CULTURE AND BEHAVIOUR OF MYXOMYCETES

A thesis submitted

by

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ABSTRACT

This thesis reports investigations on two Myxomycetes, studies being carried out on the lethal reaction which follows plasmodial fusion between certain strains of *Physarum polycephalum* and on the culture of *Didymium squamulosum*.

Under appropriate environmental conditions lethal reactions were obtained with great regularity following fusion of plasmodia of strains *i* + 15 or *i* + 32 with *i* + 29 but not with each other. The spatial distribution of killed protoplasm initially led to the view that *i* + 15 and *i* + 32 were to be regarded as "killer" strains and *i* + 29 as "sensitive". More detailed consideration of the consequences of fusing plasmodia of different sizes suggested however that it is more appropriate to regard the lethal reaction as the consequence of the mixture of plasmodial protoplasm of different genotype in certain proportions.

Cultures of *Didymium squamulosum* were established from fruiting bodies collected at two localities in the London area. Para-dichlorobenzene was shown to be

highly toxic to both plasmodia and amoebae and hence its employment for the protection of Myxomycete fruting bodies from mites and insects is inadvisable. The amoeboid phase of D.squamulosum was maintained in two-membered culture and studies on the formation and reversion of the flagellate form were carried out. These indicated that the amoeboid and flagellate states are readily inter-convertible and that it is not correct to regard the flagellate condition as a brief but obligatory phase of the life cycle.

Plasmodia were found to arise within clones of amoebae confirming that the species is homothallic or apogamic. Plasmodia from different clones fused readily with each other, and no lethal reactions were found.

ACKNOWLEDGEMENTS

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I INTRODUCTION

The organisms in the class Myxomycetes are of great interest. They have both amoebae and plasmodia in their life cycles and so offer good opportunities for the study of cellular function and behaviour. Their life cycle is as follows: the spores germinate liberating amoebae which, in wet conditions, generally develop flagella. The amoebae feed on bacteria and multiply. In some myxomycetes amoebae of different mating types fuse with each other to form the plasmodium, in other species plasmodia can arise within clones of amoebae. The plasmodia grow into large multinucleate protoplasmic structures in which there is synchronous nuclear division (fig. 1). They have no cell walls. A plasmodium has a reticulate system of veins in which protoplasm streams rhythmically back and forth. Under appropriate conditions the plasmodium differentiates into fruiting bodies and spores are formed.

More work has been done on Physarum polycephalum than on any other myxomycete. It is the only species in which the plasmodium can readily be grown on a

soluble medium in the absence of other organisms. Daniel and Rusch (1961) reported the culture of P.polycephalum on a partially defined soluble medium for over four years without an appreciable decline in growth rate. The plasmodia of P.polycephalum can be grown routinely in liquid and on agar media which resemble the one formulated by Daniel and Baldwin (1964).

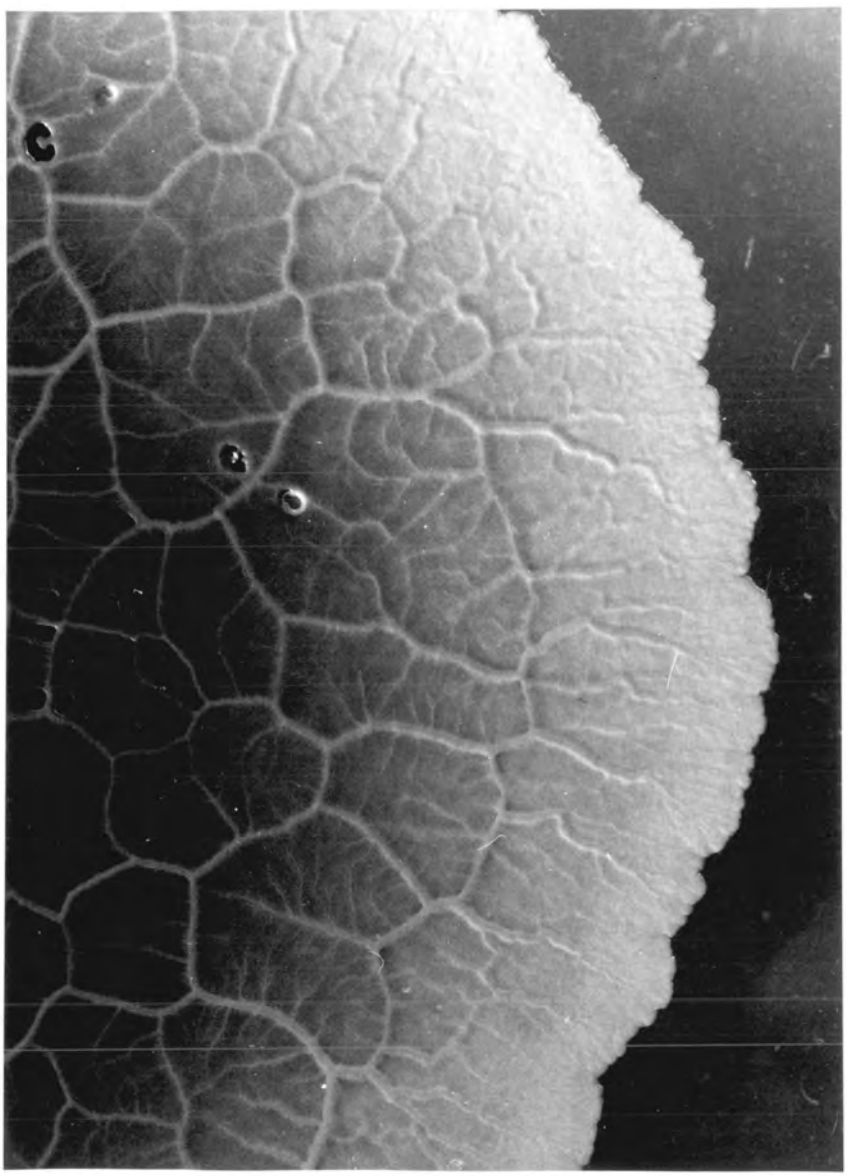
Knowledge of the genetics of P.polycephalum has been extended recently by the studies of Dee (1960, 1962, 1966a, 1966b). Carlile and Dee (1967) showed that the possession of identical alleles at the fusion (f) locus was a pre-requisite for plasmodial fusion. They found that when certain strains of plasmodia fused with each other, the final result was a plasmodium whose origin from two separate plasmodia was no longer evident, but that when other combinations of strains fuse, a lethal reaction follows a few hours later. The lethal reaction varies in its severity - one or both of the fused plasmodia may be affected by it. Sometimes there is total destruction of both plasmodia. Further investigations of the phenomenon have been made by the

present author and are reported in Section III of this thesis.

Although P.polycephalum is an organism of great interest, other myxomycetes may differ considerably from it and deserve investigation. Attempts to culture British species were therefore made; one of the first myxomycetes to be obtained was Didymium squamulosum, the subject of some intriguing reports by Mukherjee and Zabka (1965). Further studies were hence made on this species and are reported below (Section IV).

FIGURE 1

Photograph of a plasmodium of *Physarum polycephalum* on semi-defined agar (X 4½ magnification).



II MATERIALS AND METHODS

(1) Organisms

Physarum polycephalum Schweinitz.

All material employed was ultimately derived from a plasmodium supplied by Dr. H.P. Rusch (McArdle Memorial Laboratory for Cancer Research, University of Wisconsin) in 1957 to Dr. J. Dee (Department of Genetics, University of Leicester). Strains derived from this plasmodium were employed in various genetic studies (Dee, 1960, 1962, 1966a, 1966b; Carlile and Dee, 1967; Poulter and Dee, 1968).

Amoebae strains i, A7015, A7029, A7032 (Poulter and Dee, 1968); in the present study referred to as i, 15, 29 and 32; were provided by Dr. M.J. Carlile. Plasmodia i + 29, i + 15 and i + 32, prepared by mating amoebae, were also provided by Dr. M.J. Carlile.

Didymium squamulosum Fries.

Sporangia used in this study were obtained from two

localities:

(a) Wimbledon Common (7.10.67). The sporangia were on dead stems and leaves.

(b) Epping Forest (27.10.68). The sporangia were on Holly leaves and were kindly provided by Mr. S. Carter.

(2) Media

(a) Plain agar.

2% (w/v) of Oxoid agar No.1 was added to distilled water. The agar suspension was autoclaved at 15lb/in.^{2} (103.4 kN/m^2) for 15 minutes and poured into petri dishes after cooling to about 50°C.

(b) Liver extract agar.

0.045g. of Oxoid liver infusion and 2g. of agar were added to 100ml. of distilled water. The mixture was autoclaved at 15lb/in.^{2} for 15 minutes, cooled to about 50°C, and poured into petri dishes.

(c) Semi-defined medium, modified (Dr. Carlile, personal communication) from Daniel and Baldwin (1964).

Component	Concentration (g./l.of water)
Glucose	10
Peptone, bacteriological (Oxoid)	10
Citric acid. H_2O	3.54
KH_2PO_4	2
$CaCl_2 \cdot 6H_2O$	0.9
$MgSO_4 \cdot 7H_2O$	0.6
Na_2EDTA	0.224
$FeCl_2 \cdot 4H_2O$	0.06
$ZnSO_4 \cdot 7H_2O$	0.034
Thiamin hydrochloride	0.0424
Biotin	0.005
Hematin	0.005

The various components, other than hematin, were dissolved in distilled water, the pH was adjusted to 4.6 with 10% sodium hydroxide, and the medium was sterilized. Hematin solution was prepared by dissolving hemin in 1% sodium hydroxide to give 0.05% solution (e.g. 50mg. hemin to 100ml. 1% NaOH solution). The hematin solution was sterilized, stored at 5 °C, and added aseptically to sterile medium (1ml. hematin solution/100ml. medium) at room temperature immediately prior to inoculation. Autoclaving for 20 minutes at 10lb./in.^{2} (68.9 kN/m^2) was suitable for sterilizing both the hematin and other components.

(d) SM/5 medium.

This medium was devised for the two-membered culture of amoebae and plasmodia of Didymium nigripes (Kerr and Sussman, 1958).

Component	Concentration (g./l.of water)
Glucose	2
Bacteriological peptone (Oxoid)	2
Yeast extract (Oxoid)	0.2
K_2HPO_4	0.2
KH_2PO_4	0.3
$MgSO_4 \cdot 7H_2O$	0.2
Agar	20

The medium was autoclaved at 10lb./in.^{2} for 20 minutes, cooled, and poured into petri dishes.

(e) Corn meal agar.

4.4% (w/v) of Difco corn meal agar was added to distilled water. It was autoclaved at 15lb./in.^{2} for 15 minutes, cooled to about 50 °C, and poured into petri dishes.

(f) Grass extract agar.

The method for making this medium was similar to the one used by Smart (1937).

20g. of dry grass was boiled in 500ml. of distilled

water until 250ml. of the extract remained. This was filtered, placed in flasks, and autoclaved at 15lb./in.^{2} for 20 minutes. For medium preparation the stock solution was diluted with water to give a 10% solution. Agar (2% w/v) was added to the 10% solution and the mixture was autoclaved at 15lb./in.^{2} for 15 minutes. It was cooled and poured into petri dishes.

(3) Culture conditions

(a) Amoebae

Two-membered culture on agar with Escherichia coli was employed for both P.polycephalum and D.squamulosum amoebae. One or two loopfuls of the pre-grown micro-organism (for the amoebae to feed on) were added to 10ml. of sterile distilled water, and mixed. About 0.2ml. of suspension was streaked across an agar plate with a pipette and the agar surface allowed to dry. If a lawn of the micro-organism on agar was required, 1ml. of suspension was pipetted onto the agar and spread over it with a sterile bent glass rod. The plates were incubated at 30 °C for 24 hours before they were inoculated with amoebae.

After the bacteria were consumed, the amoebae encysted and remained viable for long periods.

(b) Plasmodia

Plasmodia of D.squamulosum were cultured as

above, but Bacillus thuringiensis was preferred to E.coli, as indicated later.

Plasmodia of P.polycephalum were grown in pure culture either on agar or in liquid semi-defined media. The plasmodia covered the agar in about 3 to 4 days at 24°C. At 14°C the growth rate of the plasmodia was less than at 24°C permitting the plasmodia to be transferred less often to fresh media. Micro-plasmodia of Physarum polycephalum were cultured in 500ml. Erhlenmeyer flasks containing 50ml. of semi-defined medium. The flasks were shaken at 180 r.p.m. on a rotary shaker (radius of gyration $1\frac{3}{4}$") in darkness at 24°C. Shaking ensured the cultures were aerated. Sclerotia formed in liquid cultures when nutrients were exhausted, and remained viable for some weeks.

(4) Inoculum

(a) Amoebae.

Amoebae and encysted amoebae were transferred to fresh agar media in two ways. (i) They were scraped off the surface of the agar with a wire loop and transferred to a fresh plate where the micro-organism employed for two-membered culture was growing. (ii) They were scraped off the surface of the agar and transferred to 10ml. of sterile distilled water and shaken. A loopful of the micro-organism required for two-membered culture was added to the suspension. 0.2ml. portions of the suspension were pipetted onto agar and spread over the surface of the medium with a sterile bent glass rod. This method was used to obtain plaques of amoebae in a layer of bacteria.

(b) Plasmodia.

(i) Transfer from agar to agar medium.

A plasmodium was transferred to fresh medium when it had covered most of the agar in a petri dish. A block of agar with plasmodium on it was cut out with a sterile scalpel. The block from the edge of the

plasmodial colony and about 1cm.^{2}, was transferred aseptically to a fresh plate. Later the plasmodium migrated off the block and grew on the fresh medium.

(ii) Transfer from agar to liquid medium.

A block of agar with a piece of plasmodium on it was transferred aseptically from an agar culture to liquid semi-defined medium in a 500ml. Erhlenmeyer flask. The inoculum block was carefully inserted into the flask and the block floated on the medium. The flask was kept stationary at room temperature until the plasmodium had grown away from the block over the surface of the liquid. This took from 12 to 24 hours, and then the flask was shaken on a rotary shaker to break the plasmodium up into micro-plasmodia.

(iii) Transfer from liquid to liquid medium.

Micro-plasmodia were transferred from an old liquid culture to a fresh flask of medium by withdrawing the suspension of micro-plasmodia into a sterile pipette, and introducing measured quantities of the suspension into the new medium. Usually transfers were made to give 5%, 2% and 1% inocula

(i.e. 2.5, 1 or 0.5ml. per 50ml. culture) in the fresh media.

(iv) Transfer from liquid to agar medium.

Micro-plasmodia in semi-defined medium were transferred by pipette from flasks to petri dishes containing semi-defined agar. The agar absorbed the liquid, and the micro-plasmodia grew and fused with one another forming macro-plasmodia.

(5) Spore germination technique

Two or three sporangia of the myxomycete were removed from the substratum with forceps and crushed in 10ml. of sterile distilled water. Sometimes a loopful of pre-grown bacteria was added to the spore suspension, and the suspension was mixed thoroughly. Measured portions of suspension were pipetted onto agar media and spread over the surface with a sterile bent glass rod. Plates were incubated at room temperature ($19 \pm 1^{\circ}C$).

