

SENESCENCE IN THE MYXOMYCETE PHYSARUM POLYCEPHALUM

RUSSELL T.M. POULTER

(Department of Genetics, University of Leicester)

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SUMMARY

Senescence of the Myxomycete Physarum polycephalum.

Chapter 1 consists of an introduction to the Myxomycetes (acellular slime-moulds) followed by a description of the culture techniques employed in the study of the organisms. This includes the culture of the haploid amoebae, their crossing to give diploid plasmodia, the culture of plasmodia and the induction of sporulation, and the hatching of spores to give amoebae.

Chapter 2 consists of a description of the analysis of fusion behaviour of plasmodia. The behaviour of two plasmodia when they make contact, fusion or non-fusion, was found to be controlled by two genes, four alleles of gene f ($f_1 - f_4$) being detected, and two alleles of gene n ($n_1 - n_2$). Plasmodia must have the same f genotype for fusion to be possible, and the same n phenotype, (n_2 is dominant to n_1). One exception to this rule occurs, $f_3 f_3$ homozygotes fuse with $f_4 f_4$ homozygotes. Based partly on deductions from this exceptional observation, a general model for the mode of action of the f and n genes is proposed. The analysis of fusion type was complicated by the occurrence of a recessive allele, q^- , linked to the f gene. In the homozygous state the q^- allele results in restricted nutritional behaviour, death occurring on the usual axenic culture medium. This is the first report of linkage in *Physarum polycephalum*. The genes detected during the analysis of fusion behaviour were made use of in the analysis of senescence.

Chapter 3 reports the further analysis of an actidione resistant mutant, which, it was hoped, would be of use as a marker in the study of senescence.

Chapter 4 reports the application of genetic markers to the study of the ploidy of plasmodia.

Chapter 5 begins with an introduction to the phenomenon of senescence. This is followed by the first report of senescence in a Myxomycete. Three main techniques were applied to the analysis of this phenomenon;

- a) The life-expectancy of heterokaryons formed by fusing equal quantities of plasmodia of known, and disparate, life-expectancy was studied.
- b) The f gene was used to study the stability of the nuclear ratios of such heterokaryons.
- c) Various procedures, for example mutagenesis, were applied to plasmodia as a pulse, and the life-expectancy of the treated plasmodia observed to see whether senescence had been affected by the procedure.

The data produced by these three techniques suggests the hypothesis that senescence is due to the accumulation of defective mitochondria.

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Chapter 1

1. Introduction to the Myxomycetes

A. Taxonomy

The Myxomycetes or acellular slime moulds ~~form~~ taxonomically homogeneous and isolated group of organisms. The Myxomycetes have been considered to be taxonomically close to the Acrasiales or cellular slime moulds, but this view is no longer widely held. One of the most recent classifications of the Myxomycetes (Martin, 1961) proposes a Division Mycota (Fungi) divided into two Sub-Divisions, Myxomycotina and Eumycotina. The Myxomycotina is comprised of the single class Myxomycetes, with two sub-classes Ceratiomyxomycetidae (Exosporaeae) and Myxogastromycetidae (Myxogastres), the former with the single order Ceratiomyxales, and the latter with five orders, Physarales, Stemonitales, Echinosteliales, Trichiales and Liceales. However, concerning the inclusion of the Myxomycetes in the same group as the fungi, it should be said that an increasing number of investigations seem to suggest that the Myxomycetes are more closely related to the Protozoa. For example, the mitochondria of Myxomycetes are morphologically similar to those of Protozoa, and the analysis of the unsaturated fatty acids of Physarum polycephalum suggests affinity with the Protozoa (Kudo, 1954).

B. Life Cycle

The Myxomycetes exhibit a characteristic life-cycle (Plate 1). A spore hatches to give either a single amoeba (myxamoeba) or, under certain conditions, a flagellate (swarm cell). Myxamoebae can reversibly differentiate into flagellates. Myxamoebae and flagellates are uninucleate and haploid. Myxamoebae can multiply by feeding on other microorganisms. Under suitable conditions (see F below) the haploid myxamoebae will fuse in pairs to give diploid zygotes. Zygotes grow into the macroscopic plasmodial stage, the nuclei dividing without cell division.

The plasmodia of P.polycephalum are large, bright yellow, motile and of fluid form (Plate 2). The organism consists of a single syncytial cell, vigorous oscillating protoplasmic streaming being apparent, particularly in the "veins", (thickened channels in the protoplasm.) The intricate branching network of veins is continually changing in pattern. The numerous nuclei of the syncytium are apparently physiologically identical, and exhibit synchronous mitosis. The cell is bounded by a delicate membrane and an outer layer of mucopolysaccharide slime. Plasmodia will maintain vegetative growth for a prolonged period of time, although this Thesis reports that eventually senescence occurs and the organism dies.

PLATE 1

THE LIFE-CYCLE OF PHYSARUM POLYCEPHALUM

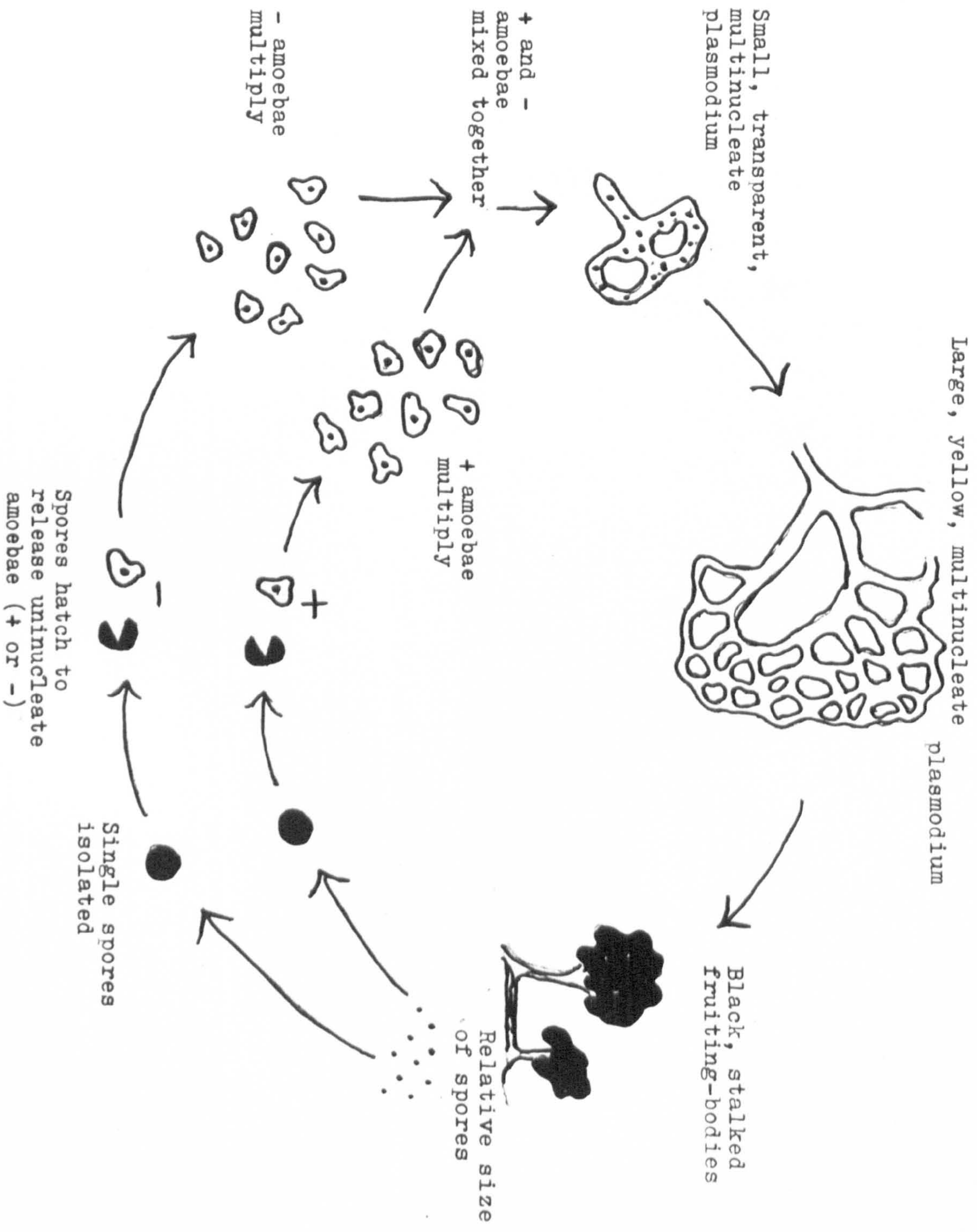
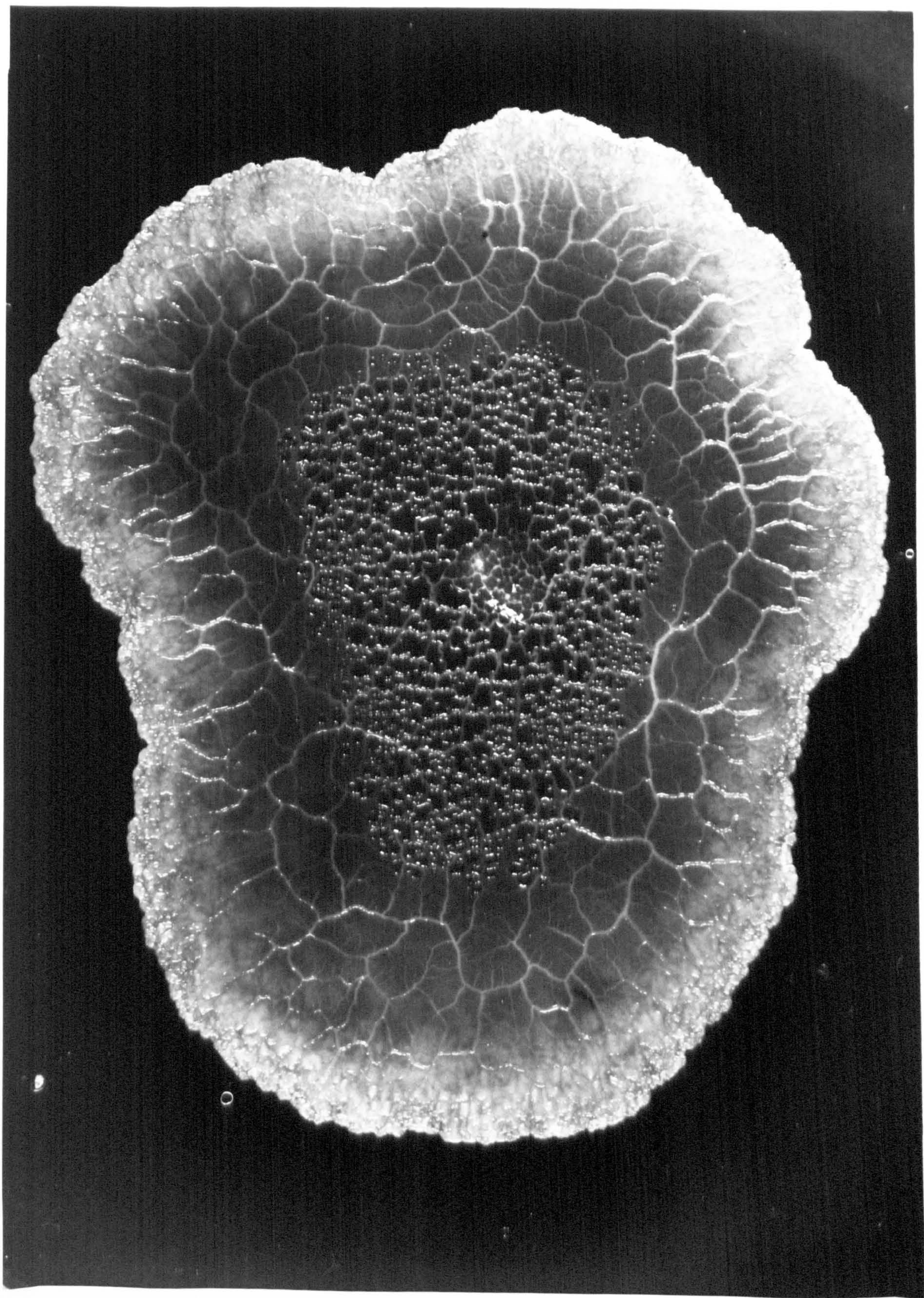


PLATE 2

A PLASMODIUM OF PHYSARUM POLYCEPHALUM (x4)



Under suitable conditions, of which starvation and light are the most important, plasmodia differentiate into spore masses. A meiotic division occurs during sporulation, the spores being haploid. The whole of the plasmodial cell takes part synchronously in this differentiation. The spores, which are uninucleate, exhibit considerable resistance to dessication and other adverse environmental conditions.

The present state of knowledge concerning the various stages of the Myxomycete life-cycle will now be briefly reviewed, with special reference to Physarum polycephalum, and the aspects relevant to this Thesis. The most recent general review of the Myxomycetes is that of Alexopoulos (1963).

C. Spores

The spore mass of P. polycephalum consists of a large number of small (approximately 10 μ diameter) black spores carried on a branched structure. The whole mass may be stalked or sessile. Cellulose (Goodwin, 1961) is characteristic of Myxomycete spore walls, and keratin-like proteids have been detected (Ulrich, 1943). Keratins have not been reported in any other group of fungi.

D. Spore Hatching

The presence of moisture is all that is required to induce spores to hatch, this process taking at least 24 hours. It is

possible that some reports of very rapid spore hatching are due to the spore masses used carrying number of encysted amoebae (see Materials and Methods; B = spore hatching). Elliot (1949) improved the frequency of spore hatching by using sodium taurocholate as a wetting agent. Spore hatching occurs over a wide pH range (pH 4 - pH 8). Although viable spores may be present in spore masses that are several years old, the age of a spore mass markedly affects the probability of hatching, possibly because of progressive dessication.

E. Flagellates and Myxamoebae

The flagellates of Myxomycetes normally develop two flagellae, but under certain conditions uniflagellate forms do occur (Kerr, 1960; Aldrich, 1968). Aldrich (1968) demonstrated that the flagella have the typical 9+2 arrangement of microtubules. Flagellum-like pseudopods have been reported (Cohen, 1959).

Flagellates can reversibly change into myxamoebae. Free moisture is sufficient to stimulate flagella formation, which is a streptomycin sensitive process. It is believed at present that flagellates are unable to divide, division occurring exclusively in the myxamoebal phase.

Myxamoebae can be grown in two-membered culture with bacteria on a variety of substrates. The most usual method is to inoculate the amoebae onto a lawn of bacteria on a firm agar medium. Only one species of myxomycete amoeba has been grown axenically, Badhamia curtisii being grown by Ross (1964) on an agar semi-defined medium

containing corn-meal and chick-embryo extract. The inability to culture myxamoebae is surprising in view of the axenic culture of the plasmodia of several species. One difficulty is the separation of amoebae from contaminating micro-organisms.

For genetic experiments it is necessary to prepare clones of amoebae which is achieved by plating on a lawn of bacteria a suitable dilution of an amoebal (or spore) suspension. An isolated amoeba (or spore) divides to give a clone of amoebae, which is visible as a plaque on the bacterial lawn (Dee, 1962). Alternatively micromanipulation may be employed. In the absence of food, myxamoebae encyst and cultures may be stored in this condition for several months without sub-culturing onto fresh media.

F. Syngamy, the Fusion of Amoebae to give a Plasmodial Zygote

The existence of a haploid/diploid cycle in the Myxomycetes has been established both by cytological and genetic analysis.

Two lines of cytological evidence have been reported; the observation of amoebal cultures particularly with respect to nuclear behaviour, and chromosome counts.

Syngamy, the fusion of amoebae, has been observed in numerous species, e.g. P.polycephalum, Physarella oblonga and Physarum oblatum (Ross, 1957). Karyogamy, the fusion of the amoebal nuclei to give a single, presumably diploid, nucleus, has also been observed in numerous

species (Ross, 1957). It is however still possible that some Myxomycetes will be found to be apogamic, plasmodial development occurring without amoebal fusion.

Ross (1966) has counted the number of chromosomes in the amoebal and plasmodia stage of Physarum flavicomum, the counts being 35 ± 2 (n) and 70 ± 2 (2n) respectively.

Dee (1960b) attempted to measure the ploidy of amoebae and plasmodia of Physarum polycephalum by Feulgen staining and microdensitometry. The DNA content of amoebal and plasmodial nuclei appeared to be the same, but the validity of interpreting these data in terms of ploidy is questionable.

The work of Dee (1962) established by genetic means that plasmodia of P. polycephalum were at some stage diploid, recombination between markers being detected. This was supported by the work of Carlile and Dee (1967) and Poulter and Dee (1968).

In Chapter 4 of this Thesis experiments will be presented which indicate that the plasmodium is diploid throughout the period of vegetative growth.

G. Homothallism and Heterothallism

Heterothallism, the existence of a mating-type system has been detected in two species of Myxomycete, P. polycephalum (Dee, 1960a) and Didymium iridis (Collin, 1961). (Clones of amoebae only giving plasmodia with other clones carrying a different mating-type.) Four

other species, which have been investigated by similar procedures, have been shown to be homothallic (or, less probably, apogamic); Didymium nigripes (Kerr, 1961), Fuligo septica (Collins, 1961), Physarella oblonga (Gehenio and Luyet, 1950) and Echinostelium minutum (Olive, 1961).

In P. polycephalum, Dee (1966a) reported that heterothallism (mating-type) was controlled by a series of four alleles of a gene mt, in the strains studied. A haploid amoebal clone carries one of these alleles, and will only produce plasmodia when mixed with clones carrying a different mt allele. Wheals (personal communication) has recently reexamined an amoebal strain of P. polycephalum which Von Stosch considered to be apogamic. Wheals reports that this strain is homothallic.

Amoebae of P. polycephalum will, if of different mating-type, fuse to give zygotes. These zygotes grow in size and become multinucleate, feeding on the bacterial lawn or on the surrounding amoebae. Mitotic divisions are apparent in these small plasmodia. Small plasmodia frequently fuse to form larger ones.

H. Meiosis

Both the time of meiosis in the life cycle, and the question of whether one or two divisions occur during meiosis, are still uncertain. Wilson and Ross (1955) and Ross (1961, 1962) studied several species of plasmodia cytologically, and reported that, in all species investigated, two meiotic divisions occurred at the time spores were delimited,

so that each spore contained one haploid product of meiosis.

Aldrich (1967), working with P.polycephalum, P.globuliferum and P.flavicomum has recently produced electronmicrographs which depict synaptenemal complexes, which are characteristic of the prophase of meiosis I in other organisms. The interpretation of Aldrich's work is however made difficult by his unnatural method of inducing sporulation, starvation on water agar for a number of hours followed by continuous exposure to fluorescent illumination (of uncertain intensity) until sporulation occurred. If Aldrich's electronmicrographs are correctly interpreted, and if they reflect the natural sequence of events, then meiosis I occurs approximately 20 hours after cleavage of the spore mass. Three of the four meiotic nuclei are believed to degenerate inside the spore. This interpretation is clearly in contradiction to the work of Wilson and Ross.

I. The Mitotic Cycle of the Plasmodium

The nuclei of P.polycephalum plasmodia are spherical and 4-5 μ in diameter (Guttes, Guttes and Rusch, 1961). A 7 cm diameter plasmodium contains more than 10^6 nuclei. All nuclei appear to be identical; each is surrounded by a membrane and contains a single large nucleolus 1 μ in diameter. Under good nutritional conditions mitotic divisions are synchronous throughout the plasmodium. During starvation nuclei continued to divide, but with less synchrony (Guttes

and Guttles, 1962).

The nuclear membrane of plasmodia may be modified during mitosis, but it never breaks down completely (Goodman, 1967), unlike the nuclear membrane of amoebae, which disintegrates completely (Koevenig, 1964).

