetous spores. Amer. J. Bot., 16: 280-286.

Gilbert, H.C. (1935). Critical events in the life history of Ceratiomyxa. Amer. J. Bot., 22: 52-74.

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

Gray, W.D. (1939). The relation of pH and temperature to the fruiting of Physarum polycephalum.
Amer. J. Bot., 26: 709-714.

Hok, K.A. (1954). Studies of the nutrition of Myxomycete plasmodia.
Amer. J. Bot., 41: 792-799.

Howard, F.L. (1931a). The life history of Physarum polycephal
Amer. J. Bot., 18: 116-133.

Howard, F.L. (1931b). Laboratory cultivation of myxomycete plasmodia.
Amer. J. Bot., 18: 624-628.

Howard, F.L. (1932). Nuclear division in plasmodia of Physarum.
Ann. Bot., Lond., 46: 461-477.

Jahn, E. (1908). Myxomycetenstudien 7. Ceratiomyxa.
Ber. Dtsch. Bot. Ges., 26A: 342-352.

Jahn, E. (1911). Myxomycetenstudien 8. Der Sexualakt.
Ber. Dtsch. Bot. Ges., 29: 231-247.

Jahn, E. (1933). Myxomycetenstudien 15. Somatische und generative Kernteilungen.
Ber. Dtsch. Bot. Ges., 51: 377-385.

Jahn, E. (1936). Myxomycetenstudien 16. Die Kernphasen und die Zahl der Chromosomen. Ber. Dtsch. Bot. Ges., 54: 517-528.

Kambly, P.E. (1939). Some physiological characteristics of myxomycete swarm-cells. Amer. J. Bot., 26: 88-92.

Kerr, N.S. and Sussman, M. (1958). Clonal development of the true slime mould, Didymium nigripes. J. Gen. Microbiol., 19: 173-177.

McLeish, J. (1959). Comparative microphotometric studies of DNA and arginine in plant nuclei. Chromosoma, 10: 686-710.

Martin, G.W. (1949). The Myxomycetes. North Amer. Flora, I,1: 1-190. N.Y. Bot. Gard., New York.

Nygaard, O.F., Guttes, S. and Rusch, H.P. (1960). Nucleic acid metabolism in a slime mould with synchronous mitosis. Biochim. Biophys. Acta, 38: 298-306.

Olive, E.W. (1907a). Evidence on sexual reproduction in the slime moulds. Science, 25: 266-267.

Olive, E.W. (1907b). Cytological studies on Ceratiomyxa. Trans. Wis. Acad. Sci. Arts Lett., 15: 754-773.

Ross, I.K. (1957). Syngamy and plasmodium formation in the Myxogastres.
Amer. J. Bot., 44: 843-850.

Rusch, H.P. (1959). Organization of growth processes.
U.N.E.S.C.O. Symposium on Biological Organization. Pergamon Press.

Schünemann, E. (1930). Untersuchungen über die Sexualität der Myxomyceten.
Planta, 9: 645-672.

Schure, P.S.J. (1949). Nuclear divisions in the fructifications of some Myxomycetes and a method of cultivation to obtain fructification.
Antonie van Leeuwenhoek Jour. Microbiol Serol., 15: 143.

Seifriz, W. and Russell, M.A. (1936). The fruiting of Myxomycetes.
New Phytol., 35: 472-478.

Skupienski, F.-X. (1917). Sur la Sexualité chez les Champignons Myxomycètes.
C.R. Acad. Sci., Paris, 165: 118-121.

Skupienski, F.-X. (1918). Sur la sexualité chez les Champignons Myxomycètes.
C.R. Acad. Sci., Paris, 167: 31-33.

Skupienski, F.-X. (1926). Sur le cycle évolutif chez une espèce de Myxomycète Endosporée, Didymium difforme (Duby).
C.R. Acad. Sci., Paris, 182: 150-152.

Skupienski, F.-X. (1927). Sur le cycle évolutif chez une espèce de Myxomycète Endosporée, Didymium difforme (Duby).
Etude cytologique.
C.R. Acad. Sci., Paris, 184: 1341-1344.

Skupienski, F.-X. (1928). Etude bio-cytologique du Didymium difforme. Première partie.
Acta Soc. Bot. Polon., 5: 225-236.

Smart, R.F. (1937). Influence of certain external factors on spore germination in the Myxomycetes.
Amer. J. Bot., 24: 145-159.

Smith, E.C. (1929). The longevity of myxomycete spores.
Mycologia, 21: 321-323.

Sobels, J.C. and Brugge, H.F.J. van der. (1950). Influence of daylight on the fruiting of two orange-yellow pigmented Myxomycete plasmodia.
Proc. Acad. Sci. Amst., 53: 1610-161

Stosch, H.A. von. (1935). Untersuchungen über die Entwicklungsgeschichte der Myxomyceten. Sexualität und Apogamie bei Didymiaceen.
Planta, 23: 623-656.

Walker, P.M.B. and Richards, B.M. (1957). A method for investigating the stoichiometry of Feulgen stain.
Exp. Cell Res., Suppl. 4: 97-102.

Wilson, C.M. and Ross, I.K. (1955). Meiosis in the Myxomycetes.
Amer. J. Bot., 42: 743-749.

Wilson, M. and Cadman, E.J. (1928). The life history and cytology of Reticularia lycoperdon Bull.
Trans. Roy. Soc. Edinb., 55: 555-608.