III STUDIES ON PHYSARUM POLYCEPHALUM

(1) Form and behaviour of amoebae.

Liver extract agar plates, seeded with E.coli, were inoculated at a few points with encysted amoebae of strain a of P.polycephalum. Cultures were incubated at room temperature in a cupboard. Amoebae were examined 5 days after inoculation of the plates.

At this stage, there were clearly defined plaques each with an advancing front consisting of numerous amoebae (Fig. 2) and some amoebae were ahead of the advancing front. Refactile contractile vacuoles were clearly seen (Fig. 3) though under bright field illumination nuclei were not visible in the amoebae. Numerous granules were present in the amoebae. Behind the advancing front, most amoebae were encysted (Fig. 3).

The diameters of six of the plaques of amoebae were recorded for four consecutive days. Measurements of each plaque were made along a line drawn on the petri dish beneath the plaque (Table 1).

Table 1

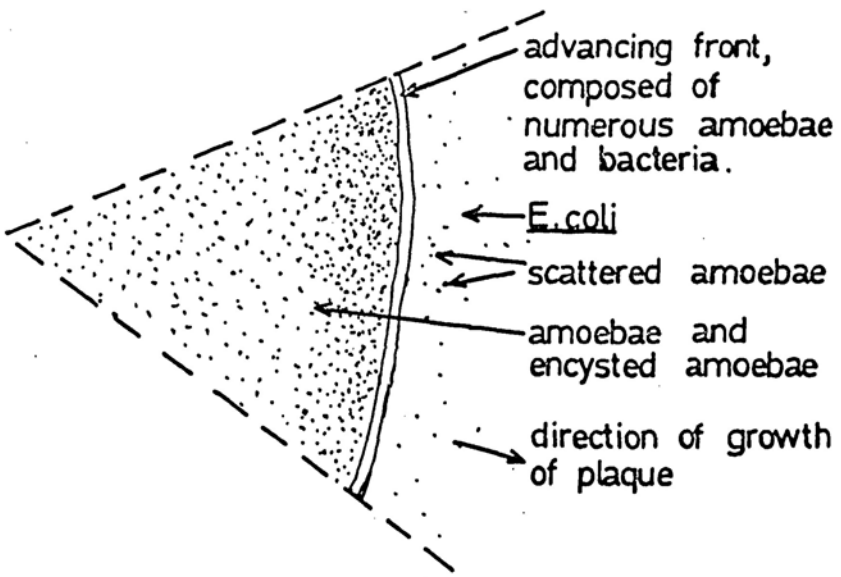
Diameter of amoeba plaques in mm.

		Days after inoculation			
		4	5	6	7
Plaque No.	1	6	8	10	13
	2	6.5	8	10.5	14
	3	9	10	10.5	13
	4	7	10	11.5	13
	5	8	9	11	15
	6	3	6	9	11
Average		6.6	8.5	10.4	13.2
Average increment during preceding 24 hours		-	1.9	1.9	2.8

The diameter of the plaques increased by 2 - 3mm./day.

FIGURE 2

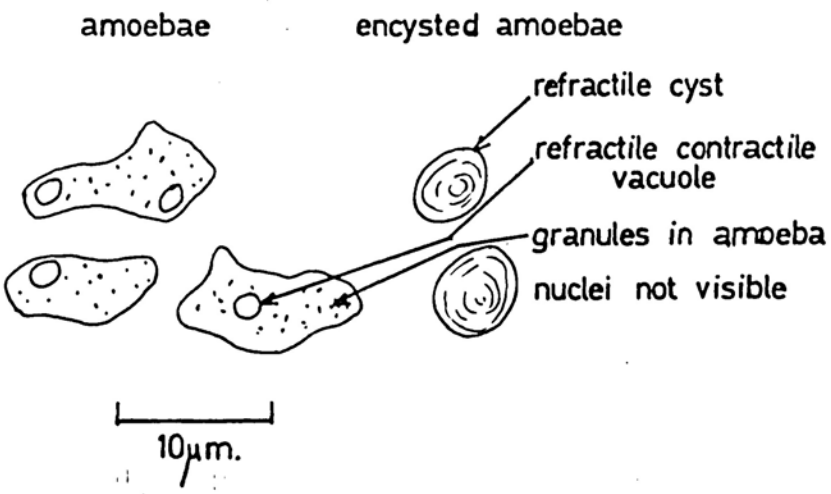
Part of a plaque of amoebae of P. polycephalum in a layer of E. coli on liver extract agar.



x 20 natural size

FIGURE 3

Drawings of amoebae and encysted amoebae of P. polycephalum.



Length of amoebae (µm.)	Diameter of encysted amoebae (µm.)
11	8
16	7
13	8
13	7
11	7
13	7

Average (to nearest µm.)

(2) The fusion reaction between plasmodia

The plasmodia used in this study were *i* + 15 and *i* + 29, each of which had alleles identical with those of the other strain at its fusion locus viz. f^2f^2 (Poulter and Dee, 1968).

When two plasmodia met on semi-defined agar, they remained separate from each other for a length of time varying from a few minutes to half an hour before fusion occurred. Protoplasm passed from one plasmodium into the other, and reversals of flow of protoplasm occurred. The surface area of protoplasm involved in fusion increased, and at the same time the connecting veins enlarged and increased in number.

The process of fusion was observed microscopically, and measurements were made of the times of streaming of protoplasm from one plasmodium into the other from the time of fusion (Fig. 4).

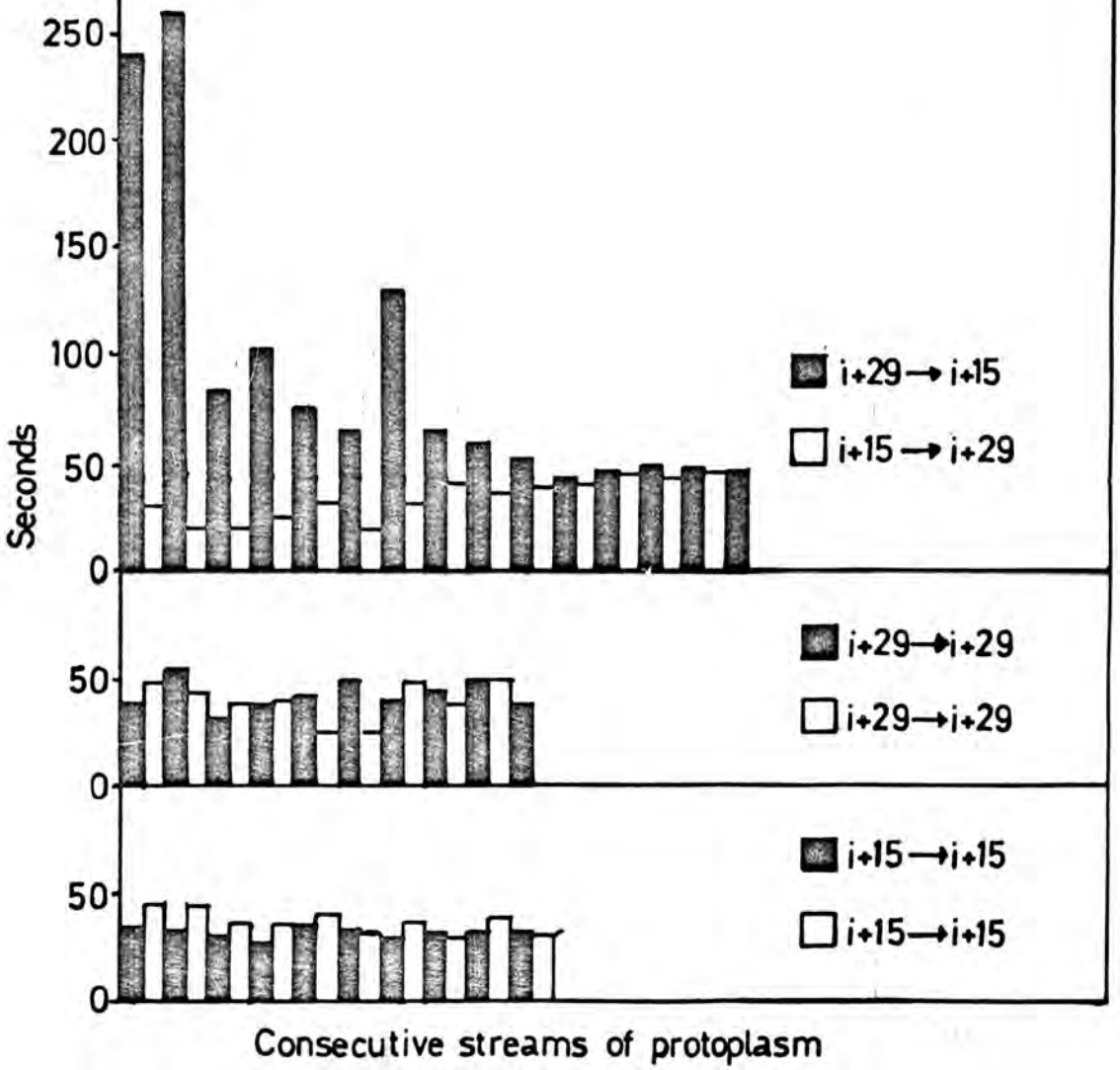
When an *i* + 15 plasmodium fused with another *i* + 15, and an *i* + 29 plasmodium with another *i* + 29, protoplasm flowed for roughly equal times from each plasmodium into the other. However, when an *i* + 29 plasmodium fused with an *i* + 15 plasmodium, protoplasm flowed from *i* + 29 into *i* + 15 for longer periods than in the reverse

direction. The periods of streaming from i + 29 into i + 15 gradually shortened whilst there was a concommitant increase in the length of periods of flow from i + 15 into i + 29. Eventually, protoplasmic flow in both directions became approximately the same. Protoplasm flowed for longer periods from i + 29 into i + 15 when fusion occurred, irrespective of the relative sizes of the two plasmodia.

Similar results were obtained when i + 15 and i + 29 micro-plasmodia fused (for method, see Section III), irrespective of the relative sizes of the fusing micro-plasmodia: protoplasm streamed from i + 29 into i + 15 for longer periods than in the opposite direction. The periods of flow from i + 29 into i + 15 gradually shortened whilst periods of flow from i + 15 into i + 29 increased until protoplasmic flow in both directions became roughly the same.

FIGURE 4

Histograms showing the lengths of time of protoplasmic streaming between two plasmodia.



(3) The lethal reaction between plasmodia.

(a) Description of the lethal reaction between strains i + 15 and i + 29.

When plasmodia i + 29 and i + 15 met, fusion occurred (Section III, 2). A few hours later, protoplasmic streaming gradually ceased over an area of variable extent. The region in which streaming stopped subsequently became sharply delimited from the rest of the plasmodium: the region was readily distinguishable to the naked eye because it was a different tint of yellow from the rest of the plasmodium, and pigment leaked from it into the medium; under the microscope, no movement was seen in it (whether protoplasmic streaming or Brownian movement) and the region became dark and globular in appearance. The non-viability of the protoplasm in which streaming had stopped could be demonstrated by attempting subculture from it (Carlile and Dee, 1967). During the following 24 hours or so the dead area was invaded and apparently digested by surviving plasmodium which was not adversely affected as a result of feeding on the dead protoplasm.

A typical distribution of dead protoplasm is indicated in Fig. 5. It will be noted that most of the

dead protoplasm is in the region occupied by plasmodium i + 29 prior to fusion as well as a little in the site of the i + 15 plasmodium. On a few plates the lethal reaction was so severe that both plasmodia died.

(b) Reaction between strains i + 29 and i + 32 plasmodia.

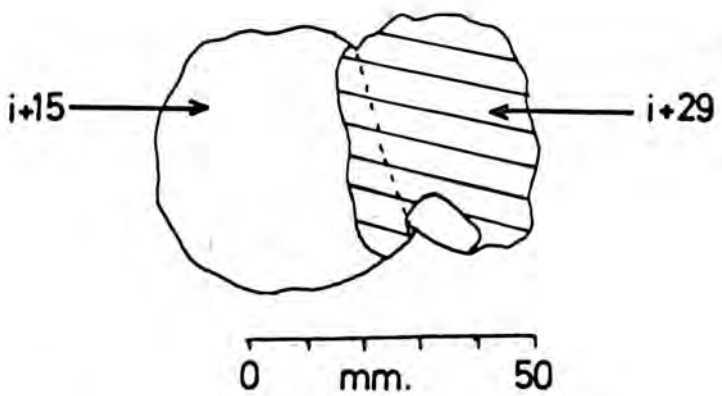
When i + 29 and i + 32 plasmodia fused, killing followed. Dead protoplasm was found in larger areas of the site of plasmodium i + 29 than in the site of i + 32.

(c) Reaction between strains i + 15 and i + 32 plasmodia.

Fusion between this remaining possible combination of strains of plasmodia was not followed by the lethal reaction.

FIGURE 5

Drawing of the typical distribution of dead protoplasm resulting from the lethal reaction between an i+15 and an i+29 plasmodium.



dead protoplasm

line of fusion between plasmodia

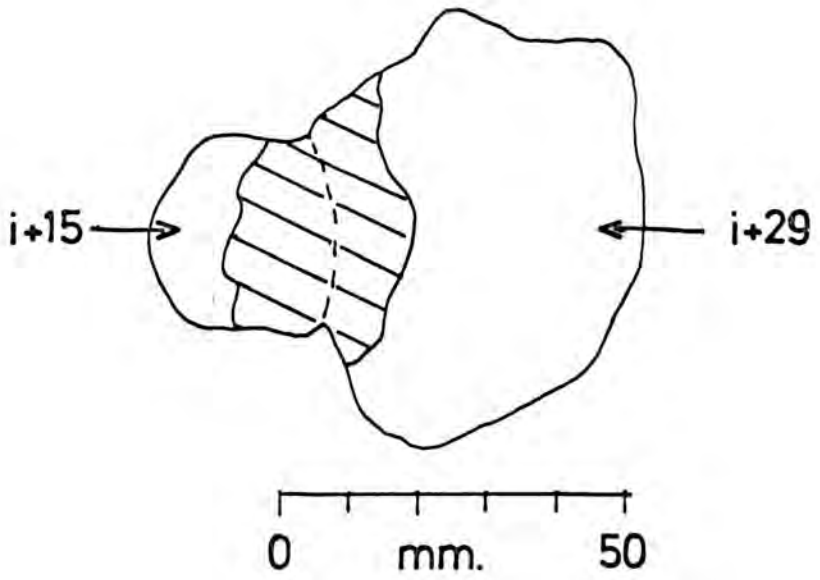
(d) The effect of varying the ratio of the sizes of *i* + 15 and *i* + 29 plasmodia.

(i) Macro-plasmodia on semi-defined agar.

When plasmodia *i* + 15 and *i* + 29 having similar sizes fused with each other, there was a lethal reaction which was mostly confined to the site of plasmodium *i* + 29. The relative size of fusing plasmodia was varied by using inoculum blocks of agar plus plasmodium cut with cork-borers of varying diameters. When a large *i* + 15 plasmodium fused with a smaller *i* + 29, the lethal reaction was confined to the site of the *i* + 29 plasmodium. When an *i* + 15 plasmodium fused with a larger *i* + 29, dead protoplasm resulting from the lethal reaction was distributed roughly equally between the sites of the two fusing plasmodia and surviving protoplasm remained at both sites (Fig. 6).