Counts of the chromosome number of P.polycephalum are variable (e.g. Ross, 1966) and probably reflect a real variation amongst strains due to polyploidy. The plasmodia used in many of the studies have been maintained vegetatively for prolonged periods and may be abnormal. It is clearly desirable that measurement should be made on plasmodia recently produced from haploid amoebal clones. The diploid chromosome number is certainly large, probably of the order of fifty.

Rusch and his associates have performed a detailed analysis of the biochemical events associated with mitosis, an analysis that was made possible by their development of an axenic medium for the plasmodium. Their conclusions will be briefly summarised.

(1) DNA synthesis occurs immediately after mitosis and lasts for about 3 hours of the 8 hour cycle (i.e. there is no G-1 period). DNA molecules replicated at one point in one S-period are replicated during a similar temporal segment of the next S-period. Nuclei from S-period plasmodia transplanted into G-2 plasmodia continued DNA synthesis. Nuclei from plasmodia in the G-2 period do not synthesise DNA when

placed in S-period plasmodia (Nygaard et al, 1960; Braun et al, 1965).

(ii) Mitochondrial DNA synthesis occurs throughout the mitotic cycle (Guttes and Guttes, 1964), but is apparently reduced during the 20 minutes preceding mitosis (Guttes et al, 1967).

(iii) RNA is synthesised in a pattern synchronous with the mitotic cycle. During mitosis very little synthesis occurs, a peak occurs about $2\frac{1}{2}$ hours after mitosis, and another peak occurs about 2 hours before mitosis (Mitttemayer et al, 1964; Braun et al, 1966). It seems likely that no transcription occurs during mitosis. The last mRNA necessary for mitosis is transcribed about 35 minutes prior to mitosis.

(iv) Thymidine kinase activity reaches a peak at the end of mitosis (Sachsenmaier and Ives, 1965) and DNA polymerase activity increases about 1 hour before metaphase (Brewer and Rusch, 1966).

(v) Histones and DNA are synthesised concurrently (Mohberg and Rusch, 1964).

J. Vacuoles and Other Protoplasmic Inclusions

Guttes, Guttes and Rusch (1961) observed various cytoplasmic particles, including pigment granules (0.2-2.0 μ in diameter), contractile vacuoles, polyphosphate granules (less than 0.5 μ in diameter) and mitochondria (1-2 μ in diameter).

There is some uncertainty about the chemical nature of the yellow pigment of P.polycephalum; it has been reported that several

pigments may be present.

Holter and Pollock (1952) detected succinic dehydrogenase activity associated with mitochondria. Ward (1956) demonstrated cytochrome oxidase activity. Yamanaka et al (1962) partially purified a cytochrome c from P.polycephalum.

K. Protoplasmic Streaming and Locomotion

This subject has been recently reviewed (Kamiya, 1959). The motive force for protoplasmic streaming in Physarum appears to be a contractile protein, myxomyosin (Loewy, 1952). This protein is the main constituent of the fibrillar structures described by Wohlforth-Botterman (1964). Plasmodial movement is the result of controlled or directed streaming (the streaming shows periodic reversals, but the net flow is in the direction of migration). The mechanism of this control of net flow is not understood, but it has been suggested that acetylcholine and acetylcholine esterase are involved (Nakajima and Hatano, 1962).

L. Sclerotium Formation

Under unfavourable conditions a plasmodium may enter a resting stage or sclerotium. This form is resistant to desiccation and is a convenient experimental means of storing plasmodia for prolonged periods of time. A sclerotium consists of microspherules, containing from 0 to 14 diploid nuclei; each microspherule being bounded by a membrane. Jump (1954) studied the conditions that induced sclerotium

formation, which included slow desiccation, high and low temperature, high osmotic pressure, sublethal concentrations of heavy metal ions, starvation and low pH.

M. Sporulation

The occurrence of meiosis during sporulation has been discussed above (H. Meiosis). A period of illumination is essential for inducing sporulation in the pigmented, but not in the non-pigmented species of the Myxomycetes (Gray, 1938). Using monochromatic light Gray established that for P.polycephalum the greatest spore inducing effect was produced with light of short wavelengths. Starvation also seems to be an absolute requirement for sporulation. It seems probable that moisture is inhibitory to sporulation, but this subject requires more study.

The biochemical events associated with sporulation are summarised by Daniel (1966). Of particular interest is the decrease in ATP activity (Daniel, 1966), the total disappearance of α amylase activity (Ward, 1958) and the increase in cytochrome-oxidase activity and decrease in ascorbic acid oxidase (Ward, 1958).

Daniel and Rusch (1962) developed a controlled procedure for inducing sporulation. Plasmodia are starved for four days on a medium consisting of a solution of salts and the vitamin niacin. During this period the glycogen content of the plasmodia is reduced (100 mcg/ml glucose inhibits sporulation). Aerobic conditions are necessary for sporulation. After the period of starvation the cultures

are exposed to 4 hours light. It appears probable that mRNA's necessary for sporulation are induced by light. Apparently this induction is completed about 2 hours after illumination, after which time addition of actinomycin D no longer inhibits sporulation (Sauer and Babcock). If the organism is returned to food within 3 to 3½ hours after illumination normal growth occurs. Approximately 13 hours after the end of illumination a mitotic division occurs, being followed almost immediately by the synthesis of cell walls. The exact times of meiosis I and II are still in some doubt (see H. Meiosis above). This sporulation procedure, while effective in inducing sporulation, is open to the criticism that it bears little resemblance to the natural induction of sporulation, and that therefore the sequence of events recorded may be atypical of "normal" sporulation. No tests of the viability of spores produced by this procedure have been reported. For the purpose of the genetic experiments reported in this Thesis a simpler procedure for inducing spores has been routinely used (see Materials and Methods section).

N. Culture of Myxomycetes

The amoebae and plasmodia of many Myxomycetes can be grown in two membered cultures with bacteria as food. So far no species has been grown axenically in both phases of its life-cycle. Daniel (personal communication) has reported that sterile spores can be

induced to hatch and fuse axenically to give vigorous plasmodia. However myxamoebae do not increase in number, and this procedure is therefore, at present, of little experimental use.

Apart from P.polycephalum the plasmodia of several other Myxomycetes have been grown axenically. Of considerable interest is the report of Cohen and Sobels (1952) that plasmodia of Badhamia utricularis can be grown on a yeast extract medium. The only species of myxamoeba that has been grown axenically is Badhamia curtisii (Ross, 1966). It may therefore prove possible to culture a species of Badhamia axenically in both phases of its life-cycle. This would provide an extremely valuable experimental system for studying the differentiation from haploid myxamoeba to diploid plasmodium.

2. Materials and Methods

A. Strains of P.polycephalum

The strains of P.polycephalum used in the work described in this Thesis are derived from two sources. The "Wisconsin" amoebal clones a and i were derived from a plasmodium supplied by Dr H.P. Rusch of the University of Wisconsin in 1957. The "Indiana" amoebal clones B173 and B174 were derived from a plasmodium collected originally in Indiana, and supplied to us by Professor C.J. Alexopoulos of the University of Texas.

B. Spore Hatching

The spore masses used throughout this work were derived from axenically grown plasmodia, and were therefore free from contaminating microorganisms. Spores were soaked in screw top bottles overnight in distilled water and then shaken vigorously to break up the spore mass into individual spores. The spore suspension was then poured off from the residue of the stalk material. Suspensions were counted in a haemocytometer, and where appropriate were diluted to the required count. A drop of the suspension (.05 ml) was then inoculated, together with an Escherichia coli suspension, onto liver-infusion agar plates, and spread. (Liver-infusion agar consisted typically of 20 g agar, 0.5 g Oxoid dried liver-infusion and 1 litre distilled water, but the concentration of liver-infusion was occasionally varied to compensate for different batches of liver-infusion.) (Sterile, disposable, 9 cm diameter, plastic petri dishes were used throughout this work.) The plates were incubated in the dark at 25°C. All free moisture was quickly absorbed into the agar surface. Within a week to ten days plaques of amoebae, clonally derived from single spores, were apparent on the lawn of E.coli. The original spore suspension was routinely plated at several dilutions, so as to inoculate from 100 to 10,000 spores per plate. (Spore viability was rarely better than 10% and occasionally was as low as .1%.) This procedure is simpler than the method of top-agar spore plating described by

Dee (1966b), and gave more reliable results.

Normally flagellates do not appear in suspensions of spores until 20-24 hours after soaking. Occasionally spore-masses gave flagellates in a matter of minutes, the probable explanation being that these had not hatched from spores, but from myxamoebal cysts produced by spores hatching and encysting at some time prior to the experiment.

C. Amoebal Culture

Amoebae were routinely recloned after being isolated from "spore" plaques. A wire loopful of amoebae was transferred to a tube of sterile distilled water, shaken vigorously and replated on liver-infusion agar (LIA) plates, together with an inoculum of E.coli sufficient to produce a confluent bacterial lawn. The plates were incubated for 1 week, and amoebae transferred from the clonal plaques onto slopes of LIA in screw top bottles, together with an inoculum of E.coli. Cultures maintained on such slopes retain their viability for several months. These procedures are those of Dee (1966a).

D. Crossing of Amoebae to Produce Plasmodia

Amoebal clones were crossed (or tested for crossing reaction) on LIA plates supplemented with 0.0004 M p-aminobenzoic acid. This substance greatly increases the frequency of successful crosses (Dee, 1966a). Loopfuls of the two clones were mixed on the centre of the

plate, together with E.coli. Cross-plates were inspected with the naked eye, plasmodia becoming apparent in one to two weeks.

E. Culture of Plasmodia

The axenic medium used to culture plasmodia (SDM, semi-defined medium) is described in Table 1. The medium is a slight modification of the media developed by Daniel and Baldwin (1964). When tiny yellow plasmodia became visible on a cross-plate, a block of agar carrying the plasmodia was transferred to an SDM agar plate and incubated in the dark at 25°C. The small plasmodia grow and migrate off the block, covering the plate in a period of about a week. (Certain plasmodia (q^- , see Chapter 2) were routinely cultured on lawns of E.coli on LIA, as the SDM agar was toxic to them.) Plasmodia were freed from contaminating E.coli by migration across acidified (pH 4) water agar. Plasmodia were sub-cultured, when they had covered the SDM agar plate, by cutting a block of agar approximately 1 cm square, and inoculating this onto a fresh SDM agar plate. The inoculum agar block can be seen in Plate 10. Plasmodia quickly recover from the physical injury of cutting. Plasmodia were routinely sub-cultured every three days for as long as the plasmodium was required.

Plasmodia can be grown in liquid shaken cultures, in which they break up and form microplasmodia (Daniel, and Baldwin, 1964). (The medium used, SDM, is shown in Table 1) Cultures were incubated in

TABLE 1

(a) Semi-defined medium (SDM)	
Glucose	10 gm
Oxoid bacteriological peptone	10 gm
Vitamin mix	100 ml
KH_2PO_4	2 gm
CaCl_2	0.9 gm
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.6 gm
$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$	0.06 gm
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.034 gm
Citric Acid. H_2O	3.54 gm
Disodium EDTA	0.224 gm
Dist. H_2O up to 1 Litre	
Adjust pH to 4.6 with 10% NaOH. Add hema-	
tin solution immediately before use: 1 ml/	
100 ml medium	
(b) <u>SDM agar</u> : Equal quantities SDM and	
melted 3% agar mixed. Hematin solution	
(1 ml/100 ml) added after cooling to 40°C.	

(c) Vitamin mix	
Inositol	119 mg
Choline chloride	85.7 mg
Biotin	158 mg
Thiamine HCl (Aneurin HCl) .	424 mg
Pyridoxal HCl	609 mg
Pyridoxine HCl	87.2 mg
Niacin (nicotinic acid) . .	42.2 mg
D-Pantothenic acid. Calcium salt	45 mg
p-aminobenzoic acid	8.16 mg
Folic acid	4.07 mg
Vitamin B ₁₂ (Cyanocobalamin)	0.049 mg
Riboflavin	43.6 mg
Dist. H ₂ O	1 Litre
(d) Hematin solution	
1% NaOH	100 ml
Hematin	0.05 g
Sterilized by autoclaving. Stored at 5°C.	

the dark on a New Brunswick Gyrotary Water Bath Shaker (model G76) at 150 revolutions per minute. Plasmodia were converted from agar culture to liquid culture by the following procedure. A block of agar carrying an inoculum of plasmodium was placed in a sterile glass flask (metal capped) and enough SDM solution added to the flask to cover the bottom, care being taken not to submerge the plasmodium. After incubation for 24 hours the plasmodium had grown as a delicate fan across the surface of the liquid. The flask was shaken vigorously to break up the fan, and the resulting suspension was added to 25 ml of SDM in a 150 ml flask (metal capped) and incubated on a shaker. The plasmodia grew as small microplasmodia of irregular shape. Plasmodia were converted from liquid to agar culture by pipetting a quantity of the microplasmodia onto an SDM agar plate. The plasmodia quickly coalesced (24 hours) and began growth as macroplasmodia.

Plasmodia were stored as sclerotia. Sclerotia often form from SDM agar cultures, if these are left in the dark. The thin sheet of sclerotium, which has a hard, slightly brittle consistency, was peeled off and stored at room temperature. Such sclerotia were induced to revert to plasmodia by placing the sheet on the surface of an SDM agar plate. The lag before growth occurred was often greater than seven days. Alternatively microsclerotia were prepared

from liquid culture microplasmodia. Cultures were left on the shaker until lack of nutrients caused microsclerotium formation. Microsclerotia were stored in this form in screw top bottles at 5°C. Such preparations retain their viability for several months. Under the microscope such preparations were seen to consist of small spherical microspherules.

F. Spore Formation

Spores were obtained from plasmodia by the following procedure. When plasmodia had grown to cover a plate of SDM agar they were exposed to natural daylight. If plasmodia were allowed to starve on the SDM plates before exposure, the incidence of sporulation was greatly reduced. Plates were placed lid downwards to prevent condensation forming, which inhibits sporulation. Exposure to direct sunlight was avoided. The sporulation room was equipped with overhead fluorescent lighting, which was used to supplement daylight on overcast days. Plasmodia were left in darkness during the night. Using this procedure sporulation occurred in almost every case. Spores usually formed during the third night following exposure, characteristic changes of morphology occurring some 5 hours after the beginning of darkness. Sporulation was apparently complete by the following morning, spores appearing morphologically normal when observed under the microscope. Spores were harvested the day after

formation and dried in sterile petri dishes to prevent hatching.

The dried spores were stored in screw-cap bottles at room temperature.

This somewhat crude procedure for inducing sporulation was preferred to the Daniel and Rusch (1962) niacin-salts technique for the following reasons:

- (i) It was simpler to induce spores directly on the culture plates, rather than transfer the plasmodia to a special sporulation medium.
- (ii) The "exhausted SDM" technique is several days quicker than the niacin-salts technique.
- (iii) The "exhausted SDM" technique involves exposure to light similar to that which might occur under natural conditions (involving both length of exposure to darkness and light, intensity and variation in intensity during the day cycle). The procedure, by approximating to the natural process, is likely to produce genetically viable sporulation. The viability of spores produced by the niacin-salts technique has never been reported.
- (iv) In our laboratory the niacin-salts technique has never produced morphologically normal sporulation, spore masses being poorly formed and large quantities of black pigment diffusing from the spore masses into the culture medium.

G. Attempts to Grow Amoebae Axenically

A procedure has not yet been found which enables amoebae to be grown axenically. Sterile spores were hatched in distilled water and

the resulting flagellates were used as the inoculum for various attempts at axenic culture. Alternatively amoebal clones grown on E.coli were plated on media containing 1000 mcg/ml streptomycin. This concentration is lethal to E.coli and resistant bacteria are extremely infrequent (1 in 10^9 - 10^{10}). The concentration is not apparently inhibitory to amoebae, one side effect being the inhibition of flagella formation. (Plasmodia can tolerate 10 mg/ml streptomycin.) Growth was obtained on E.coli which had been starved, or killed with toluene or formalin, or treated with UV or γ radiation. Various media were used in attempts to culture amoebae axenically, of which the following were used as supplements in SDM agar: chick-embryo extract, cornmeal extract, yeast extract, malt extract, potato dextrose extract, serum and whole-blood. These supplements were also added in various combinations. Diatomised earth was also added, since it was thought possible that amoebae might require particulate material for nutrition. Most of these media were also used as solutions, without the agar base. Regrettably no sustained growth occurred on any media. Work is, however, still in progress on this subject.

Spore masses will hatch on the surface of SDM agar or SDM-glucose agar, particularly if the mass is moistened. If the humidity of the plates is maintained at a high level, for example by placing the plate in an incubator containing a dish of water, the amoebae which hatch

from the sterile spore mass remain active for a considerable time, though no growth occurs. After a period of several days plasmodia are observed on the plate, quickly growing to cover the plate. In the absence of an axenic growth medium for amoebae, the system is of restricted experimental value.

H. Other Procedures

The special techniques used in the analysis of fusion, drug resistance and senescence are described in the introductions to Chapters 2, 3 and 5 respectively.

Chapter 2

THE ANALYSIS OF FUSION BETWEEN PLASMODIA OF PHYSARUM POLYCEPHALUM

1. Introduction

The fusion behaviour of myxomycete plasmodia was first studied by Towend (1937) who concluded that plasmodia of different species would not fuse, and that those of the same species would fuse. Brandza (1927) and Skupienski (1937) who worked with Didymium iridis and D.squamulosum and Gray (1945) working with Physarum polycephalum considered however that plasmodia of the same species could show non-fusion, which they interpreted in terms of physiological races within the species.

Genetically precise experiments on the control of plasmodial fusion, using cloned amoebae, were first reported in D.iridis by Collins (1966) and Collins and Clark (1968) and in P.polycephalum by Carlile and Dee (1967) and Poulter and Dee (1968). The results of the D.iridis and P.polycephalum analysis are broadly similar.

Fusion behaviour in the isolate of D.iridis which has been analysed is controlled by five genes, C, D, E, F, G, a pair of alleles being identified at each locus. At each locus one allele is dominant to the other. Plasmodia must be of the same phenotype with respect

to these five genes for fusion to be possible between them. The mating type locus, A, which controls the fusion of the haploid amoebae to give a diploid plasmodium, is not involved in the control of fusion between plasmodia. However one of the fusion genes G is linked to A, showing approximately 20% recombination. This is the first reported observation of linkage and crossing-over in a Myxomycete.

Fusion between P.polycephalum plasmodia in the isolates studied is under the control of two genes f and n, four alleles of f and two alleles of n having been identified. Dominance is apparent between the two n alleles, but not between the four f alleles. Identity of plasmodia phenotype with respect to f and n is necessary for fusion to be possible. The amoebal mating type locus mt is not involved in controlling fusion between plasmodia.

These two analyses have several important points in common. Firstly, since plasmodia derived as progeny from a single isolate (a diploid plasmodium collected from nature) show fusion and non-fusion, this behaviour is clearly not of taxonomic importance. Secondly the phenomenon of plasmodial fusion behaviour is apparently not connected with the crossing or mating behaviour of the haploid amoebae. The action of the fusion system, and of the "killing" process which frequently follows fusion, both reduce the possibility of heterokaryon formation occurring during the vegetative existence of the plasmodium.

It seems probable therefore that heterokaryon production involves some disadvantage for myxomycete plasmodia. This point will be returned to in the Discussion.

Experimentally, heterokaryon formation has been found useful for work reported in this Thesis, and potentially the technique has considerable value. Part of the interest generated by fusion control systems in plasmodia is due to the desirability of being able to produce heterokaryons predictably. The most obvious application of heterokaryons would be in the study of complementation between mutants, and potentially therefore in the genetic mapping of the organism.