FIGURE 6

Drawing of the typical distribution of dead protoplasm resulting from the lethal reaction between an i+15 and a larger i+29 plasmodium.



dead protoplasm

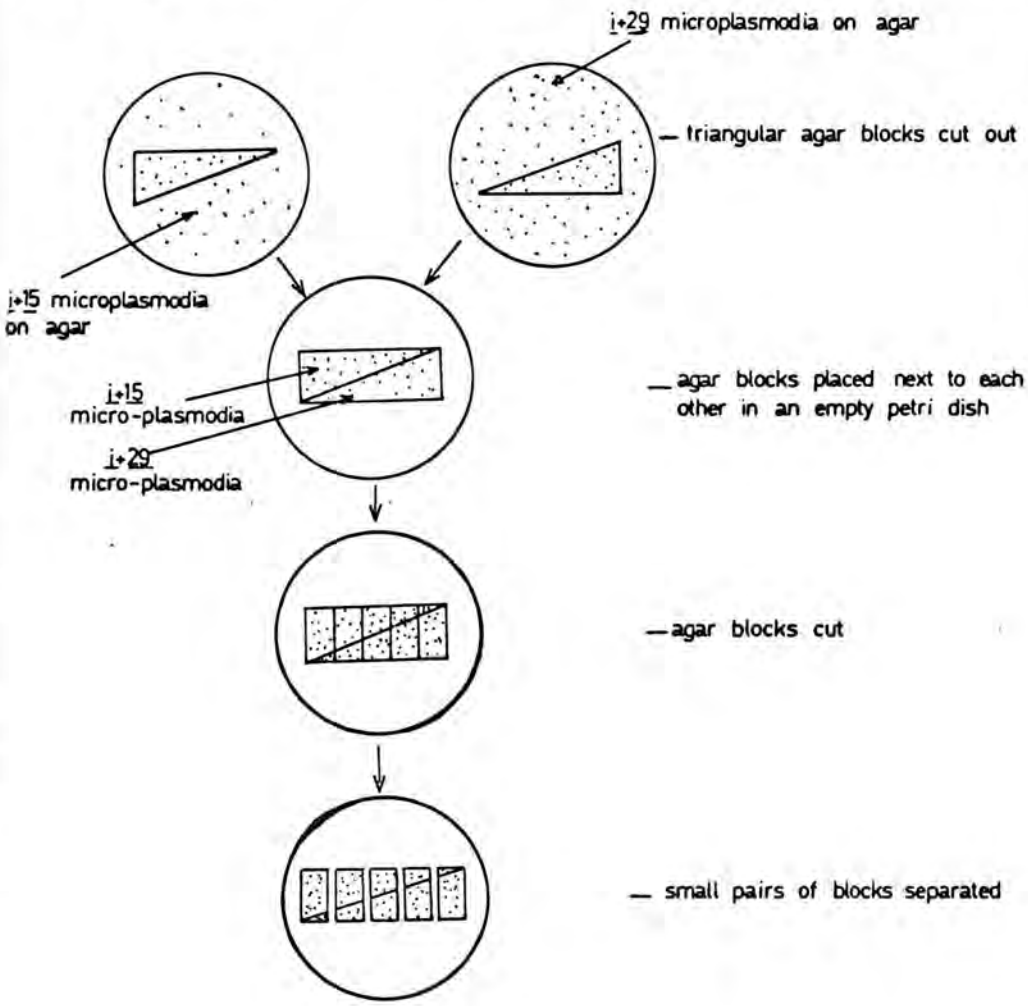
line of fusion between plasmodia

Attempts were made to fuse pairs of accurately measured plasmodia by putting inoculum blocks side by side on semi-defined agar plates. The plasmodia met and fused before they migrated off the blocks, but there were no lethal reactions.

Experiments were set up to determine the size ratios of fusing macro-plasmodia which would give a lethal reaction. Two ml. of a semi-defined liquid medium suspension of i + 15 micro-plasmodia were pipetted onto semi-defined agar plates and spread over the surface with a glass rod. Similarly, i + 29 micro-plasmodia were spread on the surface of other semi-defined agar plates. They were incubated at 24°C for 24 hours during which time most of the micro-plasmodia had fused with one another. Triangular blocks of agar, with plasmodia produced as above on their surfaces, were cut out and put into empty petri dishes so that, in each dish, a block with i + 29 plasmodia on it was adjacent to a block with i + 15 plasmodia. The pairs of blocks were cut to give five small pairs which were separated from one another, and these cultures were incubated at 24°C. The experimental procedure is summarised in Fig. 7.

FIGURE 7

Summary of method to determine which size ratios of fusing macro-plasmodia lead to the lethal reaction.



Fusion usually occurred between plasmodia thus brought together. Subsequent lethal reactions then occurred only on block pairs c (1 out of 8 trials), d (5 out of 7 trials) and e (4 out of 8 trials). On both d and e blocks the quantity of i + 29 plasmodial material exceeded that of i + 15 but accurate calculation of relative amounts of the two strains of plasmodia was not possible with this method because micro-plasmodia on the blocks did not all fuse with one another, and so the surface area of the blocks was not an exact measure of the quantities of plasmodial material involved in fusion between the genetically different plasmodia. However, the experiments showed that i + 29 and i + 15 plasmodia give the lethal reaction when an i + 29 plasmodium fuses with a smaller i + 15 plasmodium, and that the probability of the occurrence of a lethal reaction decreases when the size of the i + 15 plasmodium, relative to that of the i + 29, increases.

(ii) Micro-plasmodia on semi-defined agar.

Slide cultures of micro-plasmodia were set up to find whether the lethal reaction occurred between micro-plasmodia.

The following procedure was carried out with semi-defined liquid cultures of both i + 15 and i + 29 micro-plasmodia. One ml. of micro-plasmodial suspension was pipetted into 9 ml. of sterile distilled water to give a 10^{-1} dilution. This was repeated several times giving 10^{-2}, 10^{-3}, and 10^{-4} dilutions in water. One ml. of the 10^{-3} dilution was pipetted into a petri dish, and 1 ml. of 10^{-4} dilution put into another.

Fifteen ml. portions of semi-defined agar (containing 2.5% agar to enable easy cutting and lifting of agar) were autoclaved. The medium was cooled to 47°C and 0.15 ml. of hematin was added to each tube and shaken. A 15 ml. portion of medium was then added to each petri dish to which the suspension of micro-plasmodia has been added. The cultures were swirled, allowed to set, and put into the 24°C incubator.

After about 4 days, small plasmodia were seen growing inside and on the surface of the agar. Small plasmodia on the surface were selected and cut out with a small piece of semi-defined agar on which they were growing - one plasmodium on each piece of agar. Two pieces of agar, each bearing a small plasmodium, were put side by side on a slide in a humid petri dish.

Control plates contained agar blocks bearing the same plasmodial strain. Development and fusion of the plasmodia were observed under the microscope.

Whether or not a lethal reaction followed the fusion of i + 29 and i + 15 micro-plasmodia depended upon the relative sizes of the fusing plasmodia. No lethal reaction followed fusion of an i + 29 micro-plasmodium with a clearly larger i + 15 micro-plasmodium on the seven occasions this was attempted. There were frequent lethal reactions when micro-plasmodia were approximately equal in size (4 out of 7 trials). Killing always occurred when an i + 29 micro-plasmodium fused with a clearly smaller i + 15 micro-plasmodium.

In contrast to the lethal reaction between macro-plasmodia, micro-plasmodial lethal reactions mostly occurred in both plasmodial sites, and dead material was distributed roughly equally between the two. Generally, killing was total, whether micro-plasmodia of equal or different sizes fused. Occasionally, pieces of plasmodia survived and were found in either of the two sites and sometimes in both.

(e) Effect of varying the concentration of the medium.

A series of ten dilutions of liquid semi-defined

medium was made from x1 to x0.1 strength. Agar was added to give a 2% concentration in each, and the media were poured into petri dishes. The plates were inoculated with blocks of semi-defined agar bearing i + 15 and i + 29 plasmodia. Three replicate plates were prepared for each dilution of medium, and all cultures were incubated at 24°C. Two days later all pairs of plasmodia had fused. The frequencies with which lethal reactions occurred are given below.

Table 2

Frequency of occurrence of lethal reactions on various dilutions of semi-defined medium.

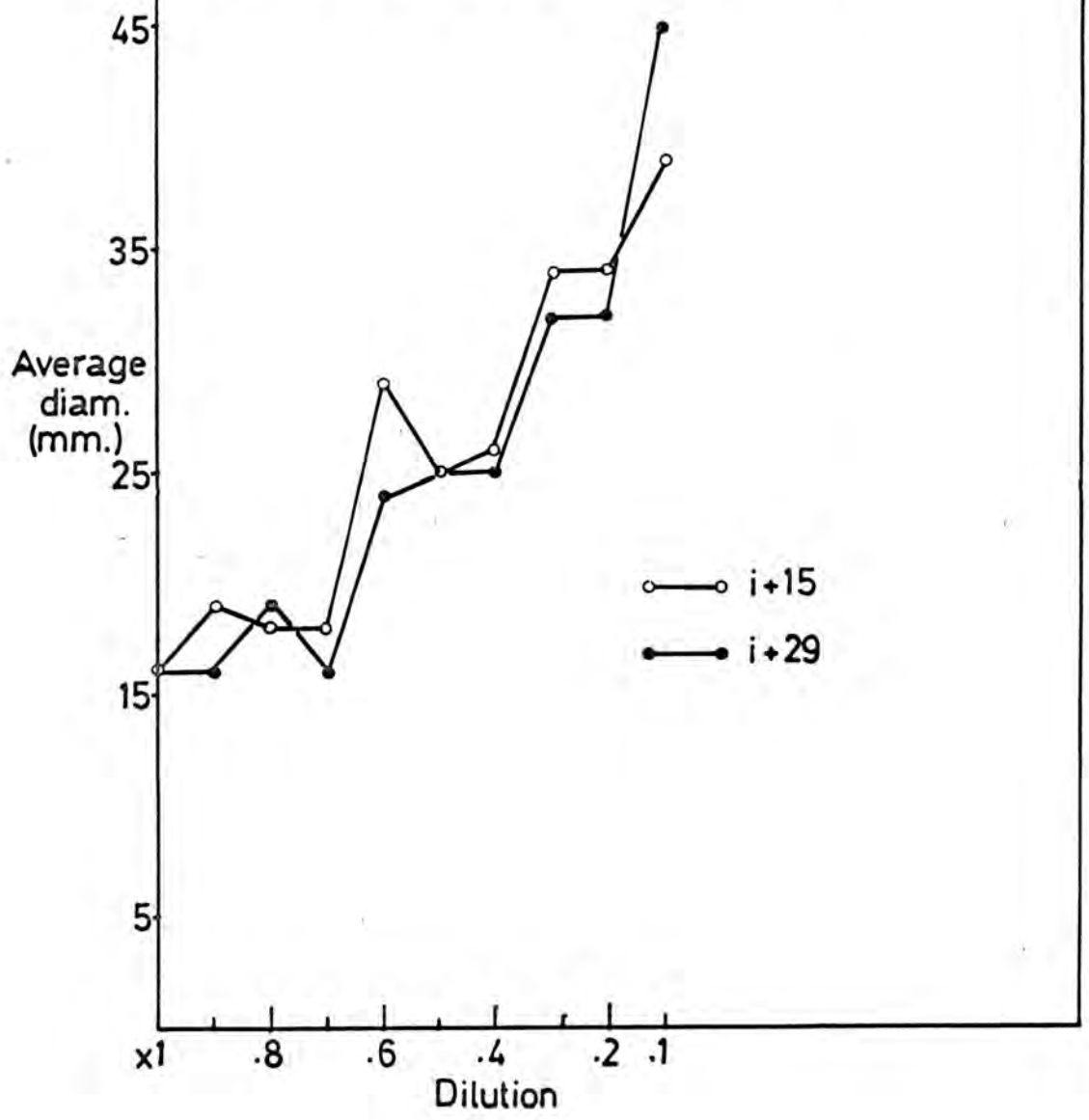
	Dilution of semi-defined medium									
	x1	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
Number of plates out of 3 on which the lethal reaction occurs.	1	2	2	1	2	1	2	2	2	0

The diameter of the plasmodia of both strains increased more rapidly as the medium employed became more dilute (Fig. 8). On 0.1 strength medium plasmodia grew rapidly and their margins broke up into several advancing fronts and the plasmodia migrated across the agar. This was somewhat similar to plasmodial behaviour on plain agar on which the lethal reaction does not occur (Carlile, personal communication).

The lethal reaction was observed on only one of the three control plates (x1 dilution) suggesting there was a defect in the medium, and on none of the 0.1 dilution plates. Little can be drawn from the failures to give the lethal reaction, because there were failures to give lethal reactions on all the media. However, the positive results are meaningful. They indicate that lower concentrations of nutrients than are provided in x1 strength semi-defined medium are sufficient for the production of lethal reactions.

FIGURE 8

Average diameters of i+15 and i+29 plasmodia 24·5 hrs. after inoculation on the range of dilutions of semi-defined agar medium.



(f) Effect of the omission of glucose.

Experiments were set up to see whether glucose was necessary for the lethal reaction to occur. Several plates of semi-defined agar (minus glucose) were each inoculated with agar blocks bearing *i* + 29 and *i* + 15 plasmodia. Plates of semi-defined agar (containing glucose), also inoculated with the two strains of plasmodia, served as controls. The cultures were incubated at 24°C and after 3 days pairs of plasmodia had fused in all of them.

There were lethal reactions on all control plates, but none on the plates lacking glucose. Hence, the omission of glucose from the semi-defined medium leads to the absence of any lethal reaction. The possibility that other sugars can replace glucose in supporting the lethal reaction has not been investigated.

(g) Antigen/antibody tests.

Experiments were carried out to investigate whether or not the lethal reaction was caused by a protein of one strain precipitating a protein of the other.

(i) Slide method.

A solution used by Chambers (1961) for injections

into Physarum was prepared. It contained three salts dissolved in water:- 0.120 M potassium chloride, 0.013 M sodium chloride, and 0.003 M calcium chloride. Oxoid Ionagar No. 2 and sodium azide were added to the solution giving 1% and 0.01% concentrations respectively. The solution was boiled, divided into 5 portions, and the pH of each was adjusted with hydrochloric acid or sodium hydroxide. The pH values of the media were 4.4, 5.2, 5.8, 6.9, and 8.1. The media were then boiled to melt the agar.

Clean microscope slides were placed on a hot plate. A few drops of molten agar medium were put onto each slide and allowed to spread over the glass surface and dry. The agar-coated glass provided a surface on which additional agar would remain more readily than on a clean glass surface. The slides were removed from the hot plate and 5ml. of molten medium were pipetted onto each slide, allowed to spread over the surface, and to harden. Two replicate slides were prepared with each portion of medium. Five circular wells were made in the agar on each slide.