The plasmodial fusion control system is also of interest for its own sake, in general terms it is a process of "self" or "non-self" identification, located at the membrane. It is therefore in general terms an analogue, of such systems as self-incompatibility in the flowering plants and sperm-egg interaction. Analogies could also be drawn with immune interactions in mammals and the heterothallism of many fungi and myxomycete gametes (haploids).

PLATE 3

TWO PLASMODIA MEETING, WITHOUT SHOWING FUSION

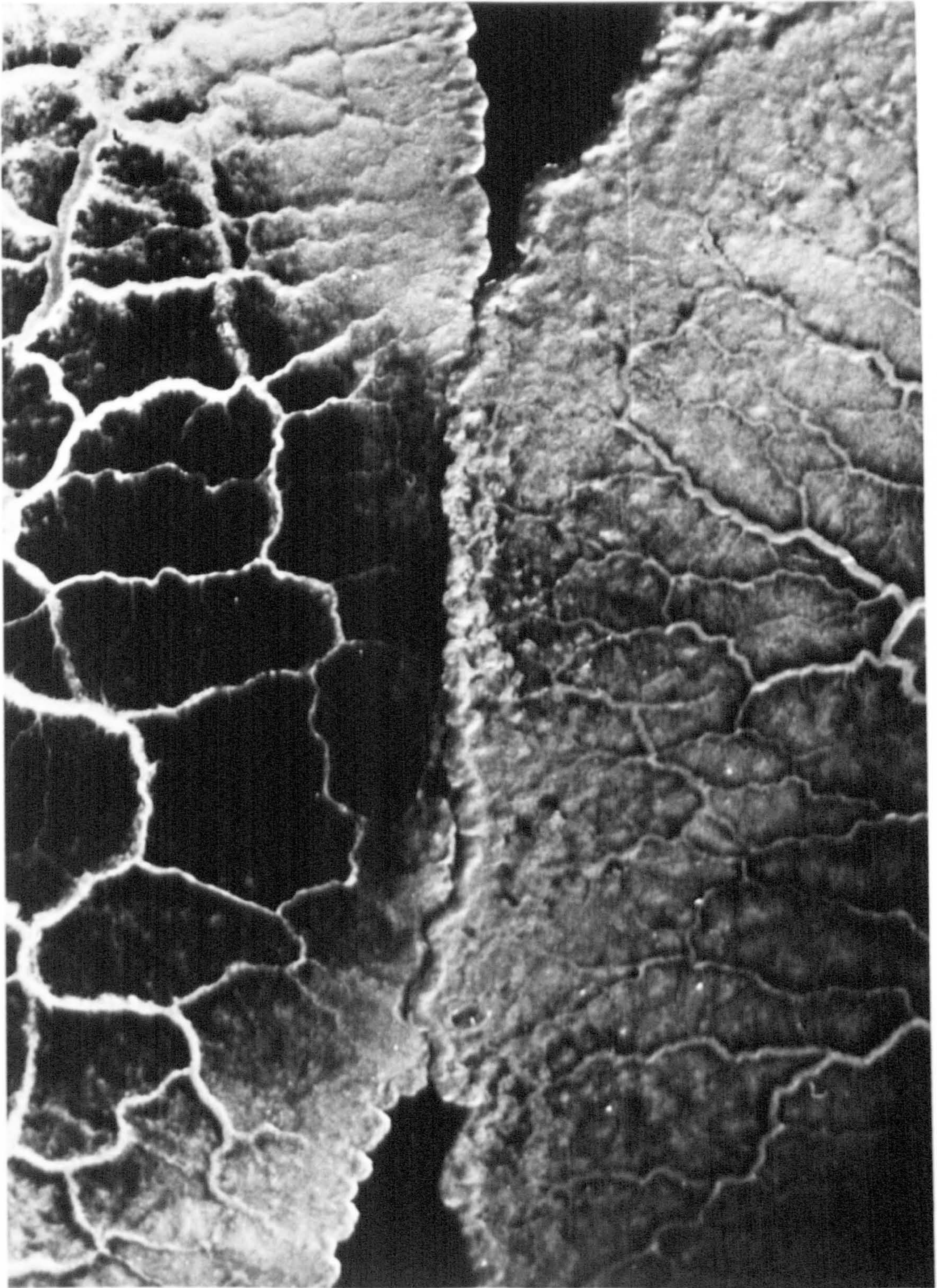
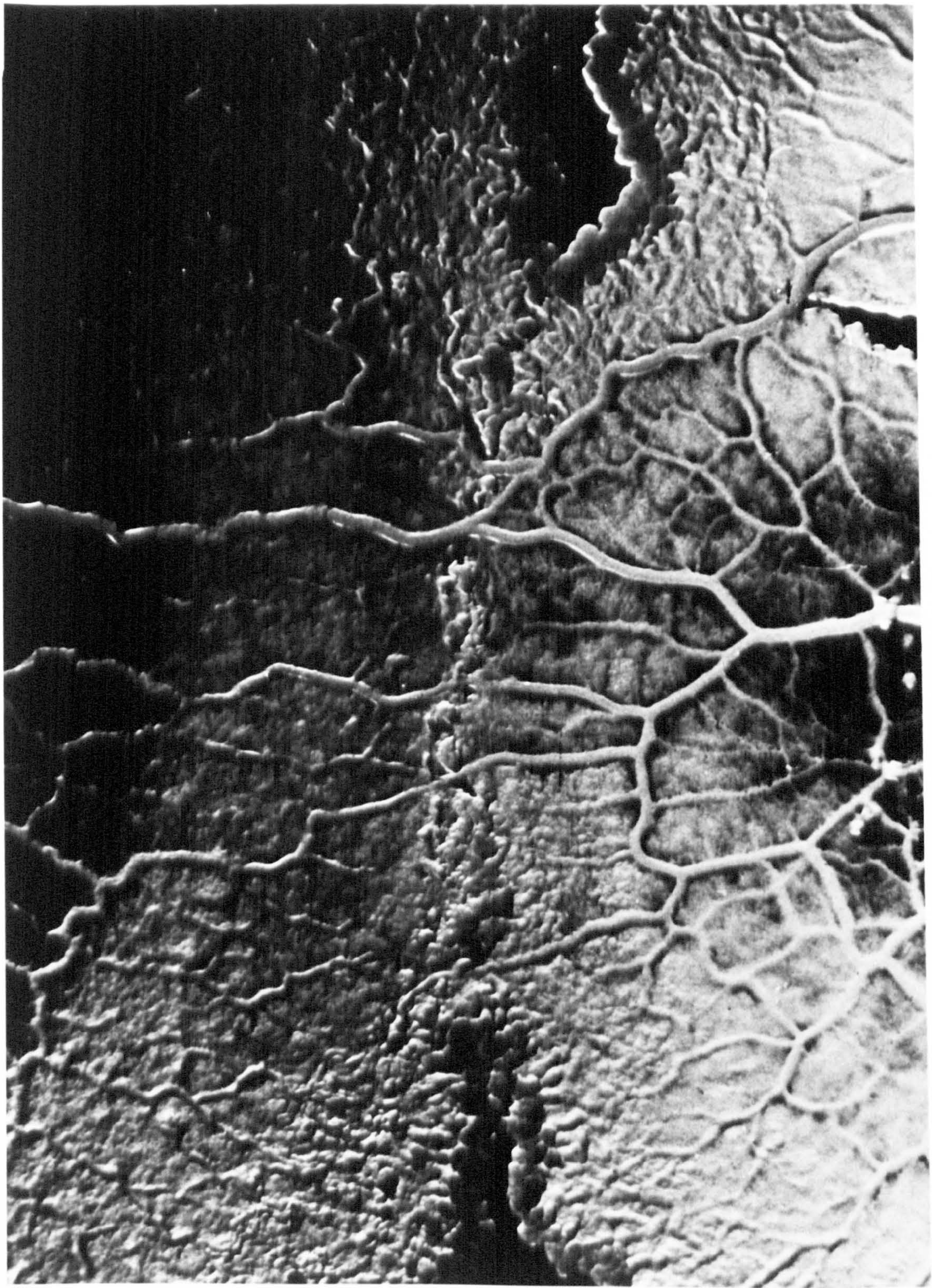


PLATE 4

TWO PLASMODIA MEETING, FUSION HAS OCCURED



2. Materials and Methods

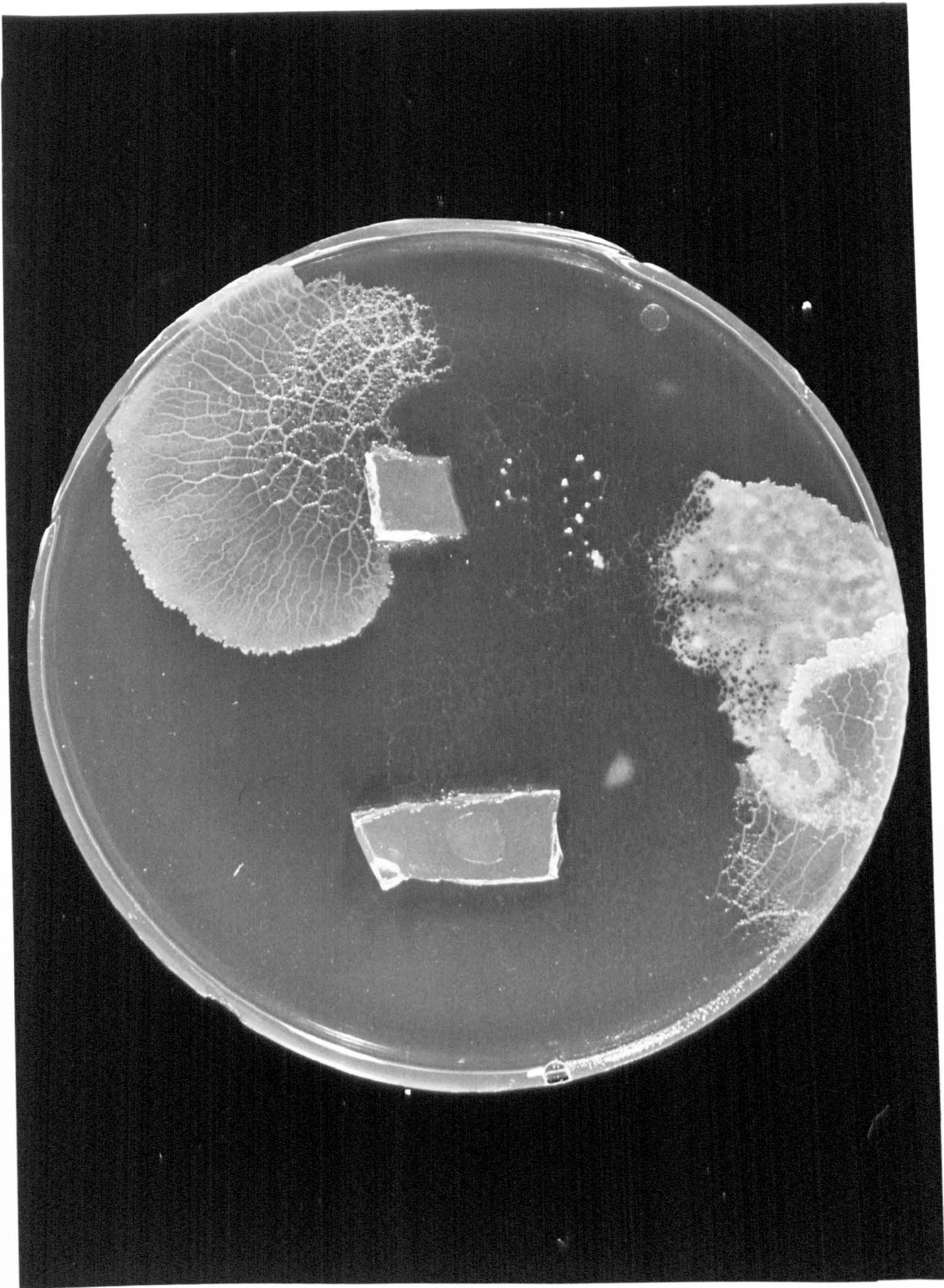
Amoebae and plasmodia were cultured by the standard procedures described in the Materials and Methods section of the Introduction. Fusion tests were performed by the procedure described by Poulter and Dee (1968) which is as follows. Plasmodia were inoculated onto a plate of half-strength SDM agar in the form of 1 x 2 cm blocks cut from plates showing vigorous plasmodial growth. The two blocks were placed 2 cm apart. The plasmodia migrated and grew onto the plate, meeting between 12 and 24 hr after inoculation. Fusion was scored when protoplasm was seen to stream between plasmodia. In most experiments, the reaction between a pair of plasmodia could be scored unambiguously as 'fusion' or 'non-fusion' soon after they met, since fusion, if it was to occur, was almost immediate and large common veins quickly developed (Plates 3 and 4). Often the fused plasmodia later showed the lethal interaction described by Carlile and Dee (1967) (Plate 5). In certain experiments, fusion was delayed and the lethal interaction occurred immediately after fusion, eliminating the small common veins which had developed. Since the small killed area was quickly overgrown, it was found necessary to observe the plasmodia in these tests continuously for 24 hr in order to obtain unambiguous scoring. Observations in all experiments were made with a Wild M5 stereomicroscope (magnifications x6, x12, x25,

PLATE 5

THE KILLING REACTION

Two plasmodia have met and fused, subsequently the mixed area has lysed. One plasmodium is in the top left hand corner, the other in the bottom right.

The area of lysis is seen on the right of the photograph, above the second plasmodium.



x50).

The analysis of fusion characters has special difficulties as a genetic problem, since it is impossible to score a plasmodium in isolation, fusion between plasmodia being the basic observation. It is therefore necessary to have a reference plasmodium which is understood to some degree. For this reason both ^{in this} the work, of Carlile and Dee and Collins and Clark used a "parental" pair of amoebal clones crossed to give a "parent" plasmodium. From this plasmodium spores and from them amoebal clones were produced. "Progeny" amoebal clones were backcrossed to the "parental" amoebal clone of the opposite mating type and the resulting "backcross" plasmodia were tested against the "parental" plasmodium and against each other. This procedure enables one to attempt an analysis of the resulting fusion pattern, since only two alleles of each gene can be present, and if two different alleles of a particular gene are present one must occur in one parental amoebal clone and one in the other, and the parental plasmodium must be heterozygous for them.

In P.polycephalum hypotheses founded on such "backcross" experiments have been tested, and where necessary modified, by analysing the fusion behaviour of "progeny clone x progeny clone" plasmodia. Such analysis has not yet been rigorously applied in the D.iridis system.

3. Results

(A) The analysis of fusion behaviour in the Wisconsin isolate

(a) This isolate has been analysed for fusion characters by Carlile and Dee (1967); however further experiments which will be reported in this Thesis have made it necessary to reconsider the interpretation of their results. Their results will be briefly considered together with certain of their previously unpublished data. Carlile and Dee crossed clones of amoebae A7 (mt₁) and i (mt₂) to give plasmodium A7+i. Spores were obtained from A7+i and these were hatched to give 50 progeny clones which were tested for mating type by using the two parent clones a and i as testers. Of the 50 clones, 13 gave plasmodia with the i (mt₂) parent amoebal clone and were therefore mt₁, and 15 gave plasmodia with the A7 (mt₁) parent clone and were therefore mt₂. (22 clones failed to cross with either parent.) Of the 13 plasmodia produced by crossing progeny clones against i, all 13 were successfully cultured onto SDM and used in the subsequent analysis. However, of the 15 plasmodia produced by crossing progeny with A7, only 5 were successfully cultured onto SDM and analysed further. This failure rate of culture is abnormally high, and further analysis has shown that it is of considerable significance for the study of the characters controlling fusion. Carlile and Dee tested the 13 progeny +i plasmodia and the 5 progeny +A7 plasmodia for fusion with the A7+i parent

plasmodium, and then for fusion with each other. The results are summarised in Tables 1 and 2. Behaviourally three fusion classes were present. Carlile and Dee proposed, as the simplest explanation that A7+i is heterozygous for a pair of alleles \underline{f}_1 and \underline{f}_2 of a gene \underline{f} , that these alleles were segregating without showing linkage to the amoebal mating-type locus \underline{mt} , and that plasmodia must carry identical \underline{f} alleles for fusion to be possible between them. Arbitrarily A7 is said to carry \underline{f}_1 and i to carry \underline{f}_2 . Table 3 is an interpretation of Table 1 on the basis of this hypothesis.

All the plasmodia used in the experiments of Carlile and Dee were produced by backcrossing progeny clones against a or i. One prediction of their hypothesis is that plasmodia produced by crossing progeny x progeny will belong to one of the three fusion groups detected in the backcross experiments, (hypothetically termed $\underline{f}_1\underline{f}_1$ (Group A), $\underline{f}_1\underline{f}_2$ (Group B) and $\underline{f}_2\underline{f}_2$ (Group C)).

(b) This prediction has not been borne out by my subsequent experiments. From spores derived from plasmodium A7+i, 45 further amoebal clones were isolated. These were tested for mating type against clone A7 (\underline{mt}_1) and i (\underline{mt}_2). Twenty six clones were found to be of mating type 2, and 16 were \underline{mt}_1 and 3 failed to react. From these clones 12 of \underline{mt}_2 and 11 of \underline{mt}_1 were taken at random and crossed against each other, the results being represented in Table 4. (Clone

TABLE 1

Results of Testing Plasmodia Derived from the Cross A7 x i for
Fusion with A7+i

Plasmodia were produced by backcrossing progeny clones of A7+i with the appropriate parental clone.

Plasmodium		Reaction* with <u>A7+i</u>		Total
Mating type of progeny clone	Mating type of parent clone	Fusion	Non-fusion	
mt ₁	mt ₂ (clone <u>i</u>)	8	5	13
mt ₂	mt ₁ (clone <u>A7</u>)	2	3	5

* Each result based on observation of at least four replicate tests.

TABLE 2

Results of Testing the Four Types of Plasmodia Present in Table 1 for Fusion with Each Other

	8 plasmodia formed from <u>i</u> + progeny clone, showing <u>F*</u> with <u>A7+i</u> in Table 1	5 plasmodia formed from <u>i</u> + progeny clone, showing <u>NF*</u> with <u>A7+i</u> in Table 1	2 plasmodia formed from <u>A7</u> + progeny clone, showing <u>F</u> with <u>A7+i</u> in Table 1	3 plasmodia formed from <u>A7</u> + progeny clone, showing <u>NF</u> with <u>A7+i</u> in Table 1
8 plasmodia represented in column 1	F	NF	F	NF
5 plasmodia represented in column 2		F	NF	NF
2 plasmodia represented in column 3			F	NF
3 plasmodia represented in column 4				F

* F = fusion, NF = non-fusion.

TABLE 3

The Interpretation of Table 1 on the
Carlile and Dee Hypothesis

No. of progeny amoebae of each genotype	Plasmodium		<u>f</u> -type deduced from reaction with <u>A7+i</u> (<u>f₁f₂</u>)	
	Genotype of progeny clone	Genotype of parent clone	Fusion	Non-fusion
8	<u>mt₁f₁</u>	<u>mt₂f₂</u> (clone <u>i</u>)	<u>f₁f₂</u> (B)*	<u>f₂f₂</u> (C)*
5	<u>mt₁f₂</u>			
3	<u>mt₂f₁</u>	<u>mt₁f₁</u> (clone <u>a</u>)	<u>f₁f₂</u> (B)*	<u>f₁f₁</u> (A)*
2	<u>mt₂f₂</u>			

* Subsequent tests (Table 2) indicate that the two types of plasmodia which fuse with A7+i (i + progeny and a + progeny) will also fuse with each other. These are both represented in the diagram as belonging to fusion group (B). Plasmodia of the other two types form two separate fusion groups (see Table 2) and are therefore represented as fusion group (A) and fusion group (C).

Backcross plasmodia derived from parental clone x progeny clone are also included.