Extracts of 3-day old i + 15 and i + 29 micro-plasmodia were made as follows. Suspensions of micro-

plasmodia in semi-defined liquid medium were centrifuged and the medium decanted. The plasmodia were suspended in Chamber's solution, re-centrifuged, and the liquid was decanted. The plasmodia (a semi-liquid mass) were put into bijou bottles and homogenized with a sonicator. The extracts in the bottles were kept in ice until they were required. The cell extracts were put into the wells in the agar on slides (Fig. 9) with drawn-out Pasteur pipettes.

The slides, which served as controls in the experiment, each had one well in the agar with one of the two kinds of extract in each well.

Each slide was put into a petri dish containing wet filter paper which maintained a humid atmosphere in the dish. The dishes were incubated at 24°C.

No lines of precipitation appeared between the wells. The amount of sedimentation in the wells was similar in test and control slides. Hence, this method revealed no protein-precipitation reaction.

(ii) Test-tube precipitation method.

Extracts of *i* + 15 and *i* + 29 micro-plasmodia were prepared in the same way as in (i). Chamber's solution, at pH 7.8 and containing 1.5% of agar, was

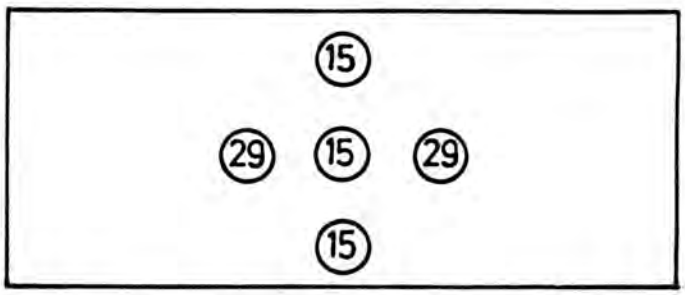
heated to boiling and kept in a hot water bath. Three replicates of each of the tubes illustrated in Fig. 10 were prepared.

Fifty ml. of the hot agar were transferred to a beaker and cooled to 45°C. One ml. of i + 29 extract was added to it and the suspension stirred gently. Five ml. of this suspension were put into tube 1 and 5ml. into tube 2, and allowed to set. One hundred ml. of the hot agar were cooled to 45°C and some was pipetted into tube 1 to give a layer 2mm. thick. Five ml. were added to tube 2, and 5ml. to tube 3. The agar was allowed to harden. Another portion of hot agar was cooled to 45°C and 1ml. of i + 15 extract added to it and stirred gently. Five ml. of this suspension were added to tube 1 and tube 3 and allowed to set. All tubes were plugged with cotton wool and kept at room temperature.

There was no precipitation in any tube. Hence, no protein-precipitation reaction was indicated.

FIGURE 9

Pattern of wells cut in agar on slide for antigen / antibody test, showing in which wells plasmodial extracts were placed.

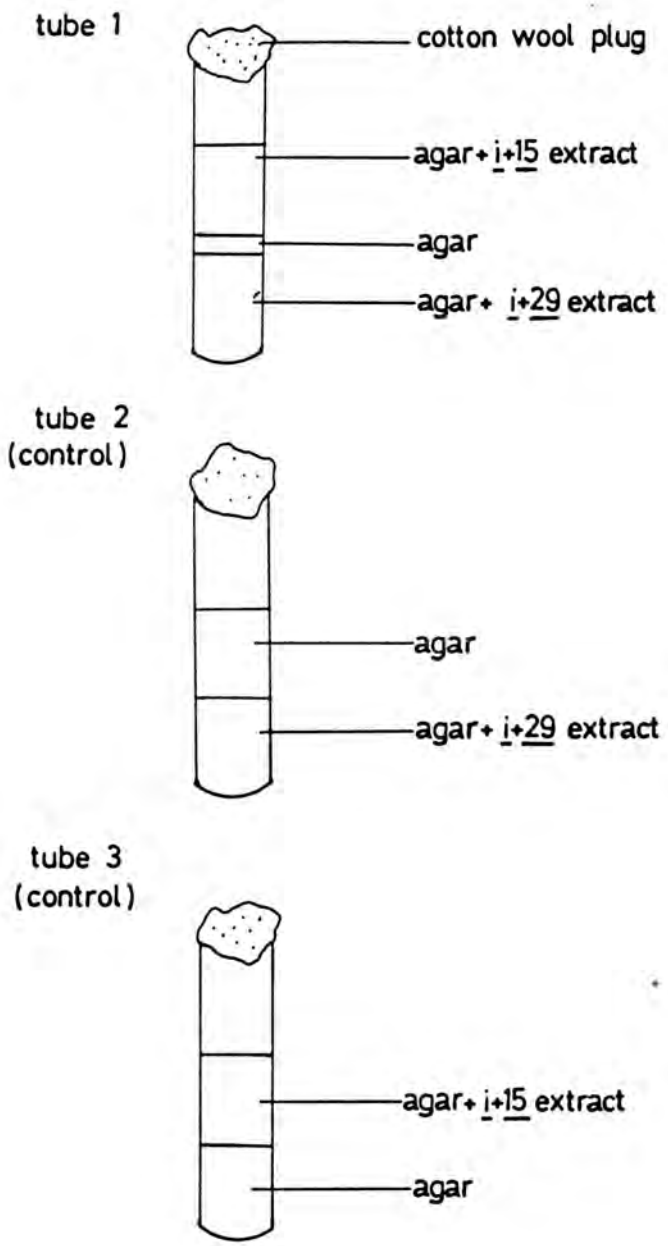


15 = i+15 plasmodial extract

29 = i+29 plasmodial extract

FIGURE 10

Antigen/antibody test:- test tube precipitation method.



IV STUDIES ON DIDYMIUM SQUAMULOSUM

(1) Collection of sporangia

Sporangia, together with some substratum, were placed in suitable containers such as hinge-lidded tins. Bead-headed pins were used to fix the specimen to a layer of cork at the base of the container. The sporangia were transported to the laboratory inside the tins which were then opened for about 24 hours to allow the specimens to dry to prevent overgrowth by moulds. The tins were then closed and kept in a laboratory cupboard.

Para-dichlorobenzene crystals should not be placed near the collection of sporangia to kill mites because it is likely that the vapour kills the spores. Experiments showing the effects of p-dichlorobenzene on D.squamulosum are recorded in sections (5)f, and (9)e.

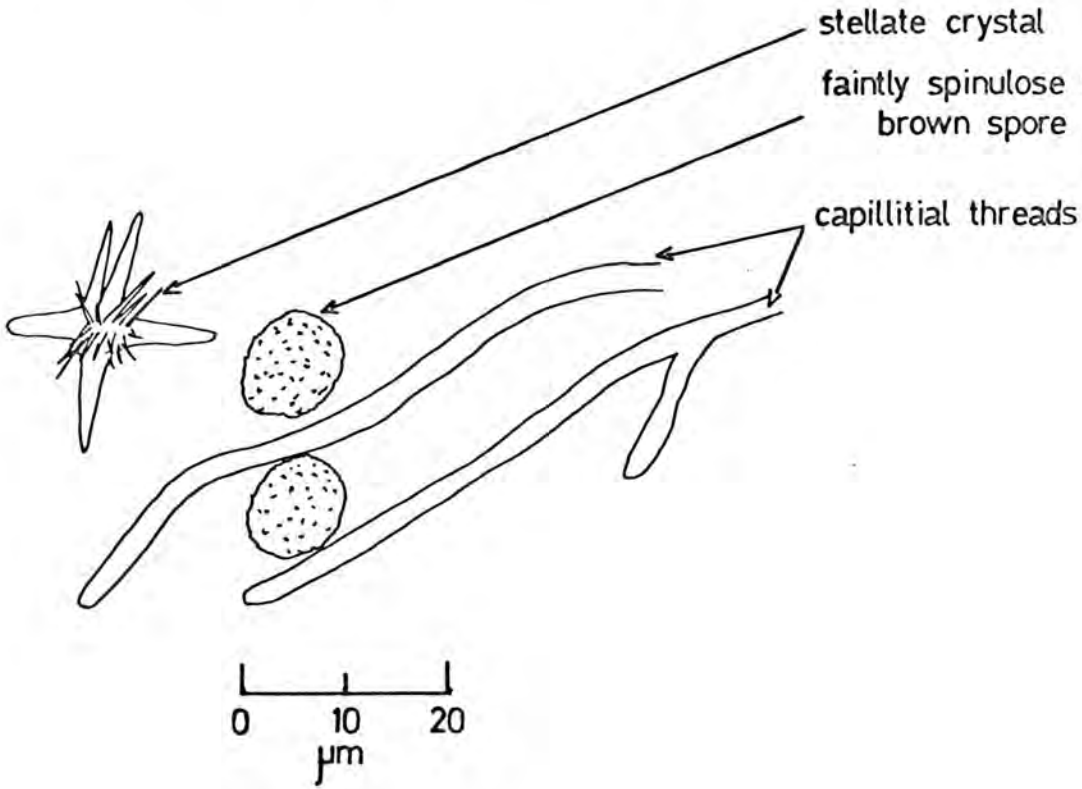
(2) Identification of *Didymium squamulosum* sporangia

Sporangia were examined microscopically (Fig.11), and their features compared with those given for *D.squamulosum* Fries by Lister (1925). Sporangia were mounted on slides using the following method: a crushed sporangium was placed on a slide in a drop of absolute alcohol, followed by a drop of 3% potassium hydroxide. After blotting the slide slightly, one drop of weak glycerine was added to the preparation and a cover slip was placed over the sample. The cover slip was sealed with nail polish.

The organism had the following morphological characteristics which agreed with those Lister gave for *D.squamulosum*. The plasmodium was colourless (Section IV, 9). The sporangia were stalked and contained stellate crystals. The capillitium was composed of slender threads and had no vesicles. The spores were violet-brown, about 9.5μm in diameter, and faintly spinulose. These observations verify the preliminary identification by Mr. B. Ing and Mr. S. Carter of the sporangia as those of *D.squamulosum*.

FIGURE 11

Drawings of spores and other inclusions of a sporangium of D. squamulosum.



(3) Spore germination

Germination was attempted in the presence of Escherichia coli on the following media:-

(a) Plain agar.

A spore/bacterial suspension was made in 10ml. of sterile distilled water by crushing two or three sporangia in the water with a sterile forceps. Then a loopful of E.coli grown on nutrient agar was added, and the suspension stirred. Two ml. portions of this suspension were pipetted onto plates of 2% plain agar and spread over the agar surface with a sterile bent glass rod.

Germination of spores occurred after at least four days' incubation at room temperature. Two or three days later plaques of amoebae were clearly visible in the bacterial layer.

(b) Corn meal agar.

The medium, similar to the one used by Mukherjee and Zabka (1965) for the germination of D.squamulosum spores, gave spore germination after about one week's incubation at room temperature. The sporangia germinated only on those parts of the agar which had dried out. No amoebae were seen on the agar if a

continuous layer of water was present.

(c) Grass extract agar.

When grass extract agar plates were inoculated with a suspension of spores, without deliberate inoculation of bacteria as well, no germination occurred. When plates were inoculated with a spore/*E.coli* suspension, germination occurred within one day of sowing.

A germination experiment employing pre-dried (50°C for 20 minutes) and undried grass extract agar plates indicated germination was more rapid on the pre-dried plates than on the undried plates. The dried plates absorbed water more quickly than the undried ones and so the former had dry patches on the agar, where the amoebae were very numerous, the day after inoculation; in contrast, it was several days before dry patches broke up the continuous film of water on the undried plates. Germination was a day later on the undried plates, on which there was still a continuous film of water over the agar. Spores will therefore germinate when they are totally immersed in water on grass extract agar, in contrast to the results for corn meal agar.

The best method found for germinating D.squamulosum spores was to sow 2ml. of spore/E.coli suspension on pre-dried plates of grass extract agar. This gave good germination of spores within one day at room temperature. The percentage germination of spores on each plate was not ascertained. The time for germination compares favourably with the results of Smart (1937) who found it took 6 days for D.squamulosum spores to germinate in hay infusion at 25°C. However, Smart's method differed considerably from the one used here; for instance, he suspended spores in dilute hay extract.

(4) Culture of amoebae.

(a) Cloning of amoebae.

Amoebae were routinely cultured on liver extract agar in association with Escherichia coli which served as food for the amoebae. Clones of amoebae were obtained as follows:- a suspension of encysted amoebae and E.coli was made in sterile distilled water and 0.2ml. portions of this suspension were pipetted onto liver extract agar plates. The suspensions were spread over the agar surfaces with a sterile bent glass rod. About 6 days later, several well-defined amoeba plaques developed in the bacterial layer. It was assumed that each plaque arose from a single amoeba cyst. Amoebae from well-separated plaques were transferred to liver extract agar plates seeded with E.coli.

The cloned amoebae quickly multiplied on the plates, and thereafter were routinely cultured with E.coli on liver extract agar. Four clones were obtained from the Wimbledon spores and nine from the Epping spores.

(b) De-contamination of amoebae.

All amoeba clones were freed from contaminating micro-organisms by successive transfers from the margin

of an amoeba colony to a fresh plate of liver extract agar with a surface layer of pre-grown E.coli on it. As the amoebae multiplied and spread outwards on the plate, contaminating micro-organisms were left behind. Such transfers were made several times before the amoebae were considered to be in 2-membered culture with E.coli.

(c) Maintenance.

Encysted amoebae were maintained on liver extract agar slopes in Universal containers (Mackie and McCartney, 1956). Encysted amoebae were suspended in a 5ml. portion of sterile distilled water and a loopful of pre-grown E.coli cells was added. Portions (0.5ml.) of the suspension were spread over the slopes of liver extract agar. After about 2 weeks numerous cysts were present on the agar, the original cysts having released amoebae which fed on the bacteria and multiplied, encysting when the food supply ran out. The bottle caps were then screwed tightly shut to ensure the humidity was high within the bottles. The cysts remained viable for some months under these conditions, and some cultures were preserved this way. Sterile medicinal paraffin oil was then added to other cultures until

the slopes, with the cysts on them, were under the oil, and the bottle caps were screwed on. This also proved to be a satisfactory way of preserving cultures. All cultures were stored at room temperature.

(5) Observations and experiments on the amoeboid phase.

(a) Form and behaviour of amoebae.

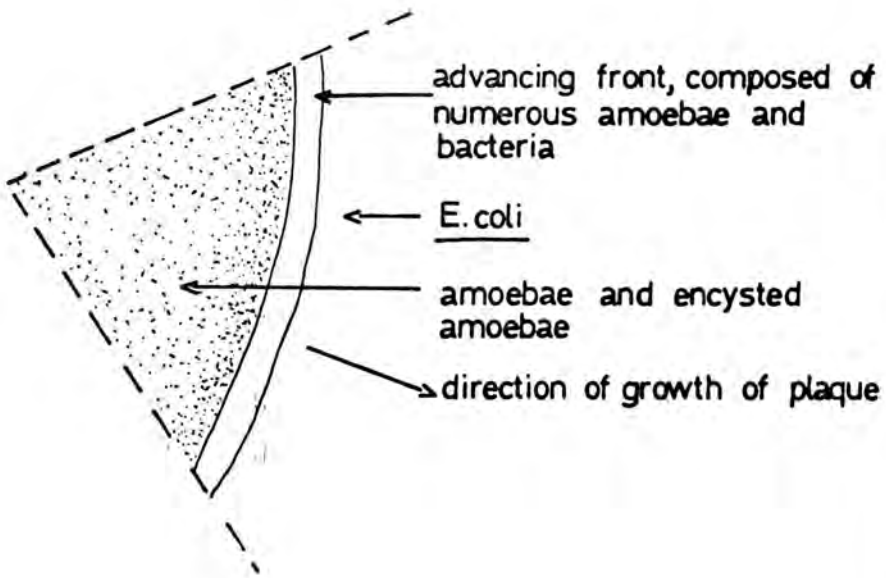
Liver extract agar plates, seeded with E.coli, were inoculated at a few points with encysted amoebae of D.squamulosum. The plates were incubated at room temperature (19 ± 1°C) in a cupboard. The following observations were made 5 days after inoculation of the plates.

At this stage there was clearly defined plaque of amoebae on each plate (Fig. 12). The advancing front of the plaque consisted of numerous amoebae, none of which were found ahead of the front. Refractile contractile vacuoles were clearly seen in the amoebae (Fig. 13). The nuclei were large and each contained a nucleolus, easily discernible from the rest of the nucleus as it transmitted less light than the rest of the nucleus. Numerous granules were present in the amoebae. Behind the advancing front, most amoebae were encysted (Fig. 13).

The diameters of five of the plaques of amoebae were recorded for four consecutive days. Measurements of each plaque were made along a line drawn on the petri dish beneath the plaque (Table 3).

FIGURE 12

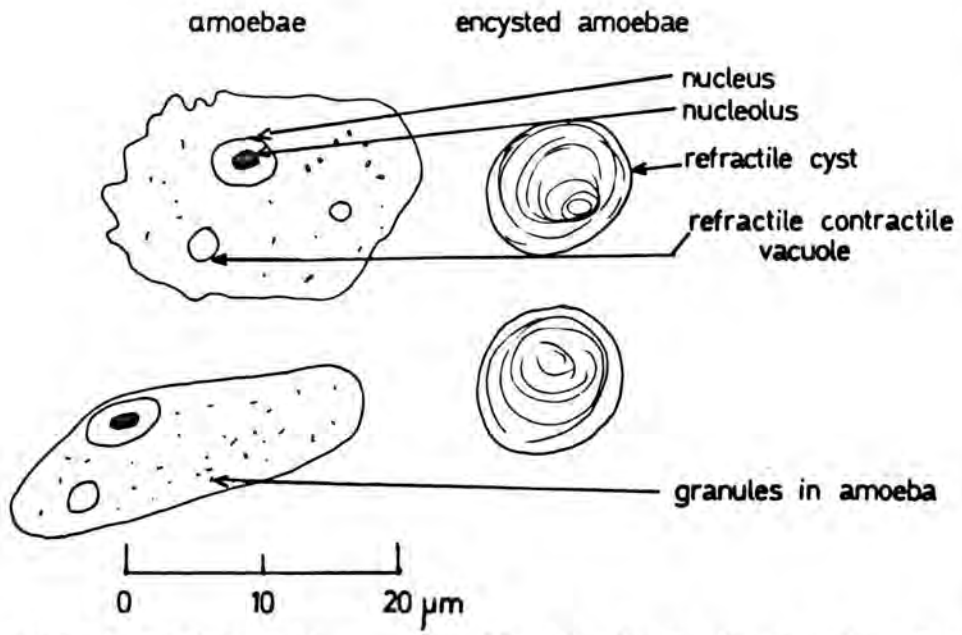
Part of a plaque of amoebae of D. squamulosum in a layer of E.coli on liver extract agar.



x 20 natural size

FIGURE 13

Drawings of amoebae and encysted amoebae of D. squamulosum.



Length of amoebae (µm)	Diameter of encysted amoebae (µm)
20	11
25	10
25	11
18	11
22	11

Average (to nearest µm)

Table 3

Diameter (mm.) of amoebae plaques

		Days after inoculation			
		4	5	6	7
Plaque No.	1	9	13	18	22.5
	2	11	15	20	25
	3	9	12	16.5	21.5
	4	10	15	17.5	22
	5	14	17	22.5	28
Average		10.6	14.4	18.9	23.8
Increment during preceding 24 hours		-	3.8	4.5	4.9

The diameter of the plaques increased by about 4 - 5 mm. per day.

The effects of different temperatures on the rate of growth of amoebae were investigated. Amoebae were grown at various temperatures in two-membered culture on a streak of E.coli on liver extract agar. Later, measurements were made of the distance of advancing fronts of amoebae from the points of their inoculation on the plates (Fig. 14).

Amoebae multiplied and migrated rapidly at room temperature (ca. 19 °C) and at 25 °C, but less rapidly at 15 °C. At 30 °C there was barely any multiplication and migration of amoebae.

(b) Encystment.

Encystment has been observed under a variety of conditions. Amoebae come to a halt, round off and secrete a thick wall (Fig. 13).

As mentioned earlier (Section (5)a) cysts form in amoeba plaques on E.coli on liver extract agar immediately behind the advancing front, and increase in frequency as the distance from the advancing front increases. This is correlated with a concomitant decrease in the

frequency of active amoebae. It would seem likely that amoebae encyst because bacteria are no longer available in sufficient quantity to support growth and activity of amoebae, i.e. that encystment is being caused by starvation.

Encystment has also been observed on plates that were drying out, but on which bacteria were still unconsumed. It is likely, therefore, that encystment was caused by desiccation.

Encystment occurred on plates incubated at 27°C. This is near the maximum temperature of the amoebae, and so encystment could have been caused by high temperature. The possibility that the amoebae were slightly desiccated, and that this contributed to their encystment, is not, however, excluded.

Amoebae which encysted as a result of the environmental factors discussed above retained their viability.

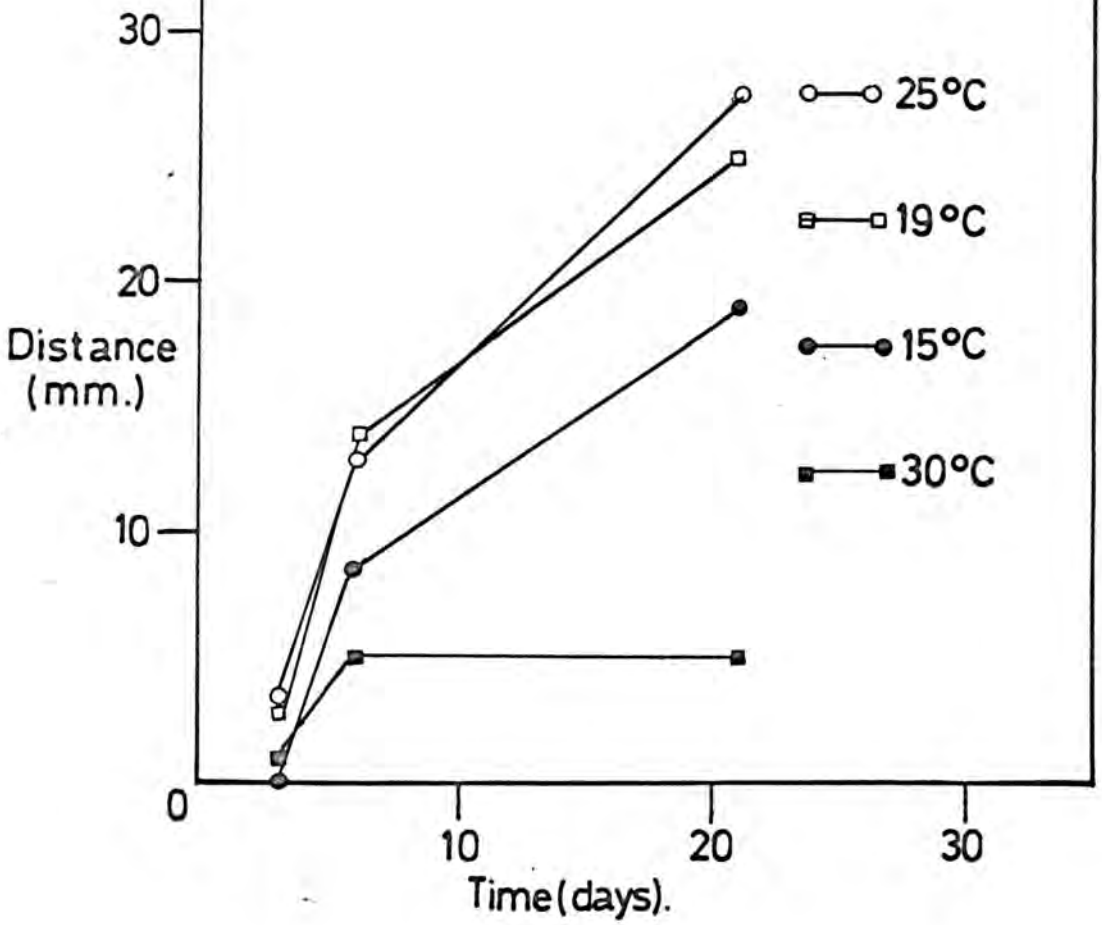
Para-amino benzoic acid has been used to improve plasmodium formation in Physarum polycephalum (Dee, 1966a). This compound, which in excessive amounts can be toxic in some organisms, was used in an attempt to improve plasmodium initiation in D.squamulosum amoebae (Section III (7) d). After 5 days' incubation on the p-amino benzoic acid - incorporated medium, the amoebae

of all Wimbledon clones had multiplied and were growing along the bacterial streaks, but after 9 days' incubation there was almost complete encystment of amoebae on all plates. A few days later encystment was complete. Neither food shortage nor, it was clear, humidity was responsible for encystment.

Hence, encystment of amoebae of D.squamulosum can be brought about by starvation, desiccation, p-amino benzoic acid, and possibly by high temperatures. It is reasonable to conclude, therefore, that encystment occurs in response to unfavourable environmental conditions.

FIGURE 14

Distance travelled by amoebae of D.squamulosum along E.coli streaks, at various temperatures.



(c) Excystment

This is the escape of the amoeba from the cyst wall. When encysted amoebae were transferred to a fresh liver extract agar plate with a pre-grown layer of E.coli, excystment occurred within 24 hours. Hence, excystment of the amoebae occurs in the presence of bacteria.

(d) Flagellates

Several plates of cloned amoebae growing on E.coli on liver extract agar were flooded with sterile distilled water so that a continuous layer of water covered the agar. The amoebae had grown on the plates for from 8 to 28 days. Many flagellates appeared within 1½ hours of adding water to the plates and were very numerous after 6 hours. At 20 hours little change was observed, but by 28 hours their numbers had greatly decreased, and after 48 hours there were very few flagellates. At 72 hours there were no flagellates but there were many round amoebae on the agar surface beneath the water. Hence, amoebae form flagellates soon after immersion in water, and after about 20 hours disappear, apparently through reversion to the amoeboid form rather than death (see over).

Further plates of amoebae of D.squamulosum were flooded with 10ml. of sterile distilled water. Three hours later, flagellates were present in the water above the agar. Drops of water containing flagellates were transferred with Pasteur pipettes to fresh plates of liver extract agar which had been spread 24 hours before with E.coli and incubated at 37°C. About 3 drops of suspension were placed at a point on the agar where bacteria were growing. These plates were examined under the microscope and the presence of flagellates and absence of amoebae on them was checked. They were incubated at room temperature. Six days later there were large plaques of amoebae in the bacterial layers, indicating that the flagellates had reverted to amoebae and the latter had multiplied. These plates were flooded in the usual way and flagellates were present in the water on all of them 4 hours later.

Hence, amoebae of D.squamulosum can, under appropriate conditions, change into the flagellate state, revert once more to the amoeboid condition and (after further multiplication) give rise yet again to flagellates.

(e) Interactions with other organisms

(i) Bacteria

Escherichia coli. As already mentioned, E.coli is ingested by amoebae of D.squamulosum and can support the amoebae in a healthy condition indefinitely.

Bacillus thuringiensis. This bacterium was employed in the two-membered culture of plasmodia of D.squamulosum; its usefulness as a food source for the amoebae of D.squamulosum was therefore investigated. Plates of grass extract agar with pre-grown B.thuringiensis on them, and plates sown with E.coli were inoculated with cloned amoebae. Amoebae feeding on B.thuringiensis multiplied much more slowly than those feeding on E.coli. In addition, the former amoebae did not produce such a sharply defined advancing front as they did with E.coli, and they advanced only 2.5mm. from the inoculation point in 11 days at room temperature compared with 15mm. from the inoculation spot on the E.coli plates. Hence, B.thuringiensis is a less satisfactory food source than E.coli for amoebae of D.squamulosum.

(ii) Fungi

Unidentified Yeast. On some spore germination plates (corn meal agar and liver extract agar), amoebae multiplied in the vicinity of a yeast. Several amoebae

had ingested yeast cells. The yeast was therefore isolated on malt agar plates containing aureomycin (2ml. of 0.2% aureomycin hydrochloride in water added to 100ml. of agar medium) to supress bacteria, and attempts were made to grow the amoebae in association with the yeast (both live and autoclaved), washed suspensions of which were streaked onto liver extract agar plates. The amoebae multiplied very slowly and encysted without migrating along the yeast streak. Hence, this yeast is not suitable for two-membered culture with the amoebae, even though it may be a useful component of the food of the amoebae in crude culture.

Trichoderma viride. This was the fungus most frequently obtained when attempting to germinate spores of D.squamulosum on plain agar plates with E.coli. An interesting observation made on germination plates contaminated with T.viride was that numerous hyphal branches of the fungus grew towards the encysted amoebae. It is possible that the hyphae were responding by tropism to nutrients released from the encysted amoebae.

T.viride was isolated from a germination plate by transfer of spores to plates of potato-carrot agar,

and was subsequently maintained on potato-carrot agar plates.

Encysted amoebae and T.viride spores were placed a short distance from each other on the surface of plain agar. Three days later, amoebae lying near the hyphae had more numerous and much larger active contractile vacuoles than other amoebae situated further from the hyphae. Furthermore the amoebae near the hyphae were larger and more rounded than usual. It is likely that metabolic products of the fungus act on the cell membrane of the amoeba and that this results in an increase in the volume of water entering the amoeba from the environment. As a result, the turgor pressure in the amoeba would rise, making the amoeba large and rounded, and contractile vacuoles would increase in size and number to deal with the large volume of water entering the cell.

(iii) Amoebae of Physarum polycephalum

Encysted amoebae of D.squamulosum and P.polycephalum were inoculated at separate points on plates of liver extract agar, previously seeded with E.coli, and the plates were incubated at room temperature. The plaques of amoebae of Didymium and Physarum grew and

came into contact with each other. At this stage the junction between the two plaques was visible to the naked eye as a distinct ridge (Fig. 15a). The two types of encysted amoeba had intermingled in this region and had no apparent ill-effect on each other. A day later the shape of the ridge had altered (Fig.15b) producing a concavity in the D.squamulosum plaque. This was probably due to the faster growth rate of the plaque of Didymium compared with that of Physarum (Section III (1) and IV (5) a). It can be shown by geometric construction that such a concavity would be expected (Fig. 16). The shape of the fusion ridge gradually changed during the subsequent days; the angle of the ridge with respect to the plaque boundary of D.squamulosum decreased further, i.e. the concavity in the Didymium plaque, produced by the ridge, was accentuated.