Clone AZ = clone a. Plasmodia are represented by one of the five symbols g, III, IV, V or VI (see text). Group III plasmodia are hypothesized to be of phenotype $f_1/f_2 n_1$,

IV $f_1/f_2 n_2$, V $f_2/f_2 n_1$, VI $f_2/f_2 n_2$ (see text). *g = found from SEM.*

4

Plasmodia Derived from Crossing Progeny x Progeny Clones of the Plasmodium A7+1

[illegible]

A7 (mt₁) was derived as anactidione (cycloheximide) resistant mutant from clone a (mt₁). No mutagenesis was used in this process, and it is a reasonable assumption, supported by experiments, that clones A7 and a are identical at mating-type loci, fusion loci etc. For this reason two amoebal clones derived from the plasmodium a+i can justifiably be included in the same table as clones derived from A7+i. a+i progeny clones are E55 and E80, the clones derived from A7+i being termed A7101 ----- A7141. The results of crossing parent clone a (= A7) and i are also included in Table 4.)

In Table 4 where a cross was successful a plasmodium is recorded in one of five ways (q, III, IV, V, VI). q represents plasmodia which failed to grow when transferred from the cross-plate to SDM agar. These will be discussed in more detail below. The other symbols (III, IV, V, VI) in Table 4 all represent plasmodia which grew on SDM and were analysed for fusion behaviour, the particular number applied to each plasmodium describing its fusion behaviour (fusion class).

The four separate fusion classes represented in Table 4 were identified as follows:

- (i) Plasmodia which fused with a+i (= A7+i) are referred to as fusion class IV plasmodia (Group B of Table 3 based on the data of Carlile and Dee).

(ii) When the plasmodia formed by backcrossing progeny clones to parent clone i (bottom row of Table 4) were tested for fusion with a+i (IV) some plasmodia fused and some did not. Those which did not fuse with the group IV tester (i.e. a+i) were referred to as fusion class VI plasmodia (Group C of Table 3). Several fusion class VI plasmodia were selected for use as testers for this group.

(iii) When the plasmodia formed by backcrossing progeny clones to parent clone a (left hand column of Table 4) were tested for fusion with a group IV tester (a+i) some fused and some did not. The plasmodia which did not fuse with group IV were tested for fusion with group VI testers (see ii). No fusions occurred. These plasmodia which would not fuse with group IV or VI testers were considered to represent a third fusion class, III (Group A plasmodia of Table 3). Several of these fusion group III plasmodia were selected for use as tester for this group.

(iv) The progeny x progeny plasmodia represented in Table 4 were classified for fusion behaviour by testing them for fusion with plasmodia representing fusion groups III, IV and VI (e.g. those that fused with fusion group III testers are considered to belong to this fusion group and are represented in the Table by "III"). Using this procedure it became apparent that some of the progeny x progeny plasmodia belonged to a fourth fusion class. These plasmodia failed

to fuse with the testers representing III, IV and VI and are considered to represent a fourth fusion class V.

It should be stressed that this fourth class, V, was not represented by any backcross plasmodia; it consisted exclusively of prgeny x progeny plasmodia. Only three fusion classes are apparent in backcross plasmodia, III, IV and VI. The observation of the fourth fusion class V thus does not conflict with the data of Carlile and Dee (which was based exclusively on backcross plasmodia). However the existence of class V means that the interpretation of Carlile and Dee is incorrect.

It was considered prohibitively laborious to perform all possible fusion tests among the plasmodia shown in Table 4 (2415). Having tentatively established the probable fusion class of a plasmodium by its reaction with the testers representing classes III, IV, V and VI, that plasmodium was then tested against approximately half of the plasmodia from the fusion class to which it was assigned. (Thus within Group VI, of the 190 possible fusion tests, 105 were done, all of which fused as expected; within Group V 90 fusions were performed, within Group IV 90 were performed, and within Group III 45). No inconsistent or ambiguous result was ever observed. Many of the tests were performed in duplicate. In summary the description of the plasmodia in Table 4 as fusion class III, IV, V or VI is based on the observation of more than 550 separate fusion tests. It is

unfortunately impossible to record each one separately here.

The plasmodia represented by "q" in Table 4 which failed to grow on SDM can be cultured for at least several weeks on a lawn of E.coli on liver infusion agar plates. In other words, they can be cultured if they are treated like amoebae. The plasmodia never grow to a size greater than a few millimetres on this medium, and they have not so far been induced to spore. It is impracticable to attempt fusion tests on such small plasmodia as it is difficult to classify interactions. When transferred to SDM agar, the q plasmodia die, showing complete lysis 24-48 hr after transfer. This is not solely due to lack of specific nutrients, as the plasmodia can survive for some time on 2% water agar. Nor is the effect due to the acid pH of the SDM agar medium, since this has been experimentally modified without allowing growth of the q plasmodia. It appears that a component of SDM agar is toxic to these plasmodia; perhaps in the absence of some essential nutrient a component allows disbalanced growth, and is therefore lethal. Attempts to identify the toxic component in SDM are at present in progress, but are too inconclusive to be reported at this time.

(c) The analysis of the data represented in Table 4

The amoebal clones in Table 4 have been grouped according to the fusion behaviour of their derivative plasmodia. For example clones 7119 and 7125 give fusion group III plasmodia when crossed

with 7126, group IV with 7131, group V with 7122 and group VI with i. Similarly clones i, 7133 and 7120 are grouped together, giving group IV with a and E55, and group VI with 7125.

It will be seen from considering the backcross plasmodia (progeny x a or i) that the conclusion of Carlile and Dee that three fusion groups are present in such plasmodia is supported by this data. However the interpretation of these three classes has been altered (see below). Thus of the plasmodia formed by backcrossing to a, a+7133 is IV and a+7108 and a+7118 are Group III. Of the plasmodia formed by backcrossing to i, 7105+i, 7129+i, E55+i, E80+i are Group IV, and 7110+i, 7119+i, 7123+i, 7125+i, 7132+i, 7140+i, 7124+i, 7141+i are Group VI.

However, the prediction of the Carlile and Dee hypothesis, that plasmodia formed by progeny x progeny crosses would fall into the three classes found in backcross plasmodia, is not borne out by the data of Table 4. The occurrence of the 13 progeny x progeny plasmodia which belong to a fourth fusion group (V), is clearly incompatible with the Carlile and Dee hypothesis, which predicts only three classes $\underline{f_1f_1}$, $\underline{f_1f_2}$ and $\underline{f_2f_2}$.

It is apparent that since some clones (for example 7122) when crossed to progeny clones of the opposite mating type produce plasmodia belonging to each of the four fusion clones (e.g. 7105+7122 is Group III, 7129+7122 is IV, 7119+7122 is V, 7124+7122 is VI), then

TABLE 5

The 9 Possible Plasmodial Genotypes Produced by Crossing in all
Combinations the Four Hypothetical Amoebal Types

$f_1n_1, f_1n_2, f_2n_1, f_2n_2$

$\frac{mt_1}{f_1n_1}$	$\frac{mt_2}{f_1n_1}$	$\frac{f_1n_2}{f_1n_2}$	$\frac{f_2n_1}{f_2n_1}$	$\frac{f_2n_2}{f_2n_2}$
$\frac{f_1n_1}{f_1n_1}$	$\frac{f_1f_1}{f_1f_1} \frac{n_1n_1}{n_1n_1}$	$\frac{f_1f_1}{f_1f_1} \frac{n_1n_2}{n_1n_2}$	$\frac{f_1f_2}{f_1f_2} \frac{n_1n_1}{n_1n_1}$	$\frac{f_1f_2}{f_1f_2} \frac{n_1n_2}{n_1n_2}$
$\frac{f_1n_2}{f_1n_2}$	$\frac{f_1f_1}{f_1f_1} \frac{n_2n_1}{n_2n_1}$	$\frac{f_1f_1}{f_1f_1} \frac{n_2n_2}{n_2n_2}$	$\frac{f_1f_2}{f_1f_2} \frac{n_2n_1}{n_2n_1}$	$\frac{f_1f_2}{f_1f_2} \frac{n_2n_2}{n_2n_2}$
$\frac{f_2n_1}{f_2n_1}$	$\frac{f_2f_1}{f_2f_1} \frac{n_1n_1}{n_1n_1}$	$\frac{f_2f_1}{f_2f_1} \frac{n_1n_2}{n_1n_2}$	$\frac{f_2f_2}{f_2f_2} \frac{n_1n_1}{n_1n_1}$	$\frac{f_2f_2}{f_2f_2} \frac{n_1n_2}{n_1n_2}$
$\frac{f_2n_2}{f_2n_2}$	$\frac{f_2f_1}{f_2f_1} \frac{n_2n_1}{n_2n_1}$	$\frac{f_2f_1}{f_2f_1} \frac{n_2n_2}{n_2n_2}$	$\frac{f_2f_2}{f_2f_2} \frac{n_2n_1}{n_2n_1}$	$\frac{f_2f_2}{f_2f_2} \frac{n_2n_2}{n_2n_2}$

Arbitrarily the n_2 allele is assumed to be dominant to the n_1 allele in columns 2 and 3, and the f_2 allele dominant to f_1 in column 3.

TABLE 6

The Number of Phenotypes Resulting from the 9 Genotypes Diagrammed in Table 5.

Assuming Dominance at Neither One or Both Loci, f and n

9 genotypic classes	Phenotype assuming no dominance	Plasmodial phenotype* assuming dominance at 1 gene n	Plasmodial phenotypes* assuming dominance at both f and n
$f_1 f_1 n_1 n_1$	$f_1 f_1 n_1 n_1$	$f_1 f_1 n_1$	$f_1 n_1$
$f_1 f_1 n_1 n_2$	$f_1 f_1 n_1 n_2$	$f_1 f_1 n_2$	$f_1 n_2$
$f_1 f_1 n_2 n_2$	$f_1 f_1 n_2 n_2$		
$f_1 f_2 n_1 n_1$	$f_1 f_2 n_1 n_1$	$f_1 f_2 n_1$	$f_2 n_1$
$f_1 f_2 n_1 n_2$	$f_1 f_2 n_1 n_2$		
$f_1 f_2 n_2 n_2$	$f_1 f_2 n_2 n_2$	$f_1 f_2 n_2$	
$f_2 f_2 n_1 n_1$	$f_2 f_2 n_1 n_1$	$f_2 f_2 n_1$	$f_2 n_2$
$f_2 f_2 n_1 n_2$	$f_2 f_2 n_1 n_2$		
$f_2 f_2 n_2 n_2$	$f_2 f_2 n_2 n_2$	$f_2 f_2 n_2$	

four distinct "fusion" genotypes must be represented by the haploid amoebal clones 7105, 7129, 7119 and 7124. (Assuming, reasonably, that non-fusion between plasmodia reflects a genetic dissimilarity.) This is of course inconsistent with the hypothesis that fusion behaviour is controlled by a single gene f represented by alleles f₁ and f₂. Since the plasmodia are derived from two parental haploid clones a and i, there can only be two alleles at any locus. To produce four amoebal "fusion" genotypes at least two genes must be involved. Arbitrarily a is termed f₁n₁ and i f₂n₂. This hypothesis predicts the four amoebal "fusion" genotypes will be f₁n₁, f₁n₂, f₂n₁ and f₂n₂. If amoebae of these four genotypes are crossed in all combinations this hypothesis predicts that nine plasmodial genotypes will be produced (see Table 5).

The nine plasmodial genotypes will give a particular number of plasmodial phenotypes, depending on whether neither, one or both loci are showing dominance. If no dominance is occurring the nine genotypes will yield nine phenotypes. If dominance is occurring at one locus, six phenotypes will occur. If dominance is occurring at both loci, four phenotypes will occur (see Table 6). The data of Table 4 enables one to choose between these three hypotheses.

The data represented by Table 4 will now be analysed by a series of deductions.

(i) As was stated above certain clones, for example 7118, when crossed to progeny clones of the opposite mating type, give rise to plasmodia representing four fusion classes (a+7118 is III, 7129+7118 is IV, 7119+7118 is V and 7141+7118 is VI). However other clones, for example i, when crossed to the same four clones (a, 7129, 7119, 7141) give only two classes of plasmodia (a+i and 7129+i are fusion Group IV and 7119+i and 7141+i are VI). This clearly establishes that clones of type i are carrying a dominant allele at one locus and not at the other. Arbitrarily n is considered to be the gene involved, and the allele n₂ carried by i is dominant to the n₁ allele carried by clone a.

(ii) Fusion Class IV contains the parental plasmodium a+i and must therefore contain plasmodia which are genotypically $\frac{f_1}{f_2} \frac{n_1}{n_2}$, that is are heterozygous at both loci. Since n₂ is dominant to n₁, this class is phenotypically $\frac{f_1}{f_2} n_2$ and may therefore contain plasmodia of genotypes $\frac{f_1}{f_2} \frac{n_1}{n_2}$ and $\frac{f_1}{f_2} \frac{n_2}{n_2}$. (Leaving the question of dominance at the f locus open for the present, if one allele is dominant it must be the f₁ allele, see (i) above)

(iii) Since clone i is $\frac{f_2}{f_2} n_2$, and n₂ is dominant, the hypothesis predicts that this clone can only give rise to two fusion classes, $\frac{f_1}{f_2} n_2$ (Group IV, see (ii)) and $\frac{f_2}{f_2} n_2$. It will be seen from Table 4 that plasmodia which have i as one parent belong either to fusion class IV or VI. Class VI is therefore considered to be pheno-

typically $\underline{f}_2/\underline{f}_2 \underline{n}_2$ (genotypically $\underline{f}_2/\underline{f}_2 \underline{n}_1/\underline{n}_2$ or $\underline{f}_2/\underline{f}_2 \underline{n}_2/\underline{n}_2$).

(iv) All clones which produce plasmodia of fusion Group VI must therefore be carrying the \underline{f}_2 allele, because VI is an \underline{f}_2 homozygote class. Therefore clones 7118 must be \underline{f}_2 (7124+7118 is VI $\underline{f}_2/\underline{f}_2 \underline{n}_2$), and 7119 must be \underline{f}_2 (7119+7120 is VI $\underline{f}_2/\underline{f}_2 \underline{n}_2$). Therefore Group V which contains the plasmodium 7119+7118 must be an \underline{f}_2 homozygote class. Since Group VI is established as representing the $\underline{f}_2/\underline{f}_2 \underline{n}_2$ phenotype (see (iii)), fusion Group V must be phenotypically $\underline{f}_2/\underline{f}_2 \underline{n}_1$ (genotypically $\underline{f}_2/\underline{f}_2 \underline{n}_1/\underline{n}_1$).

(v) Clone 7118, because it gives plasmodia of Group V (e.g. 7119+7118) $\underline{f}_2/\underline{f}_2 \underline{n}_1/\underline{n}_1$ must be genotypically $\underline{f}_2 \underline{n}_1$. Group III, which contains the plasmodium a ($\underline{f}_1 \underline{n}_1$) + 7118 ($\underline{f}_2 \underline{n}_1$) is therefore phenotypically $\underline{f}_1/\underline{f}_2 \underline{n}_1$ (genotypically $\underline{f}_1/\underline{f}_2 \underline{n}_1/\underline{n}_1$).

(vi) Since the plasmodium 7119+7115 is Group III, and 7119 is $\underline{f}_2 \underline{n}_1$ (see (iv)), clone 7115 must be genotypically $\underline{f}_1 \underline{n}_1$. It is therefore predicted that the plasmodium a ($\underline{f}_1 \underline{n}_1$) + 7115 ($\underline{f}_1 \underline{n}_1$) is of genotype $\underline{f}_1/\underline{f}_1 \underline{n}_1/\underline{n}_1$, phenotype $\underline{f}_1/\underline{f}_1 \underline{n}_1$. By similar reasoning the plasmodium E55 ($\underline{f}_1 \underline{n}_2$) + 7115 ($\underline{f}_1 \underline{n}_1$) is predicted to be genotypically $\underline{f}_1/\underline{f}_1 \underline{n}_1/\underline{n}_2$, phenotypically $\underline{f}_1/\underline{f}_1 \underline{n}_2$. It will be observed from Table 4 that in every case that a plasmodium is deduced to be an \underline{f}_1 homozygote, it is of the q type, and the fusion behaviour cannot therefore be observed experimentally. It is therefore impossible on the data of

Table 4 to determine whether the $\underline{f}_1/\underline{f}_1$ homozygotes have a phenotype distinct from the $\underline{f}_1/\underline{f}_2$ heterozygotes (i.e. whether the \underline{f}_1 allele is dominant).

In a majority of cases both clones contributing to a q plasmodium are predicted to be \underline{f}_1 , the exceptions being clones 7140 and 7124 which are of \underline{f}_2 genotype. These two clones yield q plasmodia when crossed to \underline{f}_1 clones, and normal plasmodia when crossed to \underline{f}_2 clones. A simple hypothesis to unify these observations is that the q plasmodial behaviour is due to a recessive allele of a gene \underline{q} , associated or linked with the \underline{f}_1 allele of gene \underline{f} in the parental plasmodium A7+i. The recessive allele, which in the homozygous state gives the q phenotype, is termed \underline{q}^- , the alternative dominant allele \underline{q}^+ . That the \underline{q} gene is separable (by crossing over) from the \underline{f} gene is indicated by the occurrence of the two $\underline{f}_2\underline{q}^-$ clones (7140 and 7124). (This is further supported by certain data presented later.) Table 7 is a classification of the progeny clones of A7+i on the model that this plasmodium is genotypically $\underline{mt}_1/\underline{mt}_2 \underline{f}_1\underline{q}^-/\underline{f}_2\underline{q}^+ \underline{n}_1/\underline{n}_2$, and also analyses the allele ratios and linkage. The frequency of recombination between \underline{f} and \underline{q} is of the order of 10%.

To complete the analysis of these characters, since it appeared that the \underline{f}_1 allele and the \underline{q}^- allele were separable by crossing over, it was necessary to obtain clones carrying \underline{f}_1 and \underline{q}^+ , and with them to discover if the \underline{f}_1 genotype was distinct from the $\underline{f}_1/\underline{f}_2$ genotype

TABLE 7

The Classification of A7+i Progeny Clones in Terms of Genes mt, f, n and q: The Allele Ratios and

Recombination Frequencies of the Genes Are Also Represented

Predicted progeny clone genotypes	Progeny clones of each genotype	Totals
No crossing over between <u>f</u> and <u>q</u>	<u>mt</u> ₁ <u>f</u> ₁ <u>n</u> ₁ <u>q</u> ⁺⁺	1
	<u>mt</u> ₁ <u>f</u> ₁ <u>n</u> ₂ <u>q</u> ⁺⁻	3
	<u>mt</u> ₁ <u>f</u> ₂ <u>n</u> ₁ <u>q</u> ⁺⁺	5
	<u>mt</u> ₁ <u>f</u> ₂ <u>n</u> ₂ <u>q</u> ⁺⁻	2
	<u>mt</u> ₂ <u>f</u> ₁ <u>n</u> ₁ <u>q</u> ⁺⁻	4
	<u>mt</u> ₂ <u>f</u> ₁ <u>n</u> ₂ <u>q</u> ⁺⁺	3
	<u>mt</u> ₂ <u>f</u> ₂ <u>n</u> ₁ <u>q</u> ⁺⁻	4
	<u>mt</u> ₂ <u>f</u> ₂ <u>n</u> ₂ <u>q</u> ⁺⁺	2
Crossing over between <u>f</u> and <u>q</u>	<u>a</u> (parental), 7105	
	7129, E55, E80	
	7110, 7119, 7123, 7125, 7132	
	7141, 7138	
	7115, 7126, 7114, 7137	
	7103, 7131, 7117	
	7118, 7101, 7122, 7108	
	7120, 7133, <u>i</u> (parental)	
	7140	1
	7124	1
Parental plasmodium <u>A7/i</u> <u>mt</u> ₁ / <u>mt</u> ₂ <u>f</u> ₁ / <u>f</u> ₂ <u>n</u> ₁ / <u>n</u> ₂ <u>q</u> ⁺⁺ / <u>q</u> ⁺⁻		26

Linkage (recombination) N.B. $f-q$	
$\underline{mt} - \underline{f}$ Parentals $\underline{mt_1 f_1} \underline{mt_2 f_2}$ Recombinants $\underline{mt_1 f_2} \underline{mt_2 f_1}$ RF > 50%	$\underline{f} - \underline{q}$ Parentals $\underline{f_1 q^-} \underline{f_2 q^+}$ Recombinants $\underline{f_1 q^+} \underline{f_2 q^-}$ RF > 8.3%
$\underline{mt} - \underline{n}$ Parentals $\underline{mt_1 n_1} \underline{mt_2 n_2}$ Recombinants $\underline{mt_1 n_2} \underline{mt_2 n_1}$ RF > 50%	$\underline{f} - \underline{n}$ Parentals $\underline{f_1 n_1} \underline{f_2 n_2}$ Recombinants $\underline{f_1 n_2} \underline{f_2 n_1}$ RF > 50%
$\underline{mt} - \underline{q}$ Parentals $\underline{mt_1 q^-} \underline{mt_2 q^+}$ Recombinants $\underline{mt_1 q^+} \underline{mt_2 q^-}$ RF > 50%	$\underline{n} - \underline{q}$ Parentals $\underline{n_1 q^-} \underline{n_2 q^+}$ Recombinants $\underline{n_1 q^+} \underline{n_2 q^-}$ RF > 50%

Allele Ratios	
$\underline{mt_1} : \underline{mt_2}$	12:14
$\underline{f_1} : \underline{f_2}$	11:15
$\underline{n_1} : \underline{n_2}$	15:11
$\underline{q^-} : \underline{q^+}$	13:13

(whether \underline{f}_1 was dominant to \underline{f}_2).

(d) The production of $\underline{f}_1/\underline{f}_1$ q^+ plasmodia by random crossing

The occurrence of the hypothetical $\underline{f}_1/\underline{f}_1$ \underline{n}_1 q^+ and $\underline{f}_1/\underline{f}_1$ \underline{n}_2 q^+ fusion classes was first tested for by a technique which provides large numbers of progeny plasmodia without the labour of cloning, cross-plating, etc. This simplified technique was used because the frequency of the recombinant clones was uncertain, and might have been considerably less than 10%. Spores derived from a+i were plated onto liver infusion agar + paba + bacteria, to give approximately 10 plaques per plate. These plaques grew and met at various points, giving rise to small plasmodia. From each plate a number of plasmodia could be removed on blocks of agar, and cultured onto SDM agar. More than one plasmodium was taken from a plate only if it could be clearly seen from the positions of the plaques involved that the plasmodia had separate origins. This technique provides relatively easily a large number of progeny plus progeny plasmodia of the q^+ type, the drawback of the procedure being that the amoebal clones involved are not retained, and cannot therefore be referred to for further analysis. Eighty four plasmodia produced by this method were tested against tester plasmodia representing the four recognized fusion classes (25+26 representing III, a+i IV, 40+18 V and 24+i VI). Of the eighty four plasmodia, fifty two fused with the group IV tester, eighteen with VI, seven with III and four with V. Three plasmodia failed to

TABLE 8

The Fusion Behaviour of 84 Plasmodia Derived from RandomCrossing of a+i Progeny Clones

Pheno- type	Fusion class	No. of plasmodia observed to belong to each fusion class	Expectation assuming 10% recombination between \underline{f} and \underline{g} (to the nearest whole no.)
$\underline{f}_1 \underline{f}_1 \underline{n}_1$	(I)*	(1)	1
$\underline{f}_1 \underline{f}_1 \underline{n}_2$	(II)*	(2)	4
$\underline{f}_1 \underline{f}_2 \underline{n}_1$	III	7	13
$\underline{f}_1 \underline{f}_2 \underline{n}_2$	IV	52	38
$\underline{f}_2 \underline{f}_2 \underline{n}_1$	V	4	7
$\underline{f}_2 \underline{f}_2 \underline{n}_2$	VI	18	21
		84	84

* (I) and (II) provision at this time.

The sizes of the observed classes correspond approximately with the expectation, but there appears to be some excess of class IV.

TABLE 9

Possible Genotypes of Progeny from the Plasmodium E55+i, and
Plasmodia derived from such Progeny

(E55 \underline{mt}_1 \underline{f}_1 \underline{q}^- \underline{n}_2 , \underline{i} \underline{mt}_2 \underline{f}_2 \underline{q}^+ \underline{n}_2)

Possible genotypes of progeny clones	Plasmodia resulting from cross with \underline{a} (\underline{mt}_1 \underline{f}_1 \underline{q}^- \underline{n}_1)	No. of such plasmodia
Involving no crossing over	\underline{mt}_1 \underline{f}_1 \underline{q}^- \underline{n}_2	-
	\underline{mt}_1 \underline{f}_2 \underline{q}^+ \underline{n}_2	-
	\underline{mt}_2 \underline{f}_1 \underline{q}^- \underline{n}_2	\underline{q}
	\underline{mt}_2 \underline{f}_2 \underline{q}^+ \underline{n}_2	$\underline{f}_1\underline{f}_2$ \underline{n}_2 (IV)
Resulting from crossing over between \underline{f} and \underline{q}	\underline{mt}_1 \underline{f}_1 \underline{q}^+ \underline{n}_2	-
	\underline{mt}_1 \underline{f}_2 \underline{q}^- \underline{n}_2	-
	\underline{mt}_2 \underline{f}_1 \underline{q}^+ \underline{n}_2	$\underline{f}_1\underline{f}_1$ \underline{n}_2 (II)
	\underline{mt}_2 \underline{f}_2 \underline{q}^- \underline{n}_2	\underline{q}
		8

fuse with any of the four tester strains. Of these three plasmodia, two fused with each other, and the other would not fuse with any known plasmodium. It was considered provisionally that these three plasmodia represented $\underline{f}_1/\underline{f}_1 \underline{q}^+$ types, possibly the single one being of fusion group I ($\underline{f}_1/\underline{f}_1 \underline{n}_1$) and the pair group II ($\underline{f}_1/\underline{f}_1 \underline{n}_2$) (Table 8). It was therefore accepted as a working hypothesis that the \underline{f}_1 allele was not dominant to the \underline{f}_2 allele, and that recombination occurred between the \underline{f} and the \underline{q} gene with a frequency of about 10%.

(e) The isolation of amoebae of type $\underline{f}_1 \underline{q}^+ \underline{n}_2$ and the production of $\underline{f}_1/\underline{f}_1 \underline{n}_2 \underline{q}^+$ (II) plasmodia

Amoebal clones of type $\underline{f}_1 \underline{q}^+$ could have been produced from the three probable $\underline{f}_1/\underline{f}_1 \underline{q}^+$ plasmodia derived from the random cross experiment (Table 8), but it was considered more satisfactory to use clones whose exact derivation was known.

It was therefore decided to produce progeny from an $\underline{f}_1 \underline{q}^-/\underline{f}_2 \underline{q}^+$ plasmodium, and select from amongst these those recombinants of the $\underline{f}_1 \underline{q}^+$ type. The plasmodium used was $\underline{E55+i} \underline{mt}_1 \underline{f}_1 \underline{q}^- \underline{n}_2 / \underline{mt}_2 \underline{f}_2 \underline{q}^+ \underline{n}_2$ (fusionn Group IV). Since $\underline{E55+i}$ is homozygous for \underline{n}_2 the recombinant progeny type will be $\underline{f}_1 \underline{q}^+ \underline{n}_2$. When this recombinant type is crossed against an $\underline{f}_1 \underline{q}^-$ clone plasmodia of fusion type II ($\underline{f}_1/\underline{f}_1 \underline{n}_2$) will be produced (see Table 9).

Fifty two amoebal clones were produced from $\underline{E55+i}$. These were crossed against clone a ($\underline{mt}_1 \underline{f}_1 \underline{q}^- \underline{n}_1$). Seventeen plasmodia were

produced (i.e. seventeen of the fifty two clones were \underline{mt}_2). Of these seventeen plasmodia nine were of the \underline{q}^- type ($\underline{q}^-/\underline{q}^-$ homozygotes) and eight were \underline{q}^+ ($\underline{q}^+/\underline{q}^-$ heterozygotes). The eight \underline{q}^+ plasmodia were tested for fusion type, six were of fusion class IV and two of the derived type II ($\underline{f}_1/\underline{f}_1 \underline{n}_2$). (In other words of the eight \underline{q}^+ clones which gave \underline{q}^+ plasmodia with a ($\underline{f}_1 \underline{q}^- \underline{n}_1$) six were $\underline{f}_2 \underline{q}^+ \underline{n}_2$ giving $\underline{f}_1/\underline{f}_2 \underline{n}_2$ (IV) plasmodia, and two were $\underline{f}_1 \underline{q}^+ \underline{n}_2$ giving $\underline{f}_1/\underline{f}_1 \underline{n}_2$ (II) plasmodia.)

(The fusion type of the eight plasmodia was determined by testing them against plasmodia representing the four established fusion types (a+i, IV, 7119+7137 III, 7119+7122 V and 7124+7120 VI) and also with the plasmodia in Table 8 provisionally considered to represent fusion Group I ($\underline{f}_1/\underline{f}_1 \underline{n}_1$) and II ($\underline{f}_1/\underline{f}_1 \underline{n}_2$))

One of the two group II plasmodia (a+E55138) was sclerotized for reference purposes. Clone E55138 ($\underline{f}_1 \underline{n}_2$) was crossed against clone 7119 ($\underline{f}_2 \underline{n}_1$), the resulting plasmodium being, as expected, group IV ($\underline{f}_1/\underline{f}_2 \underline{n}_2$).

To summarize this passage, plasmodia (\underline{q}^+) of type $\underline{f}_1/\underline{f}_1 \underline{n}_2$ (II) were produced following isolation of the recombinant amoebal type $\underline{f}_1 \underline{q}^+ \underline{n}_2$. The provisional description of two plasmodia in Table 8 as $\underline{f}_1/\underline{f}_1 \underline{n}_2$ was confirmed by this analysis.

(f) The isolation of amoeba of type $f_1 q^+ n_1$ and the production of $f_1/f_1 n_1 q^+$ plasmodia (fusion group I)

To obtain plasmodia of fusion group I ($f_1/f_1 n_1$) it was necessary to produce amoebal clones of type $f_1 q^+ n_1$. To do this spores were obtained from the group II plasmodium a+E55i38 ($mt_1 f_1 q^- n_1 / mt_2 f_1 q^+ n_2$) and ten clones prepared. All progeny clones of this plasmodium should be f_1 , one half of them should be q^+ and of these one half should be n_1 (since n is unlinked to q) (therefore a quarter will be of the desired type $f_1 q^+ n_1$). The ten clones were crossed to eight different amoebal strains giving the plasmodia shown in Table 10. The plasmodia were classified for fusion type (Table 10) by testing them against five plasmodia representing the established fusion types (II a+E55i38, III 7125+7126, IV a+i, V 7119+7118, VI 7124+i) and also against the plasmodium provisionally believed to represent fusion group I ($f_1/f_1 n_1$) (see Table 8). Each of the plasmodia represented in Table 10 fused with only one of these six testers. On the basis of the data of Table 10 it is possible to describe exactly the ten a+E55i38 progeny clones with respect to the genes $mt f n q$. Two plasmodia were of fusion class I ($f_1/f_1 n_1$) and the desired amoebal type $f_1 q^+ n_1$ was therefore detected. (As predicted of the ten clones, all were f_1 , the ratio of q^+/q^- was 1:1 (5:5), the ratio of mt_1/mt_2 was nearly 1:1 (6:4), and the ratio of n_1/n_2 was nearly 1:1 (6:4). One of the $f_1/f_1 n_1$ plasmodia was sclerotized for reference purposes.

TABLE 10

Detection of Group I ($f_1 f_1 n_1$) Plasmodia

10 Progeny clones of <u>a+E55138</u>	<u>a</u> $mt_1 f_1 q n_1$	<u>E55</u> $mt_1 f_1 q n_2$	<u>7132</u> $mt_1 f_2 q n_1$	<u>7124</u> $mt_1 f_2 q n_2$	<u>7126</u> $mt_2 f_1 q n_1$	<u>7131</u> $mt_2 f_1 q n_2$	<u>7118</u> $mt_2 f_2 q n_1$	<u>1</u> $mt_2 f_2 q n_2$
Clone 1 $mt_1 f_1 q n_1$					q	q	III	IV
2 $mt_1 f_1 q n_1$					q	q	III	
4 $mt_1 f_1 q n_1$					I	II	III	IV
6 $mt_1 f_1 q n_2$					q	q	IV	IV
7 $mt_1 f_1 q n_1$						q	III	
9 $mt_1 f_1 q n_2$						q	IV	
3 $mt_2 f_1 q n_2$	q	q	IV	q				
5 $mt_2 f_1 q n_1$	I	II	III	IV				
8 $mt_2 f_1 q n_2$	II	II	IV	IV				
10 $mt_2 f_1 q n_1$		II	III	IV				

Plasmodia formed by crossing progeny clones from a+E55138 ($mt_1 f_1 q n_1 / mt_2 f_1 q n_2$) against various other clones. The fusion group of the resulting plasmodia is included in the Table (see text). The deduced genotype of the progeny clones is represented in the left hand column.

The interpretation of Table 10 was supported by crossing progeny clones of aE55i38 against each other. As predicted the resulting plasmodia were q^+ , fusion group I or II.

(g) To summarize the analysis of fusion behaviour in the Wisconsin isolate of P.polycephalum:

(1) Carlile and Dee proposed that plasmodial fusion was controlled by a single gene f, two alleles being present f₁ and f₂. Plasmodia could be of three phenotypes f₁f₁, f₁f₂ and f₂f₂.

(2) Subsequent experiments have shown that this is not in fact the case, and that the three genotypes detected by Carlile and Dee in their experiments are inaccurately described by their hypothesis.

(3) It is now considered that fusion behaviour is controlled by two genes terms f and n. Plasmodia must be phenotypically identical at these loci for fusion to be possible between them. Two alleles of f (f₁, f₂) and two alleles of n, (n₁ and n₂) have been found. Dominance occurs at the n locus, n₂ being dominant to n₁. Six fusion phenotypes have been detected, I f₁/f₁ n₁, II f₁/f₁ n₂, III f₁/f₂ n₁ ("f₁f₁" of Carlile and Dee), IV f₁/f₂ n₂ ("f₁f₂" of Carlile and Dee) V f₂/f₂ n₁, and VI f₂/f₂ n₂ ("f₂f₂" of Carlile and Dee).

(4) The reason for the observation of only three types by Carlile and Dee is apparently the occurrence of an allele q⁻ of gene q, linked to the f₁ allele of gene f in the parental clones used. The q⁻ allele is recessive, in the homozygous state it produces q

plasmodia, which show inability to grow on SDM, and which cannot therefore be analysed for fusion type. Therefore in the original experiment of Carlile and Dee, although $\underline{f_1f_1}$ homozygotes were apparently produced, they are not apparent in the fusion analysis because they were of "q" type.

(5) The genes involved in fusion control, \underline{f} and \underline{n} are unlinked to the amoebal mating type locus \underline{mt} , and to each other. The \underline{q} locus is apparently some 10 map units from gene \underline{f} . This is the first report of linkage and crossing over in P.polycephalum.

(6) The amoebal mating type locus has no effect on plasmodial fusion behaviour.

(B) The killing reaction in the Wisconsin isolate

It is frequently observed that, following fusion, one or both plasmodia show lysis in the area of fusion (Plate 5). This area of lysis may, in extreme cases, involve the death of both plasmodia. Alternatively the killing may be of very limited occurrence and a heterokaryon may become established. Plasmodia may show fusion without any apparent killing reaction. The first sign of a killing reaction is the appearance of clots, or debris in the affected veins. This is followed by the cessation of streaming in the affected veins. The affected area then lyses, becoming white due to the diffusion of the yellow pigment into the agar.

It has not yet been possible to complete the analysis of the genetic control of the killing reaction. The two major difficulties are the variation in intensity of the killing reaction, which makes objective scoring difficult in cases of very mild reaction, and the obvious fact that killing can only be investigated between plasmodia which will fuse with each other. It is therefore very laborious to attempt to cross-reference killing data from the six different fusion groups.

The procedure adopted to study killing was to select one fusion class (VI), which carried the \underline{n}_2 dominant character. (This enables more amoebal clones to be studied within the fusion class than if an \underline{n}_1 group was concentrated on.) (That is, within group VI, amoebal clones of type $\underline{f}_2\underline{n}_1$ and $\underline{f}_2\underline{n}_2$ can be studied, whereas with group V only clones of type $\underline{f}_2\underline{n}_1$ could be studied.) Within the selected fusion class (VI) it was intended to produce a large series (eventually several) of plasmodia with one parent in common. By studying killing within such a large series the number of haploid amoebal "killer" genotypes should become apparent. (Fusion analysis was approached in an analogous way, see p.28). Such an experimental approach is relatively simple if no dominance is occurring, but if dominance is involved it is necessary to produce numerous large series.

Clone $\underline{1}$ ($\underline{f}_2\underline{n}_2$) was chosen as the "common parent" of the large series. The eight $\underline{1} \times$ progeny plasmodia shown in Table 4 were carefully

observed for killing reaction. These eight plasmodia fell into four groups, the members of a group showing no killing with each other, and killing with plasmodia of other killing groups. Group (i) consisted of 7132+i, 7119+i, 7140+i; (ii) 7125+i, 7123+i, 7110+i; (iii) 7141+i and (iv) 7124+i. With such a small series it would be premature to conclude that only four such groups are present in i x progeny plasmodia, but certainly at least four such groups occur, and the number of groups is unlikely to be much greater than four (since several plasmodia occur in groups (i) and (ii)). Since at least four haploid "killer" genotypes occur it can be concluded that at least two genes are involved. At present this and other series are insufficiently large for firm conclusions to be drawn concerning dominance etc.

Killing has never been observed between plasmodia of identical genotype (e.g. a+i fused with a+i). It is therefore reasonable to conclude that it is a reaction due to genetic dissimilarity and not simply a consequence of fusion. The killing reaction between particular pairs of plasmodia is repeatable. It is therefore concluded (i) that the killing reaction is a response to fusion between plasmodia which are genetically dissimilar; (ii) that at least two genes, and possibly more are involved in the control of the "killing reaction" phenotype of a plasmodium; and (iii) that at least four, and probably not more than ten "killing reaction" phenotypes are present in

plasmodia derived from the Wisconsin isolate.

The experimental importance of the killing reaction is that it may operate to prevent heterokaryon formation. It is known from numerous experiments that inbreeding reduces or eliminates the occurrence of the killing reaction. In practice therefore in view of the present incomplete understanding of the control of killing, where heterokaryon formation has been used experimentally in the work reported in this thesis, plasmodia have been chosen to have as much genetic similarity as possible, and with such plasmodia non-killing combinations have been found by trial and error.