The region where the two plaques were in contact was examined. The two kinds of amoeba were co-existing in this area, and amoebae of both species were encysting. Amoebae of Didymium were often seen to encircle encysted amoebae of Physarum, but the active amoebae soon moved away from the cysts. Hence, there was no indication

that one species of amoeba was being ingested by the other.

(f) Effect of p-dichlorobenzene

Spores of D.squamulosum stored in the presence of p-dichlorobenzene failed to germinate, therefore its effect on amoebae of D.squamulosum was investigated. Amoebae growing on plates of liver extract agar with E.coli were employed. The plates were inverted and a few crystals of p-dichlorobenzene placed on the inner surface of the lids, which were then replaced. The plates were incubated upside down at room temperature. The amoebae died within 18 hours, and ghost-like remains of amoebae were left on the agar.

FIGURE 15

Drawings showing the coalescence of amoeba plaques of P. polycephalum and D. squamulosum.

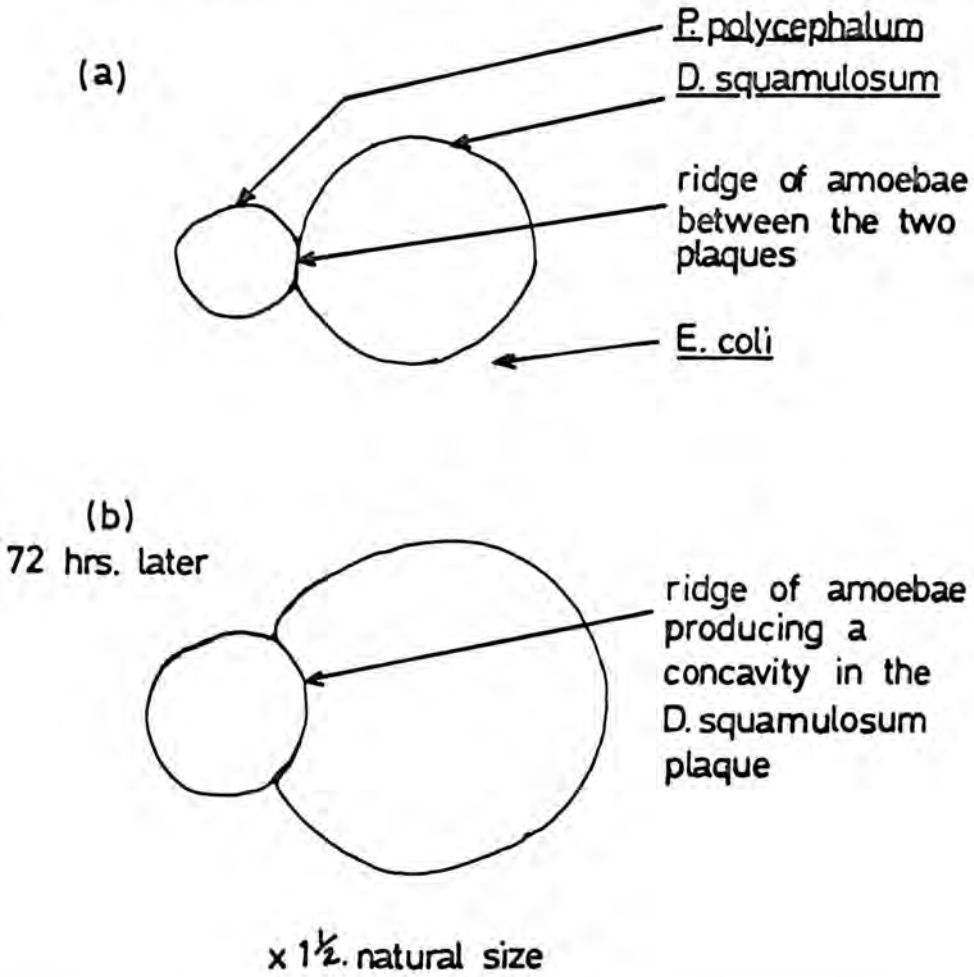
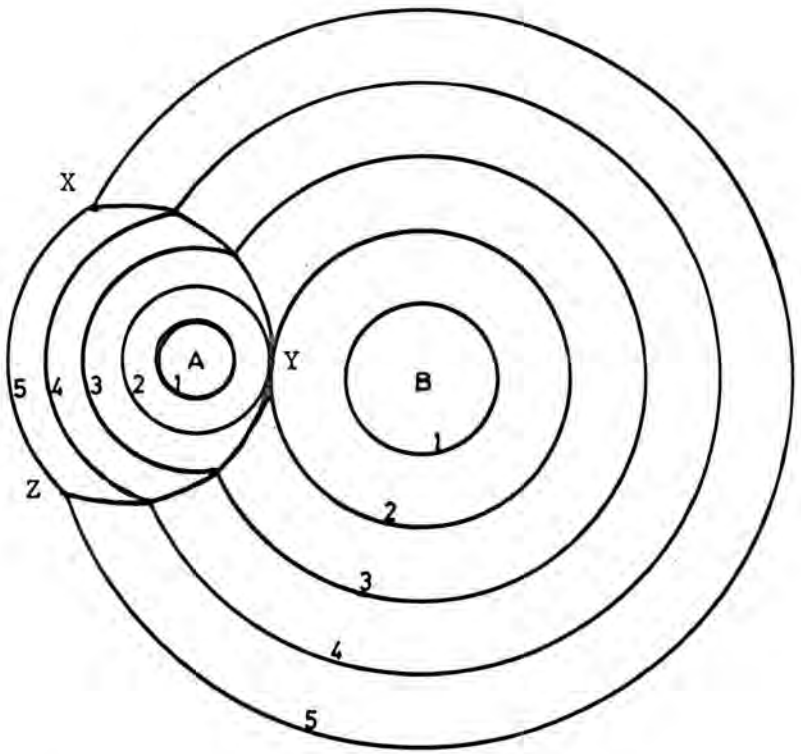


FIGURE 16

Geometric construction representing the coalescence of two colonies of amoebae, one colony enlarging at twice the rate of the other.



The diagram represents two enlarging colonies of amoebae in a layer of E.coli; 'A' a colony of P.polycephalum amoebae, and 'B' a colony of D.squamulosum. 'B' increases in a diameter at twice the rate of 'A'. The concentric circles indicate the margins of the colonies after a succession of time intervals of equal length. Circles 1 A and B represent the margins of the two colonies before meeting, circles 2 A and B the margins at the time of meeting, and partial circles 3 - 5 A and B the margins after further colony growth. It will be seen that the boundaries between the two colonies (X, Y, Z) obtained by joining up the points at which the circles would have intersected, closely resembles the boundary actually observed (Fig. 15).

(6) Plasmodium initiation

(a) The media listed below were used for plasmodium production from amoebae. The media were inoculated with encysted amoebae and bacteria in suspension, and incubated at room temperature.

Corn meal agar

A range of concentrations of the medium (0.44 - 4.4% corn meal agar in distilled water) was tested. Plasmodia formed on 4.4% corn meal agar in about 2 weeks, but at no other concentration. Plasmodia formed also on plates of 4.4% corn meal agar made with tap water, and on 4.4% corn meal agar to which 1×10^{-3}M $CaCl_2$ was added. Calcium chloride was included because Kerr (1961) showed that its addition induced plasmodium formation in Didymium nigripes. The time to plasmodium formation and the health of the plasmodia on all these media were similar.

Liver extract agar

A range of dilutions of liver extract agar were made with distilled water (0.045 - 0.0045g. liver extract in 100ml. water) and inoculated with a suspension of amoebae and bacteria. Plasmodia arose on the liver extract agar plates over the whole range of dilutions.

Wimbledon clone 2 amoebae formed plasmodia a little more rapidly than the other three Wimbledon clones. It produced plasmodia in about 12 days from inoculation of amoebae. Clones 1, 3 and 4 produced plasmodia about 2 days later. Epping amoebae formed plasmodia much sooner than Wimbledon amoebae. When the former were inoculated onto plates of liver extract agar seeded with E.coli, plasmodia were seen on all the plates after 6 days.

An experiment was carried out to investigate the importance of pH of liver extract agar for plasmodium initiation. A range of agar media was prepared having pH values from 4.1 to 7.8. All media were streaked with E.coli suspension and cloned Wimbledon amoebae were inoculated at the ends of the E.coli streaks. Four weeks later the amoebae had multiplied and advanced along the bacterial streaks on all plates, but plasmodia had formed on only some of the plates, as indicated in Table 4.

Table 4

Production of plasmodia within 4 weeks on pairs of liver extract agar plates at a range of hydrogen ion concentrations.

pH of liver extract agar				
4.1	4.9	5.9	7.1	7.8
+(+)	++	+-	(+)(+)	(+)-

\+ = Healthy plasmodium formed.

(+) = Plasmodium formed, having irregular veins; therefore it is unhealthy.

\- = Plasmodium not formed.

The results indicate plasmodium formation can occur over a wide pH range on liver extract agar, but suggest that pH 5 may be optimal.

Plain agar

Plates of plain agar (pH 7) with pregrown lawns of E.coli on them, were inoculated with 6 clones of Epping amoebae. All clones gave rise to plasmodia on this medium within 3 weeks.

Acidified plain agar (pH 4.6) plates, with pre-grown E.coli, were inoculated with 5 clones of Epping amoebae. All failed to produce plasmodia on this medium. Wimbledon amoebae, clone 2, which readily yields plasmodia on liver

extract agar, did not yield any plasmodia on plain agar at any pH (4.1 to 7.6) tested.

(b) Some environmental conditions were varied, and the effects on plasmodium initiation were examined:-

(i) Dee (1966) has used p-aminobenzoic acid to improve plasmodium initiation in Physarum polycephalum. Plain agar plates, with p-aminobenzoic acid incorporated into them (54.8mg./l. of medium) were streaked with E.coli suspension and Wimbledon amoebae were inoculated at the ends of the streaks. Plasmodium initiation did not occur; instead, the amoebae encysted prematurely (Section III, (6)b.).

(ii) Amoebae were inoculated at the ends of pre-grown E.coli streaks on liver extract agar. Pairs of plates were incubated at 4 different temperatures. The results obtained after 3 weeks are given below.

Table 5

Production of plasmodia within 3 weeks on pairs of liver extract agar plates at a range of temperatures.

Temperature, °C			
15	19 ± 1	25	30
++	++	(+)(+)	--

\+ = Large healthy plasmodium formed.
(+) = Small unhealthy plasmodium.
\- = No plasmodium formed.

Plasmodia formed from amoebae over the temperature range 15 - 25 °C. At 30 °C, plasmodia did not form.

(c) Origin of plasmodia in cloned cultures.

All clones of Wimbledon amoebae produced plasmodia on a variety of media. Similarly, all Epping clones gave plasmodia. Therefore, plasmodium initiation occurred in each strain of amoebae, each strain being composed of genetically identical cells which may or may not differ genetically from other strains. Consequently, D.squamulosum was shown to be a homothallic or apogamic myxomycete, as had been suggested by the work of Mukherjee and Zabka (1965).

(7) Morphology of the plasmodium

The plasmodium of D.squamulosum was a white or transparent macroscopic structure. The veins varied in size and appearance with conditions of culture, and in general were rather less distinct than the veins in the plasmodia of P.polycephalum.

(8) Culture of Plasmodia

Liver extract agar with E.coli

As liver extract agar with E.coli had been successfully employed for two-membered culture of amoebae, the same procedure was attempted with plasmodia. Plasmodia were inoculated at the ends of E.coli streaks on the agar and migrated along the bacterial streaks for distances up to 30mm. before they retracted or migrated across the agar. The results were not entirely satisfactory, and so the value of other media and bacteria was investigated. The plasmodial inoculum for each experiment was initially obtained from liver extract agar plates with E.coli.

Liver extract agar with Aerobacter aerogenes

Plasmodia soon left the bacterial streaks and either retracted to the inoculum blocks or migrated across the agar. Hence, the results were not satisfactory.

Plain agar with E.coli

Plasmodia migrated along the bacterial streaks up to distances of 16mm. before retracting to the inoculum block or veering off the streak and migrating across the agar. A slightly acid medium (pH 5) gave the longest

migration along the streak. However, these conditions of culture were not entirely satisfactory.

Plain agar with A.aerogenes

Plasmodia migrated only a few millimetres along the bacterial streaks. Then they left the bacteria and either migrated across the agar or, more usually, retracted to the inoculum blocks.

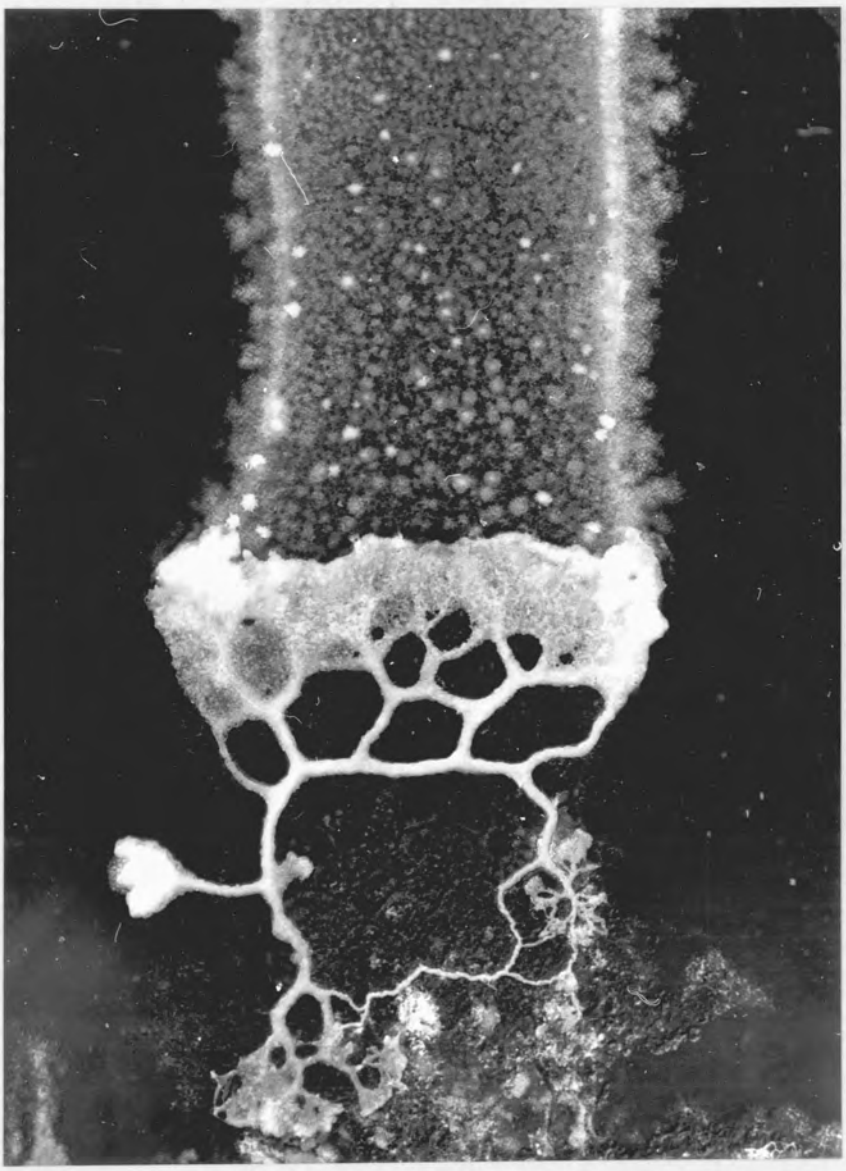
Grass extract agar with E.coli and other bacteria

Plasmodia left their inoculum blocks on all plates and migrated, often for several days, along the E.coli streaks. Eventually they veered away from the streaks and migrated across the agar. Considerable growth of the plasmodia was observed under these conditions. This was the most satisfactory result so far obtained, and so a range of bacteria, streaked onto grass extract agar were presented to the plasmodia. Citrobacter freundii, Aerobacter aeruginosa, and Proteus mirabilis were unsatisfactory - the plasmodia did not touch the bacterial streaks. The plasmodia migrated rapidly along the margins of streaks of Proteus mirabilis and Pseudomonas aeruginosa, but did not penetrate the bacterial colony.