(C) Control of fusion in the Indiana isolate

(a) The fusion behaviour of backcross plasmodia

The amoebal clones B173 (mt₃) and B174 (mt₄) were crossed and thirteen progeny clones of mt₃ and fourteen clones of mt₄ were isolated and backcrossed to the appropriate parent. Some of the resulting plasmodia were tested for fusion with B173+B174 (Table 11). As in the similar test with the Wisconsin strains (Table 1 and Table 4) some of the backcross plasmodia fused with the parent plasmodium, and some did not. Genotypes were assigned to the plasmodia, on the hypothesis that a single pair of alleles were segregating without showing dominance. (The segregating alleles are termed f₃ and f₄, since subsequent analysis showed that it was alleles of the gene f

which were segregating, (see below).) Allele \underline{f}_3 was arbitrarily assigned to B174 and allele \underline{f}_4 to B173. Those backcross plasmodia which fused with the parent B173+B174 ($\underline{f}_3\underline{f}_4$) were described as $\underline{f}_3\underline{f}_4$. (On the assumption that identity of \underline{f} type is necessary for fusion.) On the same model plasmodia failing to fuse with $\underline{f}_3\underline{f}_4$ were deduced to be $\underline{f}_3\underline{f}_3$ or $\underline{f}_4\underline{f}_4$, depending on the parent clone involved in the backcross. Three plasmodia representing the genotypes $\underline{f}_3\underline{f}_3$, $\underline{f}_4\underline{f}_4$ and $\underline{f}_3\underline{f}_4$ were then used as tester strains to classify all the plasmodia derived from B173+B174 (Table 12). Contrary to expectation, all the plasmodia which failed to fuse with the $\underline{f}_3\underline{f}_4$ tester, fused with both the deduced $\underline{f}_3\underline{f}_3$ and the $\underline{f}_4\underline{f}_4$ testers. In a further test, three of the assumed $\underline{f}_3\underline{f}_3$ and six of the assumed $\underline{f}_4\underline{f}_4$ plasmodia were tested for fusion, all combinations fusing. In other words, only two fusion classes are present in the backcross plasmodia derived from B173+B174. If the genetic interpretation is correct (Table 11) one of these two fusion classes represents the heterozygote $\underline{f}_3\underline{f}_4$, and the other consists of the two homozygous types $\underline{f}_3\underline{f}_3$ and $\underline{f}_4\underline{f}_4$. (A hypothesis to explain the unexpected fusion of genetically dissimilar $\underline{f}_3\underline{f}_3$ and $\underline{f}_4\underline{f}_4$ plasmodia will be proposed in the Discussion.)

(b) The fusion behaviour of $\underline{f}_3\underline{f}_3 + \underline{f}_4\underline{f}_4$ heterokaryons

Heterokaryon formation was used to establish definitely that $\underline{f}_3\underline{f}_3$ and $\underline{f}_4\underline{f}_4$ plasmodia, although behaviourally identical, were

TABLE 11.

Results of Testing Plasmodia Derived from the Cross B173 x B174
for Fusion with B173+B174

Plasmodium		Reaction with B173+B174 (<u>f₄f₃</u>) [*]		Total
Mating type of progeny clone [*]	Mating type of parent clone [*]	Fusion	Non-Fusion	
<u>mt₃f₃</u> <u>mt₃f₄</u>	<u>mt₄f₃</u> (strain <u>B174</u>)	3 (<u>f₄f₃</u>)	2 (<u>f₃f₃</u>)	5
<u>mt₄f₃</u> <u>mt₄f₄</u>	<u>mt₃f₄</u> (strain <u>B173</u>)	3 (<u>f₃f₄</u>)	2 (<u>f₄f₄</u>)	5

* Hypothetical f-types are incouded (see text).

Plasmodia were produced by backcrossing progeny clones B173+B174
 with the appropriate parent.

TABLE 12

Results of Testing Plasmodia Derived from the Cross BL73 x BL74 against Three Tester

Strains

Plasmodium		Reaction with tester strains			Deduced f-type of plasmodium	Deduced genotype of progeny clone	Total
Mating type of progeny clone	Mating type and f-type of parent clone	$\underline{f_3f_3}$	$\underline{f_4f_4}$	$\underline{f_3f_4}$			
$\underline{mt_3}$	$\underline{mt_4f_3}$	F	F	NF	$\underline{f_3f_3}$	$\underline{mt_3f_3}$	7
$\underline{mt_3}$	$\underline{mt_4f_3}$	NF	NF	F	$\underline{f_4f_3}$	$\underline{mt_3f_4}$	6
$\underline{mt_4}$	$\underline{mt_3f_4}$	NF	NF	F	$\underline{f_3f_4}$	$\underline{mt_4f_3}$	6
$\underline{mt_4}$	$\underline{mt_3f_4}$	F	F	NF	$\underline{f_4f_4}$	$\underline{mt_4f_4}$	8

Plasmodia were produced by backcrossing progeny clones of BL73+BL74 with the appropriate

parent.

genetically dissimilar, representing the two f homozygotes.

Several pairs of plasmodia of supposed genotypes f₃f₃ and f₄f₄ were fused. Of the various combination, one pair was selected which showed no killing reaction following fusion. This heterokaryon (f₃f₃+f₄f₄) was grown on SDM agar for several days and then tested for fusion type with f₃f₄ and f₃f₃ or f₄f₄ testers. The heterokaryon (f₃f₃+f₄f₄) fused with the f₃f₄ heterozygote, and did not fuse with f₃f₃ or f₄f₄ plasmodia. This confirms the description of plasmodia in Table 11 as f₃f₃ and f₄f₄ homozygotes, despite their being behaviourally indistinguishable.

To study the process by which the heterokaryon came to behave as a heterozygote, a modified form of fusion test was used.

(i) Four plasmodia were inoculated onto a single large (15 cm) petri dish containing SDM agar. The four blocks carrying the plasmodia were placed in a row around the edge of the dish. The four plasmodia were of the following types, firstly an f₃f₄, near to this an f₃f₃, some way further round the edge an f₄f₄, and then near to it an f₃f₄.

(i.e. f₃f₄, f₃f₃ f₄f₄, f₃f₄)

(ii) The f₃f₄ and f₃f₃ pair of plasmodia grew and made contact, no fusion occurring. Some distance away the f₄f₄ and f₃f₄ pair made contact, no fusion occurring. At this point four discrete plasmodia were present on the petri dish.

(iii) The four plasmodia continued to grow, eventually the $\underline{f_3f_3}$ and $\underline{f_4f_4}$ plasmodia coming into contact. Fusion occurred between them and a heterokaryon ($\underline{f_3f_3} + \underline{f_4f_4}$) was established. (Plate 6 and 7). The time of this fusion was accurately recorded. The heterokaryon, from its time of origin, was in intimate contact with $\underline{f_3f_4}$ plasmodia, and it was therefore possible to observe how long it took for the heterokaryon to adopt the heterozygote $\underline{f_3f_4}$ fusion character (since when this occurs fusion will be observed between the heterokaryon and the two $\underline{f_3f_4}$ plasmodia) (Plate 8). The heterokaryon, if formed from roughly equal quantities of $\underline{f_3f_3}$ and $\underline{f_4f_4}$ plasmodia, took about 24 hours before it behaved as a heterozygote. During this time the heterokaryon and the two $\underline{f_3f_4}$ plasmodia flanking it grew across the plate, maintaining intimate contact but showing no fusion. After a period of approximately 24 hours, small areas of rather atypical fusion occurred simultaneously between the heterokaryon and the $\underline{f_3f_4}$ plasmodia on either side of it. (The atypical fusion is termed pseudo-killing, since it bears some resemblance to killing at a cursory inspection. However under a microscope no cessation of streaming is apparent and no lysis, and the phenomenon probably bears no physiological resemblance to killing.) After a short period the atypical fusions develop into normal fusions, and the three plasmodia fuse into a single organism. (No killing occurred between the heterokaryon and the $\underline{f_3f_4}$ plasmodium.)

PLATE 6

THE FUSION OF AN f_3f_3 and an f_4f_4 PLASMODIUM (1)

The plasmodia from the two middle blocks (an f_3f_3 and an f_4f_4) have fused. The heterokaryon (f_3f_3/f_4f_4) has met one of the f_3f_4 tester plasmodia, and has not fused with it.

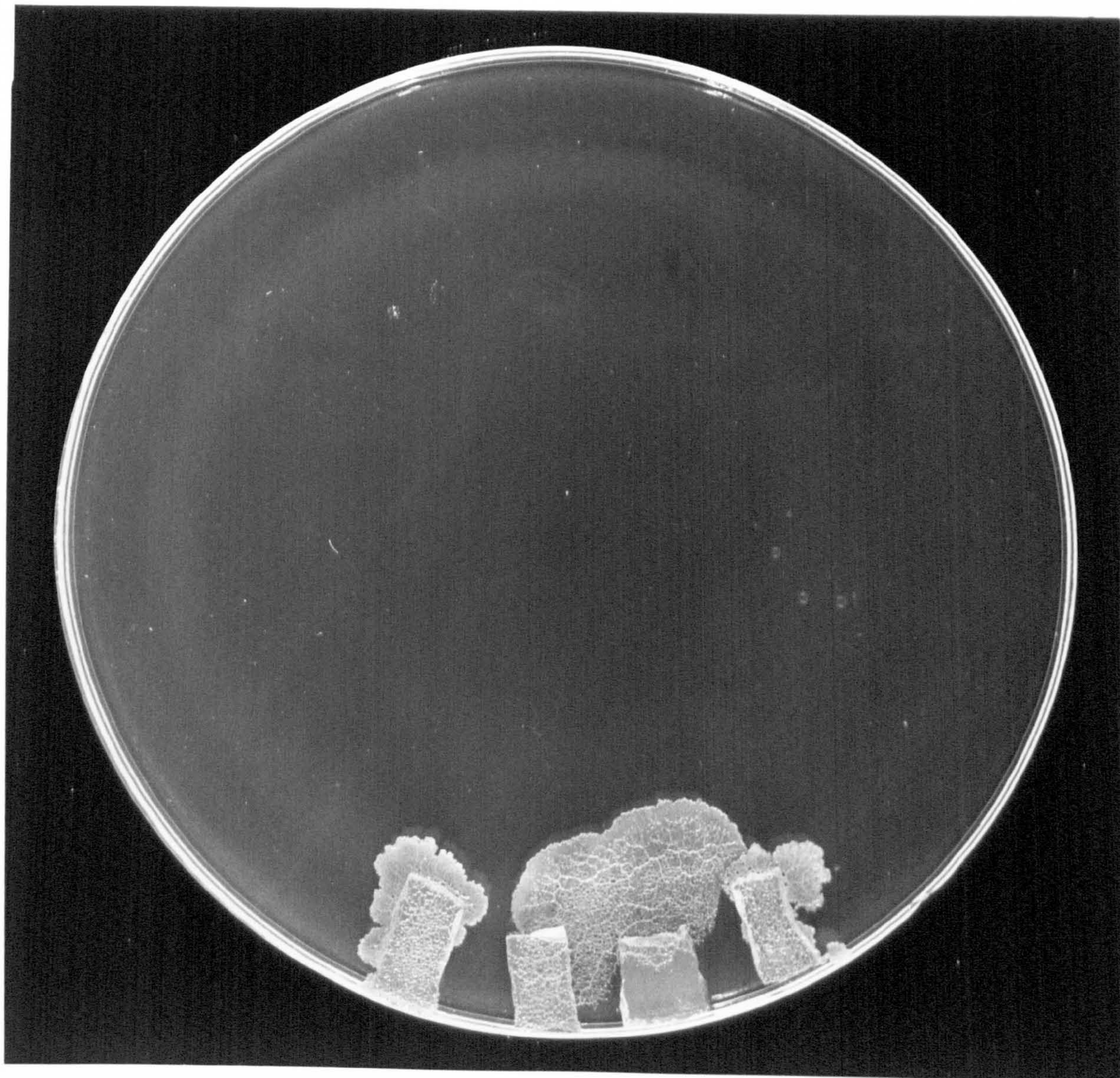


PLATE 7

THE FUSION OF AN $f_3 f_3$ WITH AN $f_4 f_4$ PLASMODIUM(2)

The plate shows a later stage of the experiment shown in PLATE 6. The $\frac{f_3 f_3}{f_4 f_4}$ heterokaryon (the central plasmodium) has still not fused with the $\frac{f_3 f_4}{f_3 f_4}$ testers (the two outside plasmodia)

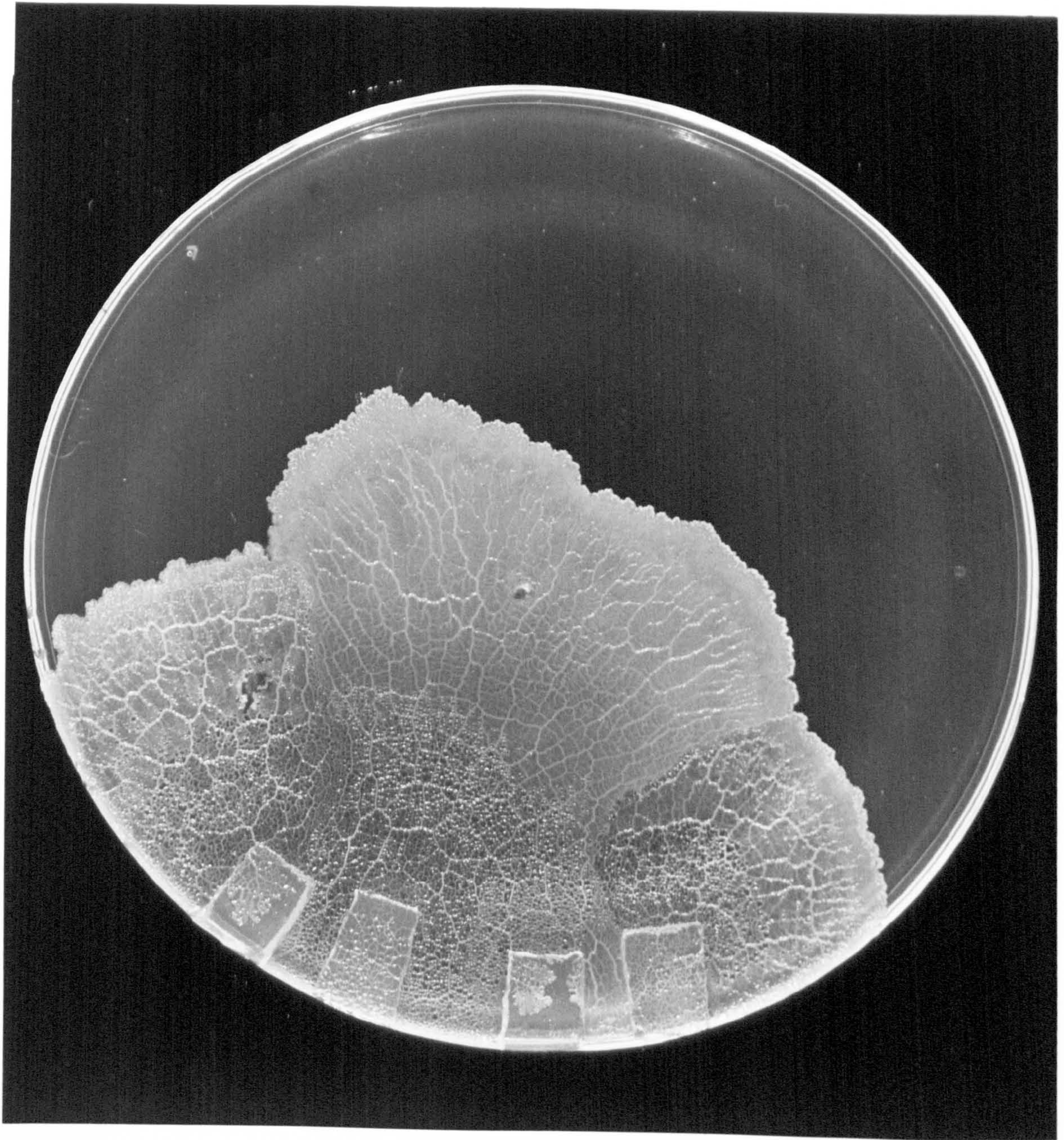
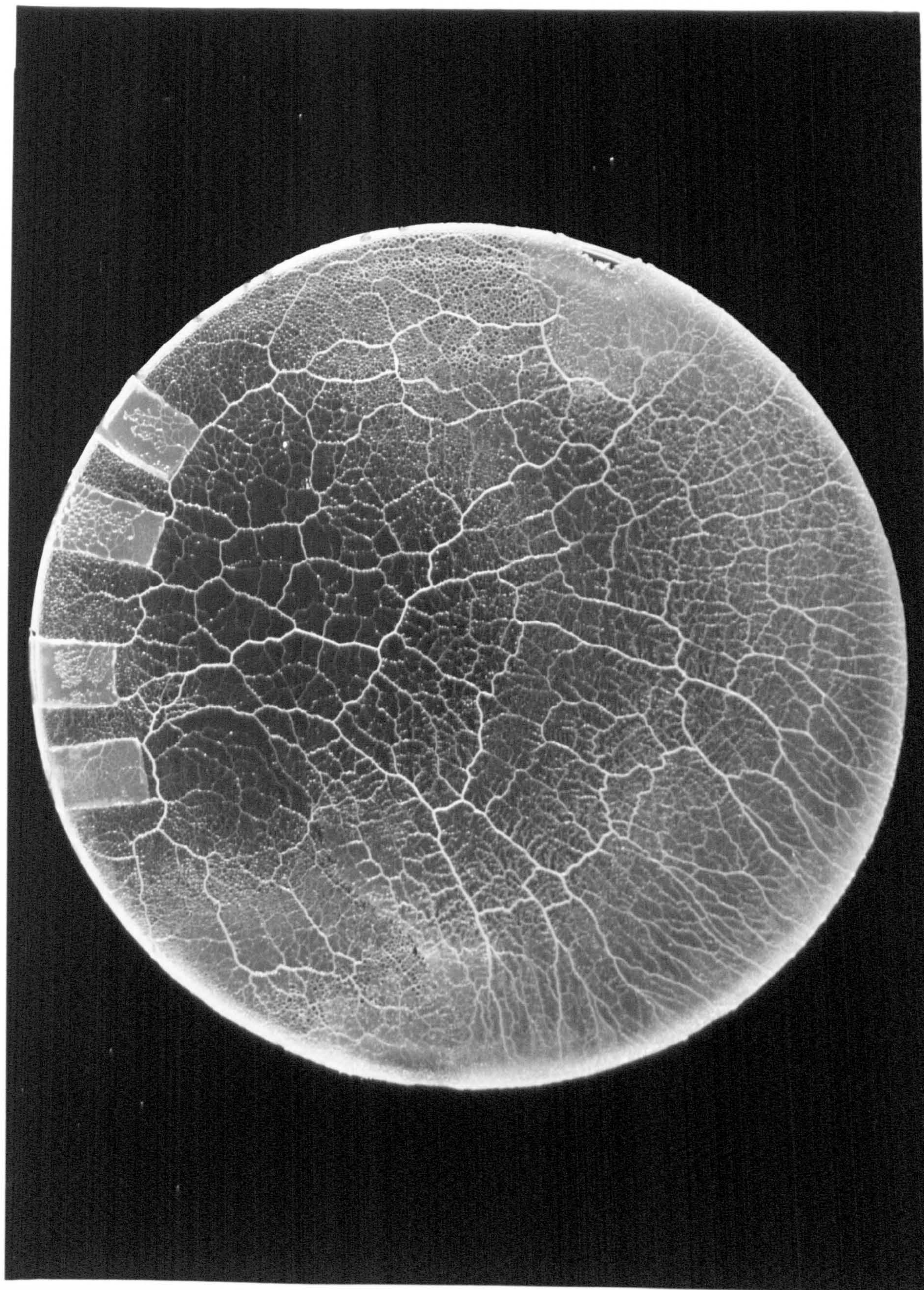


PLATE 8

THE FUSION OF AN $f_3 f_3$ AND AN $f_4 f_4$ PLASMODIUM

This plate shows a later stage of the experiment shown in PLATES 6 and 7. The $f_3 f_3 / f_4 f_4$ heterokaryon has now fused with the $f_3 f_4$ testers. The fusion with the lower tester is still incomplete, non-fusion still being apparent over some of the area of contact.



Experiments were also done to see what was the limiting proportion of $\underline{f_3f_3}:\underline{f_4f_4}$ in a heterokaryon which allowed heterozygous behaviour to develop. The proportions of the two homozygotes entering the heterokaryon were modified just prior to fusion by cutting away a quantity of one of the plasmodia. It was found that if the ratio of the two types in the heterokaryon was approximately 10:1, or more nearly equal, then the heterokaryon came to behave as a heterozygote ($\underline{f_3f_4}$). If the proportions were more unequal than 10:1 the heterokaryon retained the homozygous behaviour ($\underline{f_3f_3}$ or $\underline{f_4f_4}$). The heterokaryons formed from ratios less equal than 1:1 took longer than 24 hours to change over to heterozygote behaviour. This could take in excess of 48 hours for a heterokaryon formed from 10:1 proportions of the two homozygotes. (This experimental system has been made use of in the analysis of senescence, see below, Chapter 5.)

- (c) Confirmation that $\underline{f_3f_3}$ and $\underline{f_4f_4}$ plasmodia carry different f alleles, despite the two plasmodia classes being functionally indistinguishable

From the progeny amoebae of B173+B174 three clones of assumed genotype $\underline{mt_3f_3}$ and three of $\underline{mt_4f_4}$ were crossed in all possible combinations. These had, in the previous backcross experiment (Table 11) produced plasmodia of the $\underline{f_3f_3}$ and $\underline{f_4f_4}$ classes. The nine

resulting plasmodia fused with an $\underline{f_3f_4}$ tester and did not fuse with $\underline{f_3f_3}$ and $\underline{f_4f_4}$ testers (i.e. the nine plasmodia were $\underline{f_3f_4}$). This result confirmed the assumed genotypes of the amoebal clones and demonstrated that the $\underline{f_3f_3}$ and $\underline{f_4f_4}$ plasmodia carried different \underline{f} alleles.

(D) Allelism of f_1 and f_2 with f_3 and f_4

In the preceding discussions of the Indiana plasmodia the pair of segregating alleles have been referred to as being \underline{f} alleles ($\underline{f_3}$ and $\underline{f_4}$). This hypothesis, the allelism of $\underline{f_1}$, $\underline{f_2}$, $\underline{f_3}$ and $\underline{f_4}$ was based on the following experiments. (These experiments will be described briefly and summarized in Table form.)

(i) (Table 13) The amoebal clones \underline{a} ($\underline{f_1n_1}$), \underline{i} ($\underline{f_2n_2}$), $\underline{7132}$ ($\underline{f_2n_1}$) and $\underline{7131}$ ($\underline{f_2n_2}$) were crossed with both $\underline{B173}$ and $\underline{B174}$. The eight resulting plasmodia were tested for fusion with each other and with testers representing the six Wisconsin fusion groups (I, II, III, IV, V, VI). No fusions occurred. This demonstrated that neither of the alleles segregating in the Indiana isolate was represented in the Wisconsin strains.

(ii) (Table 14) Progeny clones from the plasmodium $\underline{B174+7132}$ ($\underline{f_2n_1}$) were analysed by backcrossing them to the appropriate parent and observing the fusion behaviour of the resulting plasmodium. The results indicate that only two "fusion" genotypes were present in

TABLE 13

Hypothetical Phenotypes of the Plasmodia Resulting
from Crossing Clones Representing the Four Genotypes
Detected in the Wisconsin Strains with B173 and B174

			Indiana clones	
			<u>B173</u> (<u>f₄n₁</u>)	<u>B174</u> (<u>f₃n₁</u>)
Wisconsin clones	<u>a</u>	<u>f₁n₁</u>	<u>f₁f₄ n₁</u>	<u>f₁f₃ n₁</u>
	<u>7131</u>	<u>f₁n₂</u>	<u>f₁f₄ n₂</u>	<u>f₁f₃ n₂</u>
	<u>7132</u>	<u>f₂n₁</u>	<u>f₂f₄ n₁</u>	<u>f₂f₃ n₁</u>
	<u>i</u>	<u>f₂n₂</u>	<u>f₂f₄ n₂</u>	<u>f₂f₃ n₂</u>

None of the 8 plasmodia have the same fusion phenotype, and therefore no fusions occur.

Progeny clones from 7132+B174 were backcrossed to B174 (mt₄f₃n₁) and also crossed with a (mt₁f₁n₁) and B173 (mt₃f₄n₁). Where plasmodia were obtained they are represented on the Table by their deduced fusion phenotype. This was determined by testing for fusion with the following plasmodia: 7119+7126, f₁f₂n₁; a+B174, f₁f₃n₁; 7140+B173, f₂f₄n₁; 7140+B174, f₂f₃n₁; B173+B174, f₃f₄n₁; B174+BB128, f₃f₃n₁ (BB128 is of progeny clone of B173+B174)

TABLE 24

Fusion Behaviour of Plasmodia Derived from 7132+B174

 $(\underline{f}_2/\underline{f}_3 \quad \underline{n}_1/\underline{n}_1)$

Progeny clone	Crossed with <u>a</u> (<u>mt</u> ₁ <u>f</u> ₁ <u>n</u> ₁)	Crossed with B173 (<u>mt</u> ₃ <u>f</u> ₄ <u>n</u> ₁)	Crossed with B174 (<u>mt</u> ₄ <u>f</u> ₃ <u>n</u> ₁)	Deduced genotype of progeny clone
4		<u>f</u> ₃ <u>f</u> ₄ <u>n</u> ₁		<u>mt</u> <u>f</u> ₃ <u>n</u> ₁
5		?	?	<u>mt</u> ₁ <u>f</u> <u>n</u>
8	<u>f</u> ₁ <u>f</u> ₂ <u>n</u> ₁	<u>f</u> ₂ <u>f</u> ₄ <u>n</u> ₁		<u>mt</u> ₄ <u>f</u> ₂ <u>n</u> ₁
9			<u>f</u> ₃ <u>f</u> ₃ <u>n</u> ₁	<u>mt</u> ₁ <u>f</u> ₃ <u>n</u> ₁
10		<u>f</u> ₃ <u>f</u> ₄ <u>n</u> ₁		<u>mt</u> <u>f</u> ₃ <u>n</u> ₁
11	<u>f</u> ₁ <u>f</u> ₂ <u>n</u> ₁			<u>mt</u> ₄ <u>f</u> ₂ <u>n</u> ₁
12		<u>f</u> ₂ <u>f</u> ₄ <u>n</u> ₁		<u>mt</u> <u>f</u> ₂ <u>n</u> ₁
15		<u>f</u> ₃ <u>f</u> ₄ <u>n</u> ₁		<u>mt</u> <u>f</u> ₃ <u>n</u> ₁
16		<u>f</u> ₂ <u>f</u> ₄ <u>n</u> ₁		<u>mt</u> ₁ <u>f</u> ₂ <u>n</u> ₁
17		<u>f</u> ₂ <u>f</u> ₄ <u>n</u> ₁		<u>mt</u> <u>f</u> ₂ <u>n</u> ₁
18	<u>f</u> ₁ <u>f</u> ₃ <u>n</u> ₁			<u>mt</u> ₄ <u>f</u> ₃ <u>n</u> ₁
19	<u>f</u> ₁ <u>f</u> ₃ <u>n</u> ₁			<u>mt</u> ₄ <u>f</u> ₃ <u>n</u> ₁
20			<u>f</u> ₂ <u>f</u> ₃ <u>n</u> ₁	<u>mt</u> ₁ <u>f</u> ₂ <u>n</u> ₁
21		<u>f</u> ₃ <u>f</u> ₄ <u>n</u> ₁		<u>mt</u> <u>f</u> ₃ <u>n</u> ₁
24		<u>f</u> ₃ <u>f</u> ₄ <u>n</u> ₁		<u>mt</u> <u>f</u> ₃ <u>n</u> ₁

Ratio f₃:f₄, 8:6; Ratio mt₄:mt₁, 4:4.

the amoebal progeny clones, corresponding to the two parental types B174 and 7132. This result suggests that B174 and 7132 differ with respect to "fusion" genotype at one gene.

(iii) (Table 15) Amongst the progeny clones of B173+7132 only the parental "fusion" genotypes were found. This suggests that B173 and 7132 differ at only one "fusion" gene.

(iv) (Table 16) Amongst the progeny clones of B174+E55 ($\underline{f}_1\underline{n}_2$) two recombinant types occurred, one of which was identifiable as $\underline{f}_1\underline{n}_1$, which suggests that B174 and E55 differ at two "fusion" genes.

These four experiments establish that the segregating alleles detected in the Indiana plasmodia are \underline{f} alleles (\underline{f}_3 and \underline{f}_4). The Indiana plasmodia are homozygous for the \underline{n}_1 allele of gene \underline{n} (which is also carried by parent clone a of the Wisconsin strain). The four experiments described above are represented on this hypothesis in the following manner:

(i) See Table 13.

(ii) B174 ($\underline{f}_3\underline{n}_1$) x 7132 ($\underline{f}_2\underline{n}_1$) gives progeny clones of the two parental types ($\underline{f}_3\underline{n}_1$, $\underline{f}_2\underline{n}_1$).

(iii) B173 ($\underline{f}_4\underline{n}_1$) x 7132 ($\underline{f}_2\underline{n}_1$) gives progeny clones of the two parental types ($\underline{f}_4\underline{n}_1$, $\underline{f}_2\underline{n}_1$).

(iv) B174 ($\underline{f}_3\underline{n}_1$) x E55 ($\underline{f}_1\underline{n}_2$) gives progeny clones of parental type $\underline{f}_3\underline{n}_1$, $\underline{f}_1\underline{n}_2$) and also two recombinant types ($\underline{f}_1\underline{n}_1$ and $\underline{f}_3\underline{n}_2$).

TABLE 15.

Fusion Behaviour of Plasmodia Derived from 7132+B173

$$(\underline{f_2}/\underline{f_4} \quad \underline{n_1}/\underline{n_1})$$

Progeny clone	Crossed with <u>a</u> (<u>mt₁f₁n₁</u>)	Crossed with <u>B173</u> (<u>mt₃f₄n₁</u>)	Crossed with <u>B174</u> (<u>mt₄f₃n₁</u>)	Deduced genotype of progeny clone
4	<u>f₂f₁n₁</u>		<u>f₂f₃n₁</u>	<u>mt₃ f₂ n₁</u>
8			<u>f₄f₃n₁</u>	<u>mt f₄ n₁</u>
9	<u>f₄f₁n₁</u>			<u>mt₃ f₄ n₁</u>
13			<u>f₂f₃n₁</u>	<u>mt f₂ n₁</u>
15		<u>f₄f₄n₁</u>	<u>f₄f₃n₁</u>	<u>mt₁ f₄ n₁</u>
16			<u>f₂f₃n₁</u>	<u>mt f₂ n₁</u>
19	<u>f₄f₁n₁</u>			<u>mt₃ f₄ n₁</u>
21			<u>f₂f₃n₁</u>	<u>mt f₂ n₁</u>
22		<u>f₂f₄n₁</u>		<u>mt₁ f₂ n₁</u>

Progeny clones from 7132+B173 were backcrossed to B173 (mt₃f₄n₁) and also crossed with a (mt₁f₁n₁) and B174 (mt₄f₃n₁). Where plasmodia were obtained they are represented on the Table by their deduced fusion phenotype. This was determined by testing for fusion with the following "tester" plasmodia: a+B173, f₁f₄n₁; 7140+B174, f₂f₃n₁; 7140+B173, f₂f₄n₁; B173+B174, f₃f₄n₁; B174+BB128, f₃f₃n₁; 7119+7126, f₁f₂n₁.

Progeny clones from E55+B174 were backcrossed to B174 (mt₄f₃n₁) and also crossed with a (mt₁f₁n₁) and B173 (mt₃f₄n₁). Where plasmodia were obtained they are represented on the Table by their deduced fusion phenotype. This was determined by testing for fusion with the following plasmodia; a+E55i38, f₁f₁n₂; a+5aE55i38, f₁f₁n₁; 7131+B174, f₁f₃n₂; a+B174, f₁f₃n₂; 7131+B173, f₁f₄n₂; a+B173; f₁f₄n₁; B173+B174, f₃f₄n₁; B174+BB128, f₃f₃n₁. No tester of type f₃f₄n₂ was available; plasmodia which fused with each other but with none of the known testers were provisionally classified as having this phenotype. Certain plasmodia failed to show fusion with any other plasmodium, these are recorded as "?". The "q" plasmodia were probably f₁f₁ homozygotes.

TABLE 16

Fusion Behaviour of Plasmodia Derived from E55+B174 $(\underline{f}_1/\underline{f}_3 \ \underline{n}_2/\underline{n}_1)$

Progeny clone	Crossed with \underline{a} ($\underline{mt}_1 \underline{f}_1 \underline{n}_1$)	Crossed with B173 ($\underline{mt}_3 \underline{f}_4 \underline{n}_1$)	Crossed with B174 ($\underline{mt}_4 \underline{f}_3 \underline{n}_1$)	Deduced genotype of progeny clone
1		?		$\underline{mt} \ \underline{f} \ \underline{n}$
2	\underline{g}			$\underline{mt}_4 \ \underline{f} \ \underline{n}$
3	$\underline{f}_1 \underline{f}_1 \underline{n}_1$			$\underline{mt}_4 \ \underline{f} \ \underline{n}$
5	\underline{g}	$(\underline{f}_3 \underline{f}_4 \underline{n}_2)$		$\underline{mt}_4 \ \underline{f}_3 \ \underline{n}_2$
7			?	$\underline{mt} \ \underline{f} \ \underline{n}$
9	$\underline{f}_1 \underline{f}_3 \underline{n}_2$?		$\underline{mt}_4 \ \underline{f}_3 \ \underline{n}_2$
10	$\underline{f}_1 \underline{f}_3 \underline{n}_2$	$\underline{f}_3 \underline{f}_4 \underline{n}_1$		$\underline{mt}_4 \ \underline{f}_3 \ \underline{n}_1$
13		$(\underline{f}_3 \underline{f}_4 \underline{n}_2)$		$\underline{mt} \ \underline{f}_3 \ \underline{n}_2$
14			?	$\underline{mt} \ \underline{f} \ \underline{n}$
15		$\underline{f}_1 \underline{f}_4 \underline{n}_1$	$\underline{f}_1 \underline{f}_3 \underline{n}_1$	$\underline{mt}_1 \ \underline{f}_1 \ \underline{n}_1$
17		$\underline{f}_1 \underline{f}_4 \underline{n}_2$	$\underline{f}_1 \underline{f}_3 \underline{n}_2$	$\underline{mt}_1 \ \underline{f}_1 \ \underline{n}_2$
18		?	?	$\underline{mt}_1 \ \underline{f} \ \underline{n}$
19		?		$\underline{mt} \ \underline{f} \ \underline{n}$
20		?		$\underline{mt} \ \underline{f} \ \underline{n}$
21			$\underline{f}_3 \underline{f}_3 \underline{n}_1$	$\underline{mt}_1 \ \underline{f}_3 \ \underline{n}_1$
24		$(\underline{f}_3 \underline{f}_4 \underline{n}_2)$		$\underline{mt} \ \underline{f}_3 \ \underline{n}_2$

(E) Further analysis of the B173+B174 progeny, making use of the f_2 allele.

From the plasmodium B173+B174 three progeny clones of each of the assumed genotypes, $\underline{mt_3 f_3 n_1}$, $\underline{mt_3 f_4 n_1}$, $\underline{mt_4 f_3 n_1}$ and $\underline{mt_4 f_4 n_1}$, were crossed with 1 ($\underline{mt_2 f_2 n_2}$). The resulting plasmodia were tested for fusion with 1+B174 ($\underline{f_2 f_3 n_2}$) and 1+B173 ($\underline{f_2 f_4 n_2}$). The plasmodia fused with either one or the other tester strain (~~Table 17~~) indicating that it was correctly hypothesised that only two allelic factors ($\underline{f_3}$ and $\underline{f_4}$) were segregating among the progeny of B173+B174 (see Tables 11 and 12).

4. Discussion

Summary

(i) The occurrence of fusion between plasmodia produced from amoebal clones of P.polycephalum was found to be strain-dependent. The responsible factors were found to segregate amongst the progeny clones of heterozygous plasmodia, which agrees with the analysis of Dee and Carlile (1967) and Poulter and Dee (1968). Two isolates were studied, "Indiana" and "Wisconsin". In these isolates plasmodial fusion is controlled by two genes, n and f.

(ii) The n gene. Two alleles of gene n have been detected, the n₂ allele being dominant to n₁. Plasmodia must have the same phenotype with respect to gene n for fusion to be possible between them. After considering the possible mode of action of the f gene, a hypothetical model for the mode of action of the n gene will be proposed (see ix below).

The genes hypothesised by Collins and Clark (1968) to explain the fusion behaviour of D.iridis plasmodia all resemble the n gene in that one of the two alleles shows dominance.

(iii) The f gene. Four alleles of gene f have been detected (f₁, f₂, f₃ and f₄). With the exception of fusions between f₃f₃ and f₄f₄ plasmodia (see viii below) fusion is possible only between plasmodia carrying the same f alleles.

(iv) Mating type and plasmodial fusion. The mating type locus apparently does not affect fusion behaviour, since some plasmodia carrying the same mt alleles fail to fuse, and some plasmodia carrying different mt alleles fuse. Viewed as a differentiation phenomenon this is extremely interesting. The haploid amoebae apparently have one process controlling cell fusion, represented by the mt gene, and the plasmodia have another process controlling cell fusion, represented by the f and n genes. It would be of interest to observe the development of expression of the f and n genes following the crossing of two amoebal clones to give a diploid zygote. It might be expected that for some while after zygote formation the f and n genes will not be fully expressed, during this period heterokaryons between plasmodia of different fusion types might become established. If this prediction is experimentally verified it might provide a useful system for studying the phenomenon of the differentiation of the haploid uninucleate amoebae into diploid macroscopic plasmodia.

(v) The q gene. A gene q has been detected, being represented by two alleles q^+ and q^- . q^- is recessive; in the homozygous state it gives plasmodia which are capable of growing on bacteria, but incapable of surviving when transferred to the standard axenic nutrient medium, SDM agar.

This q heterozygosity was found in the natural isolate, no

mutagenesis having been used. It is of interest to speculate on the point that neither the \underline{g}^- plasmodia nor the haploid amoebae will grow on the axenic SDM medium. This could be a superficial similarity or it could reflect an underlying similarity in the metabolic organisation. The \underline{g}^- plasmodia could possibly reflect not a single metabolic block, but rather a failure in differentiation from the amoebal to the plasmodial physiology. Further analysis of the nutritional requirements of the amoebae and \underline{g}^- plasmodia is needed to test this interesting possibility.

(vi) Linkage. It has been established that the gene \underline{g} is linked to \underline{f} ; recombinants between these two genes occur with a frequency of about 10%. This is the first reported example of linkage and crossing over in P.polycephalum.

Genes \underline{f} , \underline{n} and \underline{mt} are unlinked to each other.

(vii) The killing reaction. Some preliminary experiments concerning the killing reaction have been performed. The only certain point which has so far been established is that several genes are involved, and that the responsible factors segregate amongst the progeny of a plasmodium.

There is a theoretical point of considerable interest concerning the killing reaction. The killing reaction is apparently a reflection of genetic dissimilarity. It is known that "backcross" plasmodia may show the killing reaction on fusing with each other or with the

"parental" diploid plasmodium. Therefore the genetic dissimilarity which is resulting in killing between backcross plasmodia is contained in the diploid "parental" plasmodium without apparently affecting the organisms viability. The system therefore presents a conceptual problem which might be summarised as, why does not the heterozygote mimic the lethal consequences of the heterokaryotic state? The analysis of the killing phenomenon is at too early a stage for speculation to be profitably taken very far, but at present two working hypotheses are being considered.