The most satisfactory results were obtained with grass extract agar, streaked with Bacillus thuringiensis suspension. Epping plasmodia migrated along the pre-grown bacterial streaks and grew (Fig. 17). Plasmodia were transferred repeatedly to fresh plates of grass extract agar plus B.thuringiensis, and they migrated along the streaks. Repeated attempts to grow Wimbledon plasmodia under these conditions failed; the plasmodia left the streaks and died on the agar a few days later. Epping plasmodia were maintained as follows: they were grown on plates of grass extract agar with B.thuringiensis at 6 °C. The plasmodia were healthy but their migration and growth rates were slow, enabling the frequency of subculture to be reduced to once every 2 - 3 weeks.

FIGURE 17

Photograph of a plasmodium of *Didymium squamulosum* on a streak of *Bacillus thuringiensis* on grass extract agar (X 5 magnification).



(9) Observations and experiments on plasmodia of D.squamulosum

(a) Migration

Plasmodia were incubated at various temperatures on streaks of B.thuringiensis on grass extract agar, and the rates of migration of plasmodia along the streaks were measured (Fig. 18). The plasmodia kept at 24°C migrated most quickly. At 27°C the plasmodia failed to leave their inoculum blocks. At 6°C plasmodial migration was slow and roughly linear. At this temperature, plasmodia did not form fan-like advancing fronts, instead they were irregularly lobed and occasionally retracted off the B.thuringiensis streak.

(b) The effect of the addition of sugar on morphology and growth of plasmodia.

Grass extract agar plates containing 1% glucose and streaked with B.thuringiensis were inoculated with D.squamulosum plasmodia. The plasmodia, which migrated along the bacterial streaks, were compared with those on media containing no glucose.

Plasmodia on the medium lacking glucose gouged the agar, but plasmodia on glucose-incorporated medium did not. The veins of the plasmodia on agar lacking glucose

were more distinct than those of plasmodia supplied with glucose; the location of the veins in the latter was indicated only by protoplasmic movement, whereas the veins were clearly demarcated in plasmodia on agar lacking glucose. Plasmodia supplied with glucose were almost overwhelmed by bacteria 18 hours after leaving their inoculum blocks, but they had recovered 24 hours later, then migrated over the bacteria and grew slowly. Plasmodia on agar lacking glucose grew more rapidly, probably because the bacteria did not reach sufficient numbers to overwhelm the plasmodia.

Similarly, when D.squamulosum plasmodia fed on E.coli on liver extract agar, the agar was gouged, but when 1% glucose was incorporated into the medium the plasmodia ceased gouging the agar. The plasmodia on liver extract agar containing glucose had regular outlines and straight veins, unlike the other plasmodia (on medium lacking glucose) which had less regular outlines and convoluted veins.

(c) Fusion experiments

Experiments were carried out to see whether plasmodia derived from cloned amoebae would or would not fuse with one another. This was done by inoculating two pieces

of plasmodia, one at each end of a pre-grown B. thuringiensis streak, on grass extract agar. Plates were incubated at room temperature and results were obtained after a few days when plasmodia had grown along the streaks and met. The results of the experiments show that all strains of plasmodia tested fused with one another. There were no killing reactions following fusion.

Table 6

Results of fusion experiments

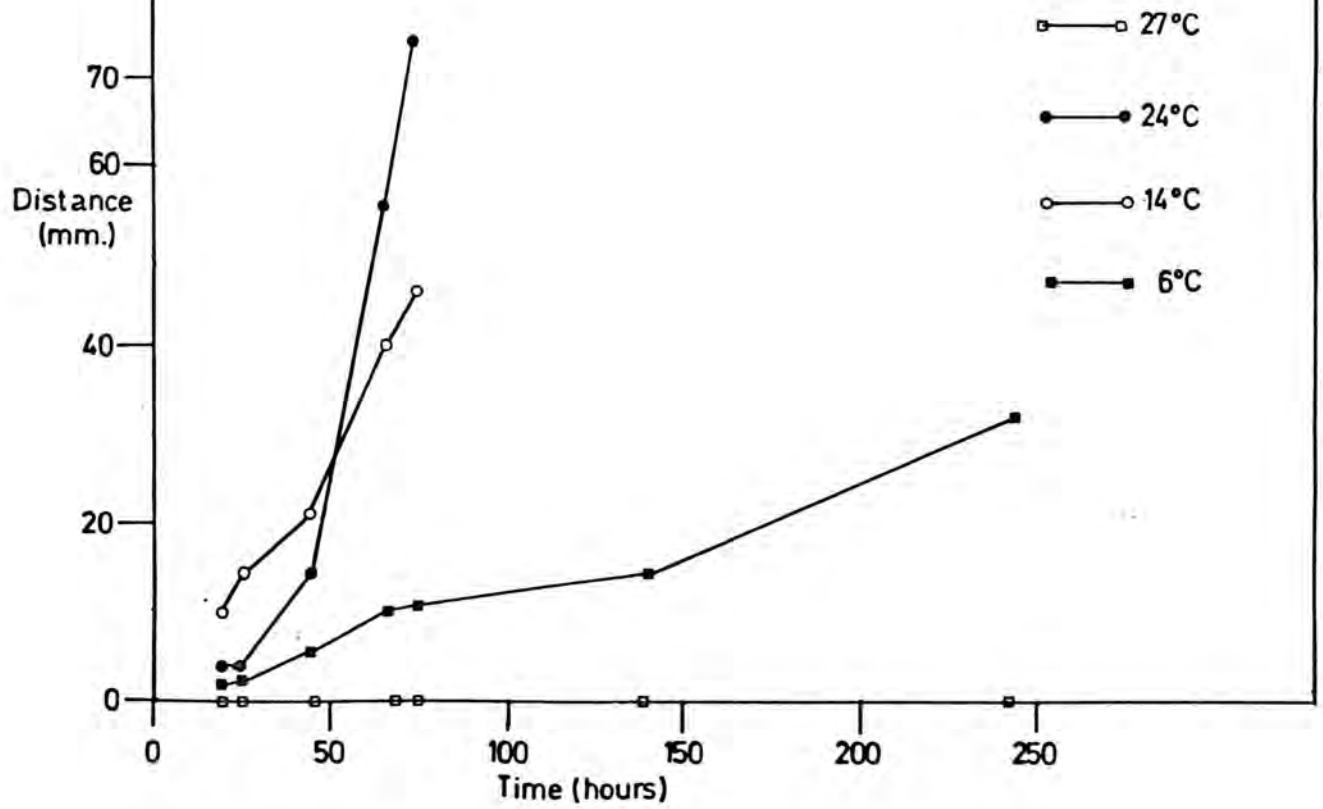
CLONE	1	2	4	6	8
8	o	+	o	+	+
6	o	+	o	+	
4	+	+	+		
2	+	+			
1	+				

\+ = fusion
\- = no fusion
o = not attempted

The numbers in the table refer to the clone of Epping amoebae from which plasmodia were derived.

FIGURE 18

Graph of the distance migrated by plasmodia of D. squamulosum with time, at various temperatures.



(d) Interaction with other organisms

Bacteria.

Results of plasmodial interaction with some bacteria are given in Section IV (B).

An alga: Chlorella vulgaris

Plain agar plates were streaked with suspensions of washed and unwashed Chlorella cells, inoculated with D.squamulosum plasmodia, and incubated at room temperature. Some plates were kept in the light and others in darkness. There was no apparent difference in behaviour of the plasmodia with these different treatments.

Many Chlorella cells were ingested by plasmodia: some cells were in vacuoles moving with the streaming protoplasm in the veins or lying motionless in gel protoplasm. There were some vacuoles containing two or more algal cells. Plasmodia left the algal streaks after about one day and migrated over the agar. During this time the numbers of algal cells within the plasmodia dropped until none were seen, and algal cells were left behind on the plasmodial tracks in the agar. It was not clear whether the algal cells were ingested and later

digested, or whether after they were ingested the algal cells were eliminated unharmed from the plasmodium.

(e) Effect of para-dichlorobenzene

A plasmodium, grown for ten days on B.thuringiensis on grass extract agar at room temperature, was exposed to the fumes of p-dichlorobenzene by the same method used for amoebae (Section III (6) (f)). The plasmodium was observed continuously as soon as it was exposed to p-dichlorobenzene vapour.

Streaming stopped in all veins after 40 seconds' exposure. After one minute's exposure, the lid and crystals were removed and so the plasmodium was exposed to the air. Five minutes later there was some resumption of streaming in a few small areas of the plasmodium though most of it appeared to be dead. After twenty minutes' exposure to fresh air there were only the few surviving parts seen earlier. Most of the plasmodium was dead.

Experiments with plasmodia of different ages confirmed the results given above.

V DISCUSSION

(1) Physarum polycephalum

(a) Introductory remarks

Carlile and Dee (1967) gave a preliminary account of fusion between plasmodia of P.polycephalum and of a lethal reaction which follows some fusions. They showed that there is fusion between pairs of plasmodia which are of different strains but of identical genotype at the fusion (f) locus. With some combinations of strains, the final result of fusion was a healthy plasmodium of which the origin from two separate plasmodia was no longer evident, but with other combinations of strains fusion was followed by a lethal reaction which varied in its severity. Further investigations on the lethal reaction are reported earlier in the present thesis. These will now be discussed further, in relation to the biological significance and the mechanism of the reaction.

(b) Cultural conditions

The lethal reaction was discovered in plasmodia growing on semi-defined agar (Carlile and Dee, 1967), but does not occur in plasmodia on plain agar (Dr. M.J. Carlile, personal communication). As reported earlier

the present author has shown that no lethal reaction occurs on dilute semi-defined medium (x0.1 of the original concentration), nor on semi-defined agar lacking glucose.

Hence, the lethal reaction occurs in a limited range of nutritional conditions and so its occurence in nature is uncertain. If it does occur its function may be to act as a barrier preventing vegetative fusions of genetically unlike plasmodia.

(c) Evidence from other strains of plasmodia

i + 29, i + 15 and i + 32 plasmodia are identical with respect to their genotype at the fusion locus, and as a result they all fuse with one another. The result of fusion between andi + 15 and an i + 29 plasmodium was a lethal reaction which generally affected the i + 29 site more severely than the i + 15. Similarly, a fusion between an i + 32 and an i + 29 plasmodium resulted in a lethal reaction which affected the i + 29 site more severely than the i + 32. When an i + 32 plasmodium fused with an i + 15 no lethal reaction followed. Hence, i + 32 and i + 15 plasmodia resemble each other closely and differ from i + 29; the latter is the more severely affected by the lethal reaction following a fusion with

either the i + 32 or i + 15 plasmodium.

Two other plasmodia, having the same genotype at the fusion locus as i + 29, i + 32 and i + 15 plasmodia, were tested (Dr. M.J. Carlile, personal communication) and each gave the lethal reaction after fusing with i + 29, but not with other strains. The i + 29 plasmodium was more severely affected by the lethal reaction than the one that interacted with it. Hence, only two classes of plasmodia have yet been found within this group of fusable plasmodia viz. i + 29, and the rest. Hence, so far the lethal reaction may be regarded as the result of the interaction of two factors; patterns of interaction that might indicate a more complex basis for the lethal reaction have not been found.

(d) Observations on streaming between plasmodia after fusion

It has been shown that when an i + 15 plasmodium fuses with an i + 29, protoplasm streams from i + 29 into i + 15 for longer periods than in the reverse direction, irrespective of the relative sizes of the two plasmodia. The periods of flow from i + 29 into i + 15 gradually shortened, whilst periods of flow from i + 15 into i + 29

increased until times of protoplasmic flow in both directions became roughly the same. This suggests that an i + 29 plasmodium has a higher internal hydrostatic pressure than i + 15.

(e) Conclusions from the interaction of plasmodia of various sizes.

Lethal reactions between micro-plasmodia were always obtained when a small i + 15 fused with a large i + 29, the resulting plasmodium commonly being wholly destroyed at both i + 15 and i + 29 sites. Sometimes small areas survived, but there was no tendency for them to be in one site rather than the other. Lethal reactions sometimes followed the fusion between micro-plasmodia of i + 15 and i + 29 of roughly equal size, the dead areas being uniformly distributed between i + 15 and i + 29 sites. Lethal reactions were not observed when the micro-plasmodium of i + 29 was smaller than that of i + 15 (Table 7).

Table 7

The occurence and distribution of the lethal reaction.

	In micro-plasmodia	In macro-plasmodia
Large *i* + *29* and small *i* + *15*	Lethal reactions always obtained. Uniform distribution of dead material in both plasmodia.	Lethal reactions regularly obtained. Dead areas in both *i* + *15* and *i* + *29* zones.
i + *29* and *i* + *15* roughly equal in size.	Lethal reactions obtained in about ½ of the experiments. Uniform distribution of dead material in both plasmodia.	Lethal reactions regularly obtained. Dead area mainly in the *i* + *29* site.
Small *i* + *29* and large *i* + *15*.	No lethal reactions obtained.	Lethal reactions regularly obtained. Dead protoplasm entirely in the *i* + *29* site.

It is reasonable to assume, in view of the high rates of protoplasmic streaming observed and the small amounts of protoplasm involved, that when micro-plasmodia fuse, mixing of protoplasm is rapid and complete. This would explain the

uniform distribution between the i + 15 and i + 29 sites of dead material on occasions when the lethal reaction was observed. The consistent absence of lethal reactions when i + 15 was bigger than i + 29 and its consistent presence when i + 15 was smaller than i + 29 suggest that the lethal reaction is the consequence of the mixing in certain proportions of two different types of protoplasm.

Unless there was reason to regard the medium as being defective, lethal reactions were regularly obtained when macro-plasmodia were fused, regardless of whether i + 15 was smaller than, equal to, or bigger than i + 29. The differences observed concerned not the presence or absence of the reaction but its distribution between sites. When a small i + 15 macro-plasmodium fused with a large i + 29 the lethal reaction occurred in areas of roughly equal size in both sites. When the two fusing plasmodia were roughly equal in size, the lethal reaction subsequently occurred mainly in the i + 29 site, but some dead material was present also in the i + 15 site. The lethal reaction resulting from the fusion of a large i + 15 macro-plasmodium with a small i + 29 was confined to the i + 29 site (Table 7).

The occurrence of the lethal reaction in interacting micro-plasmodia of i + 15 and i + 29 of different sizes was interpreted as indicating that the mixing of the two types of protoplasm in certain proportions was lethal. This hypothesis can also account for the distribution of the lethal reaction in macro-plasmodia.

When macro-plasmodia fuse, mixing of protoplasm is not complete for several hours, as has been demonstrated both by isotope studies (Kerr and Waxlax, 1968; and Miller, Anderson and Peterjohn, 1964) and cytological observation (Rusch, Sachsenmaier, Behrens and Gruter, 1966). Hence, in different regions the proportions of the two types of protoplasm will differ. When the i + 15 plasmodium is larger than the i + 29, a mixture in which i + 29 protoplasm is in excess of i + 15 will be confined to the original site of i + 29, and hence we may expect the lethal reaction to be confined to the i + 29 site; this in fact occurs. When the i + 29 plasmodium is larger than the i + 15, a mixture of protoplasm in which i + 29 exceeds i + 15 may be expected to extend into the i + 15 site and to produce a lethal reaction there, as is in fact observed.

It seems best, therefore, to regard the lethal reaction between i + 15 (and similar strains) and i + 29

as a consequence of the mixing of particular proportions of the two types of protoplasm rather than in terms of "killer" and "sensitive" genotypes, an interpretation consistent with lethal reactions in Paramecium (Beale, 1954) and yeast (Woods and Bevan, 1968). The present phenomenon does however resemble the lethal reactions studied in Neurospora by Wilson, Garnjobst and Tatum (1961).

(2) Didymium squamulosum

(a) Spore germination

Much effort has been devoted to the study of germination of myxomycetes. Hawker (1952) has assessed some of the internal and external factors influencing the spore germination of myxomycetes. Gray and Alexopoulos (1968) have recently provided a critical assessment of the literature on spore germination, and showed that in spite of the great amount of work done, the initiation of spore germination in myxomycetes is still ill-understood.

Pinoy (1907) suggested that bacteria induced spore germination of myxomycetes, but Gray and Alexopoulos (1968) cited considerable evidence to support their view that the presence of bacteria is not always necessary for spore germination. The present author found that there was no germination of spores of D.squamulosum on grass extract agar unless bacteria (e.g. E.coli) were also present. Hence, there is a need for further investigation of the factors affecting spore germination, particularly the effects of other micro-organisms.

In the present study, spores of D.squamulosum, suspended in distilled water with E.coli, were inoculated onto a variety of media. Germination occurred after 4 days at

room temperature on plain agar, whereas on corn meal agar germination took place after about 7 days. There was much bacterial development on the corn meal agar plates and this may have resulted in a retardation of spore germination. Germination of D.squamulosum spores occurred on all plates of grass extract agar sown with the suspension. Amoebae and empty spore cases were seen after only one day. This was, in the experience of the present author, the most rapid and reliable method for the germination of D.squamulosum spores. This contrasts favourably with the results of Smart (1937) who found, admittedly employing rather different techniques, that the germination of D.squamulosum spores in grass extract took 6 days.

Gray and Alexpoulos (1968) advocate the use of p-dichlorobenzene to protect myxomycete spores from mites and insects. However, it is inadvisable to use p-dichlorobenzene for protecting spores because the present author found that none germinated after storage in its presence. The substance has been shown in the present study to be lethal to amoebae and plasmodia of D.squamulosum.

(b) The amoeboid phase

(i) Morphology and growth

Much attention is given to the plasmodial phase of the myxomycete life cycle in the literature, but there is little discussion of amoebae. Gray and Alexpoulos (1968) have written a short chapter on amoebae of myxomycetes, but they too emphasise the importance of plasmodia at the expense, the present author feels, of the amoebae.

In the present study, the amoebae of D.squamulosum were exposed to a range of temperatures. The greatest rate of amoeba advance along a streak of E.coli was in the range 20 - 25°C. Growth was very poor at 30°C. The rate of advance was slower at 15°C than at 20°C, and at 6°C there was virtually no multiplication of the amoebae. There is much scope for further physiological studies on the amoebae of myxomycetes.

When Didymium and Physarum amoeba colonies met, the two kinds of amoeba co-existed without any apparent harm to either. Since, however, D.squamulosum amoebae migrate faster than those of P.polycephalum, simultaneous inoculation of the two species onto a lawn of E.coli leads to the greater part of the bacteria being consumed by Didymium. Hence, in nature, competition for the limited nutrients could occur between myxomycete species with large swiftly moving amoebae and those with small slow amoebae, even

though there may be no direct antagonism.

The study of amoebae of Didymium and Physarum revealed some interesting differences between the two types. Some comparisons are given below in Table 8.

Table 8

A comparison of amoebae of D.squamulosum and P.polycephalum

	Didymium amoebae	Physarum amoebae
Nucleus	Visible in living amoebae with bright field illumination (x400 magnification) 5 m diameter.	Not visible under these conditions.
Shape of cells	Generally smooth outline.	Generally irregular, lobed outline.
Length of cells	18 - 25µm.	11 - 16µm.
Diameter of encysted amoebae	10 - 11µm.	7 - 8µm.

The colony margins of both kinds of amoeba are sharply defined to the naked eye. Under the microscope, the advancing front of D.squamulosum is seen to consist of a wide, clearly defined band of amoebae. That of P.polycephalum is narrow and less sharply defined, there being many solitary amoebae in advance of the majority. Perhaps the most striking difference between the colonies is that the growth rate of the D.squamulosum colony is approximately twice that of the P.polycephalum colony. It is possible that the morphology of myxomycete amoebae and their plaques might be of value in identification and classification. There is clearly a need for further study of amoebae of myxomycetes.

(ii) The flagellate phase

Ross (1957) observed spores of D.squamulosum germinate to give myxamoebae. Within 5 hours these amoebae had produced flagella and hence had become swarm cells. The flagellate state did not last long because no swarm cells (flagellates) were seen in 24 - 30 hour-old cultures, only amoebae being present. These cells were described as "irreversibly amoeboid" and D.squamulosum as a representative

of his "briefly flagellate" type.

The present author has shown, however, that amoebae of D.squamulosum in 8 - 28 day-old cultures, when immersed in water, change into flagellates, and that flagellates were present in such cultures up to 48 hours after immersion. It has also been demonstrated (Section IV, 5 (d)) that amoebae of D.squamulosum can, under appropriate conditions, change into the flagellate state, revert once more to the amoeboid condition and (after further multiplication) give rise yet again to flagellates. D.squamulosum cannot therefore be regarded as "irreversibly amoeboid" and the utility of such categories as "briefly flagellate" is questionable.

(c) The plasmodial phase

(i) Plasmodium initiation

In the present study, all clones of Wimbledon and Epping amoebae produced plasmodia, confirming the tentative conclusions of von Stosch (1935) and Mukherjee and Zabka (1965); hence, D.squamulosum is like D.nigripes in which it has been firmly established by Kerr and Sussman (1958) that plasmodia can arise in cultures of cloned amoebae. D.nigripes was hence regarded as homothallic. On the

other hand, in some members of the genus Didymium plasmodium initiation only occurs when amoebae of differing mating types are brought together. Collins (1963), for example, has shown that in D.iridis plasmodium formation results from the mating of amoebae of differing mating type; although in addition to the normal heterothallic strains, homothallic strains have been reported (Collins, 1961). This latter conclusion has, however, been criticised by Dee (1966a).

Recently, increasing evidence has been produced indicating that in D.nigripes plasmodia arise from amoebae without sexual fusion occurring, i.e. by apogamy rather than by homothallic mating. Kerr (1967) traced by cinemicrography the origin of plasmodia in a clone of amoebae, and Sylvia Kerr (1968) discovered that the ploidy levels of nuclei in amoebae and plasmodia were the same. Hence, the question of whether D.squamulosum is a homothallic or apogamic myxomycete is an open one. The large size of amoebae of D.squamulosum might be significant, i.e. they might be diploid like the plasmodium and hence plasmodium initiation would be apogamic. Further cytological studies on plasmodium initiation in D.squamulosum are therefore desirable.

Mukherjee and Zabka (1965) obtained plasmodia on 4.4% corn meal agar plates only a few days after inoculation with amoebae and cysts. Although the present author achieved considrable success in the production of plasmodia from amoebae - a range of concentrations of corn meal agar (4.4 - 0.44% corn meal agar) and of liver extract agar (0.045 - 0.0045% liver extract agar) supported plasmodium initiation - plasmodia were not visible until about two weeks after inoculation of the plates.

(ii) Growth in two-membered culture; interaction with other species.

Plasmodia could be grown only in two-membered culture with certain bacteria. Escherichia coli and Aerobacter aerogenes on liver extract agar provided conditions which allowed limited growth of the plasmodia: the plasmodia migrated along streaks of these bacteria for a few days, then left the streaks, migrated over the agar, and eventually died. Only Bacillus thuringiensis on grass extract agar supported continuous and healthy development of plasmodia. These observations provide evidence that some bacteria are more suitable than others for supporting the growth of myxomycete plasmodia.

The plasmodium of D.squamulosum ingested the cells of the alga Chlorella vulgaris but it was not clear whether this was later followed by digestion of the algae or elimination of the algae unharmed from the plasmodium. Lazo (1961) failed to observe the "incorporation" of Chlorella vulgaris into the plasmodium of D.squamulosum but was more successful with some other myxomycetes in which incorporation of the alga occurred. In a few, it apparently persisted in the plasmodium, multiplied, and led to enhanced growth of the plasmodium.

(iii) Interaction with other plasmodia.

In view of the work carried out on P.polycephalum (Section III) it was thought worthwhile to attempt the fusion of plasmodia of D.squamulosum to see whether there were any barriers to fusion, and whether killing reactions followed fusion.

All Epping isolates employed in these tests fused with one another and no killing reactions occurred. Fusion failures have been observed in plasmodia of D.squamulosum (Mukherjee and Zabka, 1965) but appear to be a consequence of degeneration of some plasmodial lines after prolonged subculture. Kerr and Waxlax (1968) make it clear that

such degeneration was responsible for the fusion failures they observed between plasmodia of D.nigripes. In the present study such degenerative changes were not observed, nor were the morpholcgical changes reported by Mukherjee and Zabka (1965) seen.

REFERENCES

BEALE, G.H. (1954). The Genetics of *Paramecium aurelia*. Cambridge University Press.

CARLILE, M.J. and DEE, J. (1967). Plasmodial fusion and lethal interaction between strains in a Myxomycete. Nature, Lond. 215, 832 - 834.

CHAMBERS, R. and CHAMBERS, E.L. (1961). Explorations into the nature of the living cell. Harvard University Press.

COLLINS, O'Neil R. (1961). Heterothallism and homothallism in two Myxomycetes. Amer. J. Bot. 48, 674 - 683.

COLLINS, O'Neil R. (1963). Multiple alleles at the incompatibility locus in the Myxomycete *Didymium iridis*. Amer. J. Bot. 50, 477 - 480.

DANIEL, J.W. and RUSCH, H.P. (1961). The pure culture of *Physarum polycephalum* on a partially defined soluble medium. J. gen. Microbiol. 25, 47 - 59.

DANIEL, J.W. and BALDWIN, H.H. (1964). Methods of culture for plasmodial Myxomycetes. in Methods in Cell Physiology. 1, ed. Prescott, D.M. Academic Press.

DEE, J. (1960). A mating-type system in an acellular slime mould. Nature, Lond. 185, 780 - 781.

DEE, J. (1962). Recombination in a Myxomycete, Physarum polycephalum Schw. Genet. Res. 3, 11 - 23.

DEE, J. (1966a). Multiple alleles and other factors affecting plasmodium formation in the true slime mould, Physarum polycephalum Schw. J. Protozool. 13, 610 - 616.

DEE, J. (1966b). Genetic analysis of actidione - resistant mutants in the Myxomycete Physarum polycephalum Schw. Genet. Res. 8, 101 - 110.

GRAY, W.D. and ALEXOPOULOS, C.J. (1968). Biology of the Myxomycetes. The Ronald Press Company.

HAWKER, L.E. (1952). The physiology of Myxomycetes. Trans. Brit. mycol. Soc. 35, 177 - 187.

KERR, N.S. and SUSSMAN, M. (1958). Clonal development of the true slime mould, Didymium nigripes. J. gen. Microbiol. 19, 173 - 177.

KERR, N.S. (1961). A study of plasmodium formation by the true slime mould, Didymium nigripes. Exp. Cell Res. 23, 603 - 611.

KERR, N.S. (1967). Plasmodium formation by a minute mutant of the true slime mould, Didymium nigripes. Exp. Cell Res. 45, 646 - 655.

KERR, N.S. and WAXLAX, J.M. (1968). A yellow variant of the Eumycetozoan Didymium nigripes which exhibits aging. J. Exp. Zool. 168, 351 - 362.

KERR, N.S. and WAXLAX, J.N. (1968). The fusion of plasmodia of a true slime mould grown on various nutrients and the mixing of fused plasmodia. Trans. Amer. Microsc. Soc. 87, 197-200.

KERR, S. (1968). Ploidy level in the true slime mould Didymium nigripes. J.gen. Microbiol. 53, 9 - 15.

LISTER, A. (1925). A Monograph of the Mycetozoa. British Museum N.H.

LAZO, W.R. (1961). Growth of green algae with Myxomycete plasmodia. Amer. Mid. Nat. 65, 381 - 383.

MILLER, D.M.; ANDERSON, J.D. and PETERJOHN, C.W. (1964). Protoplasmic distribution and plasmodial fusion in a Myxomycete Physarum polycephalum. Trans. Illinois State Acad. Sci. 57, 94 - 97.

MUKHERJEE, K.L. and ZABKA, G.G. (1965). Developmental stages in the laboratory culture of the homothallic myxomycete Didymium squamulosum. Mycopathol. et Mycol. Appl. 27, 209 - 222.

PINOY, E. (1907). Role des bacteries dans le developpement de certains Myxomycetes. Ann. Inst. Pasteur. 21, 622 - 656, 686 - 700.

POULTER, R.T.M. and DEE, J. (1968). Segregation of factors controlling fusion between plasmodia of the true slime mould Physarum polycephalum. Genet. Res. 12, 71 - 79.

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

RUSCH, H.P.; SACHSENMAIER, W.; BEHRENS, K. and GRUTER, V. (1966). Synchronization of mitosis by the fusion of the plasmodia of Physarum polycephalum. J. Cell Biol. 31, 204 - 209.

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

STOSCH, H.A. von. (1935). Untersuchungen uber die Entwicklungsgeschichte der Myxomyceten. Sexualitat und Apogamie bei Didymiaceen. Planta 23, 623 - 656.

WILSON, J.F.; GARNJOBST, L. and TATUM, E.L. (1961). Heterocaryon incompatibility in Neurospora crassa - Microinjection studies. Amer. J. Bot. 48, 299 - 305.

WOODS, D.R. and BEVAN, E.A. (1968). Studies on the nature of the Killer Factor produced by Saccharomyces cerevisiae. J. gen. Microbiol. 51, 115 - 126.