(a) It is possible that the "incompatible " lethal genes are active in the heterozygous plasmodium, but the intensity of the process is very mild, being proportional to the rate of gene translation. The "incompatible" products of killer genes are assumed on this hypothesis to be neutralised following interaction. When two homozygous plasmodia fuse they will each possess pools of unneutralised killer substances, which will give an acute interaction, resulting in the killing of the plasmodia. This hypothesis, in its simplest form, would predict that since, a heterozygote (e.g. a+i) should be in a "neutralised" condition, it should never produce a killer reaction on fusion with another plasmodium. In fact a+i does show killing interactions, and therefore further ad hoc assumptions have to be made, which for reasons of space will not be elaborated on here.

(b) Alternative hypotheses could be based on the assumption that either one or both "killer alleles" are blocked at transcription or translation, such a control becoming effective at an early stage after zygote formation. If one allele prevents the transcription of the other it will appear as dominant during analysis. This would fit with the observation that heterozygotes become involved in killing reactions, and has the advantage over theories of type (a) that the heterozygotes are not assumed to suffer a mild chronic killing reaction.

(viii) The unexpected fusion of f_3f_3 and f_4f_4 plasmodia; the general model for the mode of action of the f gene

The experiments reported in section 3 (C) (a), (b) and (c) demonstrated that the f_3f_3 and f_4f_4 plasmodia, although indentical in fusion behaviour, in fact carried different f alleles. The experiments reported in section 3 (E) in which progeny clones of B173+B174 were crossed to an f_2 clone confirmed this analysis.

The heterozygote f_3f_4 behaves throughout in accordance with the expectation that identity of f allele is necessary for fusion, as do f_2f_3 and f_2f_4 plasmodia. The heterokaryon resulting from fusion between f_3f_3 and f_4f_4 plasmodia behaves, after a short delay, as an apparent f_3f_4 . The unusual behaviour of the f_3 and f_4 alleles is thus confined to the homozygotes. It is apparent from the non-fusion of f_2f_3 and f_2f_4 plasmodia (or f_3f_3 and f_3f_4) that these

alleles are distinct and carry sufficient "information" for them to produce characteristic phenotypes in all plasmodia other than the homozygotes.

The model proposed to explain the fusion of the genetically dissimilar $\underline{f}_3\underline{f}_3$ and $\underline{f}_4\underline{f}_4$ plasmodia is as follows. The genotypes of \underline{f}_3 and \underline{f}_4 amoebal clones are assumed to carry defects which result in the homozygous state in inactivity of the \underline{f} gene. \underline{f}_3 clones carry one defect and \underline{f}_4 clones another. The defect could be in \underline{f} or in another locus but the evidence suggests that, if the defect is not in \underline{f} , the site must be very close to \underline{f} (see below (a)). In the homozygotes $\underline{f}_3\underline{f}_3$ and $\underline{f}_4\underline{f}_4$ these defects are expressed as inactivity on the part of the \underline{f} gene, in the heterozygote $\underline{f}_3\underline{f}_4$ they complement each other and the \underline{f} gene acts normally.

Since the defective or "inactive" homozygotes show unexpected fusions, their defects must be in a process which normally prevents fusion between dissimilar plasmodia. The model therefore requires that the \underline{f} gene, in its normal functioning, acts to prevent fusion between dissimilar plasmodia.

(The alternative and incompatible hypothetical choice was that the \underline{f} gene acted as a recognition factor, catalysing the fusion of similar strains. If this hypothesis were correct then plasmodia showing inactivity of the \underline{f} gene would show unexpected non-fusions.)

If the complementation in the $\underline{f}_3\underline{f}_4$ heterozygote were due to two loci, recombinant classes of amoebae should occur. When crossed these clones would give, for example, "f active" $\underline{f}_3\underline{f}_3$ plasmodia showing complementation, which would not fuse with $\underline{f}_4\underline{f}_4$ testers. No such recombinants have been found in the thirty seven progeny clones analysed. The complementing sites are therefore closely linked to each other and to the f locus, (which could in fact be one of the complementing sites) or, more probably, the complementation is actually between the \underline{f}_3 and \underline{f}_4 alleles of the single gene f.

If intragenic complementation is operating, the action of the f gene probably involves the production of dimers (or higher polymers). Since recognition of f type is most likely to occur at the surface of two plasmodia in contact, it is at the membrane that the dimers are hypothesised to occur. It is supposed that this surface barrier of dimers inhibits fusion of a plasmodium with any other plasmodium with which it may be in contact, unless the barrier is cancelled or neutralised by identical dimers carried by the other plasmodium. When two plasmodia showing "deficient" or "inactive" barriers meet (e.g. $\underline{f}_3\underline{f}_3$ and $\underline{f}_4\underline{f}_4$) they fuse. When a deficient plasmodium meets a plasmodium carrying an inhibitory coat (e.g. an $\underline{f}_3\underline{f}_3$ meets an $\underline{f}_3\underline{f}_4$) the barrier remains uncanceled and fusion cannot occur. The cancelling process could be envisaged as the polymerisation of identical dimers to give tetramers. If this model for the action of

the f gene is correct, it presents interesting parallels and contrasts with the suggested mode of action of the S (self-incompatibility) gene in some higher plants (Lewis, 1964). Lewis proposed that the S gene produces dimers which exist in both pollen and style. Self-pollination brings together tissues carrying identical dimers, which combine to give a tetramer inhibitory to pollen tube growth. In the proposed model for the f system the dimers are the biologically active form, inhibiting fusion, and the formation of tetramers removes this inhibition.

A physiological test of this model is of course desirable, but has not so far proved possible. Various procedures have been used in the attempt to alter plasmodial fusion behaviour while leaving the organism still viable, amongst them being

- (a) Low Ca^{++} and Mg^{++}
- (b) Detergents
- (c) Urea
- (d) Various proteolytic enzymes.

None of these treatments has so far produced the desired manipulation of fusion behaviour. One difficulty experimentally is the delicate nature of the plasmodial membrane.

(ix) The n gene can be considered in a similar way to the f gene as far as mode of action is concerned. To account for the dominance of n₂ over n₁, n₁ can be considered as inactive, or alternatively the

action of the \underline{n}_1 allele may be inhibited by the \underline{n}_2 allele. The \underline{n}_2 allele could be considered as creating an inhibitory coat on the surface of the plasmodium, which prevents fusion with other plasmodia unless it is cancelled by an identical coat.

(x) Ecological implications. The \underline{f} and \underline{n} genes are considered to be a mechanism discouraging the formation of heterokaryons by preventing fusion between distantly related plasmodia and reducing the chance of fusion between plasmodia of the same population. The frequency of heterokaryons in a natural population will be in inverse proportion to the number of \underline{f} or \underline{n} alleles carried by that population. The populations from which our two original plasmodia were isolated may well have carried more than two pairs of alleles. The operation of the \underline{f} and \underline{n} genes and the killing reaction which often follows fusion must result in heterokaryons being infrequent in natural populations. Caten and Jinks (1966), considering the action of similar factors in the $\underline{f}\underline{n}\underline{g}\underline{i}$, have also concluded that heterokaryosis is rarer in natural populations than previously supposed.

The adaptive value of preventing heterokaryosis is not obvious. It might be argued that heterokaryons should have increased vigour, by analogy with heterosis. However for some, as yet uncertain, reason it must be assumed that heterokaryon formation involves disadvantages to the plasmodia. One possible disadvantage would be the exchange of infection, another possibility is that the plasmodial

senescence process reported in this Thesis (Chapter 5) would spread between plasmodia and seriously affect their viability.

The interpretation of the hypothetically "f inactive" $\underline{f}_3\underline{f}_3$ and $\underline{f}_4\underline{f}_4$ plasmodia requires that the f gene functions to prevent fusion of dissimilar plasmodia. By analogy, the n gene could be considered to perform the same role. An alternative interpretation of the action of the n gene is however tenable. It is possible that this gene performs two functions preventing the fusion of dissimilar strains and catalysing the fusion of similar strains. It is necessary to modify the model for the mode of action of the n gene slightly to accommodate this hypothesis. Plasmodia, on this hypothesis, would carry on their membranes distinctive "fusion dimers". These dimers would inhibit fusion with other plasmodia, unless cancelled or neutralised by identical dimers on the membrane of the other plasmodium.

(The process of neutralization is viewed as possibly the formation of tetramers between identical dimers.)

In the modified hypothesis for the functioning of the n gene the formation of these tetramers would bring together the two membranes into intimate contact, thus catalysing fusion between similar strains.

It is possible to propose such a dual functioning for the f gene, if various ad hoc assumptions are allowed. The point will not however be elaborated on here.

(xi) Finally, although the occurrence of non-fusion between two plasmodia is now established as being insufficient evidence for the taxonomic separation of those plasmodia into physiological races, the possibility remains that the actual f and n alleles carried by populations of P.polycephalum may be characteristic of those populations. It would be possible by establishing an extensive series of "tester" plasmodia to classify any isolate for fusion character. This might enable one to differentiate distinct populations or races of this Myxomycete. The Myxomycetes provide favourable material for such a study because of the improbability of them being widely redistributed by human or natural means. The behaviour of natural populations of microorganisms is as yet scarcely studied, and such a study might be of considerable interest.

Chapter 3

THE SELECTION AND STUDY OF MUTANTS

For various reasons it would be valuable to be able to select mutants of P.polycephalum. Potentially such mutants could be of value for making a map of the linkage groups of the organism, as genetic markers in senescence experiments, or to enable the organism to be of much greater use for biochemical analysis of such problems as the regulation of nucleic acid synthesis, the regulation of enzymes or differentiation. However such projects are at present impeded by the extreme difficulty of selecting any form of mutant in P.polycephalum.

1. Mutagenesis of Amoebae

Two methods were devised to mutagenise amoebae, both using N-methyl-N-nitro-N-nitrosoguanidine (NMG) (Adelburg et al, 1965).

(i) Amoebae were washed off liver-infusion agar slopes and suspended in distilled water. NMG was then added to give a final concentration of 62.5 mcg/ml. The suspension was left for 15 minutes, and then bacteria were added. The mixed suspension was then centrifuged down, the solution poured from the pellet which was then resuspended in distilled water and centrifuged again. The pellet of amoebae and bacteria was then plated on water-agar containing the drug under consideration. (The bacteria were added before centrifuging to enable a firm pellet of moderate size to be formed.) The concentration of

NMG used, 62.5 mcg/ml and the time of treatment were selected so as to achieve very weak mutagenesis, since for various purposes it was desired to avoid multiple mutations. A range of NMG concentrations were tested, 62.5 mcg/ml being the highest concentration that produced no appreciable drop in viable amoebal count.

One unfortunate characteristic of this procedure is that no growth occurs between mutagenesis and exposure to the drug, and therefore mutations may not have time to be phenotypically expressed. This procedure is therefore only useful if the concentration of inhibitory drug used retards but does not completely inhibit amoebal growth.

(ii) An alternative method, which involves amoebal growth during mutagenesis, has been employed in some experiments. Amoebae were cultured on liver-infusion agar slopes in which .1 mcg/ml NMG had been incorporated. (This level of NMG produced no appreciable drop in amoebal viable count when amoebae were plated onto it.) After 5 or 6 days incubation the mutagenised amoebae were harvested and plated on the drug under consideration.

2. Auxotrophic Mutants in the Amoebae

The inability to grow amoebae axenically prevented the selection of auxotrophic mutants.

3. Drug Resistant Amoebal Mutants

The method described by Dee (1966a) for culturing amoebae involved using growing bacteria. This method required that, before amoebae could be tested for sensitivity to a drug, the bacteria had first to be grown in its presence. Assuming that this could be achieved the difficulty arose that the bacteria might degrade the drug. These difficulties were overcome by the introduction of a modification of the amoebal culture technique.

In this modified procedure amoebal clones were routinely cultured on LIA slopes with a multiple auxotrophic strain of E.coli, which showed no apparent back-mutation rate to wild type. Amoebae were tested for sensitivity to a drug by inoculating them onto 2% water agar/drug plates which had been spread with a thick lawn of non-growing auxotrophic bacteria. This lawn was prepared by growing up the auxotrophic E.coli in nutrient broth, centrifuging and spreading the resulting pellet on the 2% water agar/drug plate. This procedure, the use of a lawn of non-growing E.coli, resulted in a considerable improvement in the repeatability of drug tests. (The auxotrophic strain showed no growth in this procedure. Wild-type bacteria when plated on the water agar plates showed some slight growth, due presumably to impurities in the agar.) However despite this innovation very little progress has been made, the remaining difficulties still being considerable. The amoebae seem to be

naturally highly resistant to a variety of antibiotics, perhaps because of the ecology of the organism. For example, streptomycin (10 mg), nalidixic acid (1000 mcg/ml), colchicine (1000 mcg/ml) and sulphonamide (1000 mcg/ml) all fail to inhibit the amoebae. Several drugs which are effective against the plasmodium are only partially inhibitory to the amoebae, presumably because the E.coli lawn provides an alternative and preferentially used supply of nutrients. For example, parafluorophenylalanine inhibits the plasmodia at 10^{-3} M but fails to inhibit the amoebae completely at higher concentrations.

Of the various inhibitory drugs that have been investigated the results concerning the seven used most intensively in attempts to select resistant amoebae will be briefly summarised.

(i) Cycloheximide (Actidione)

This drug greatly retards amoebal growth at 3 mcg/ml. Plasmodial growth is inhibited by 2-3 mcg/ml. A spontaneous amoebal mutant resistant to 3 mcg/ml has been isolated by Dee (1966a), and further analysed by Dee and Poulter (in press). This analysis will be described in detail below (10).

(ii) Emetine

This substance is an alkaloid amoebicide.(Robinson,1948). The drug is sensitive to heat and light. Amoebae are inhibited by 25 mcg/ml. Rather curiously a concentration of 350 mcg/ml is necessary to inhibit the plasmodia. An amoebal mutant resistant to 25 mcg/ml

was isolated and analysed by Dee (1962) but subsequently lost. The mutant showed rather variable expression. Attempts to reselect mutants have failed, one unstable or spurious mutant being found in 5×10^6 amoebae plated (type i) mutagenesis).

(iii) Neomycin

Amoebae are inhibited by 125 mcg/ml. No resistant mutants have been detected in 10^7 amoebae plated. (mutagenesis type ii)), two apparently spurious mutants being found.

(iv) Kanamycin

Amoebae are inhibited by 75 mcg/ml. No resistant mutants have been detected in 10^6 amoebae plated (mutagenesis type ii)).

(v) 5-Fluorouracil

This analogue of uracil inhibits protein synthesis (Hignett, 1966). Amoebae are inhibited by 50 mcg/ml. Preliminary experiments with this drug have failed to produce any resistant mutants.

(vi) Potassium Dichromate

This substance blocks the activity of various enzymes through the action of the heavy metal anion. Amoebae are inhibited by 150 mcg/ml. 10^7 amoebae, mutagenised by procedure (ii) have been plated on this level without stable mutants being found, one unstable or spurious mutant being detected.

(vii) Tetrazolium (Triphenyltetrazolium chloride (Zweifach, Black and Shorr, 1951))

This substance is used as a vital dye to detect, in bacteria, the ability to ferment certain sugars, deep red formazan granules being formed intracellularly as a degradation product. The amoebae show unexpected sensitivity to this substance, being inhibited by 50 mcg/ml. No resistant amoebae were detected after plating 2×10^7 , using mutagenesis procedure (i).

4. Mutants selected for, or expressed, in the Plasmodia

The existence of an axenic medium for plasmodia provides a possibility of detecting nutritional mutants. One such, the g^- mutant, which in the homozygous state results in inability on the part of the plasmodium to survive on SDM, has been detected (see Chapter 2) because of its chance linkage to the f gene. It is at present impracticable to look for mutants in the plasmodia for the following reasons.

- (i) Plasmodia are diploid, and normally multinucleate. Mutagenesis of plasmodia is therefore unlikely to result in mutant phenotypes.
- (ii) The amoebae are heterothallic, and therefore mutagenised homozygotes cannot be synthesised in one generation, it being necessary to out-cross the mutagenised amoebal clone, and self the progeny clones of the resulting plasmodium. This procedure is prohibitively laborious.

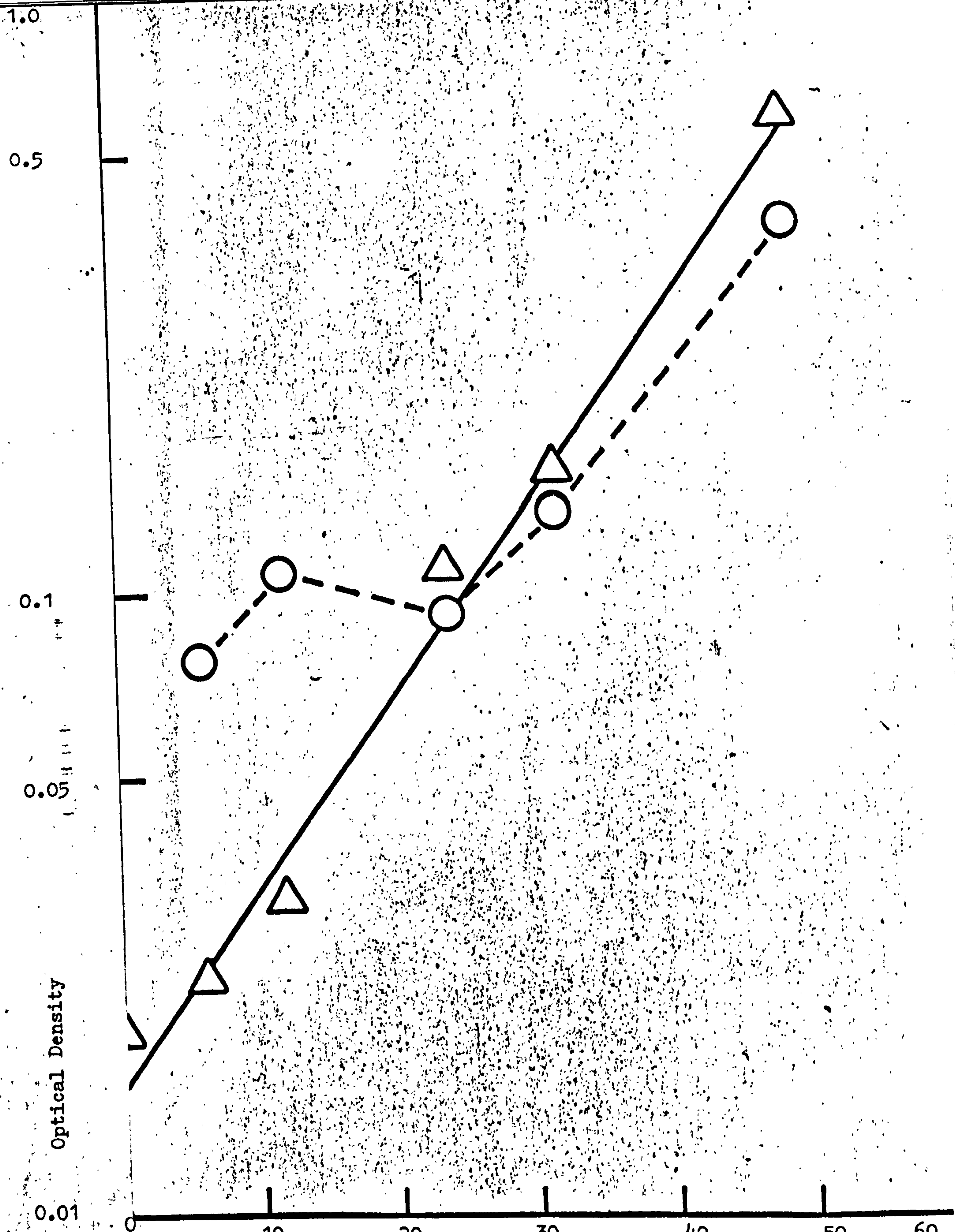


Figure 1 Comparison of measurement of growth rate by pigment(O), and protein(Δ), estimation.

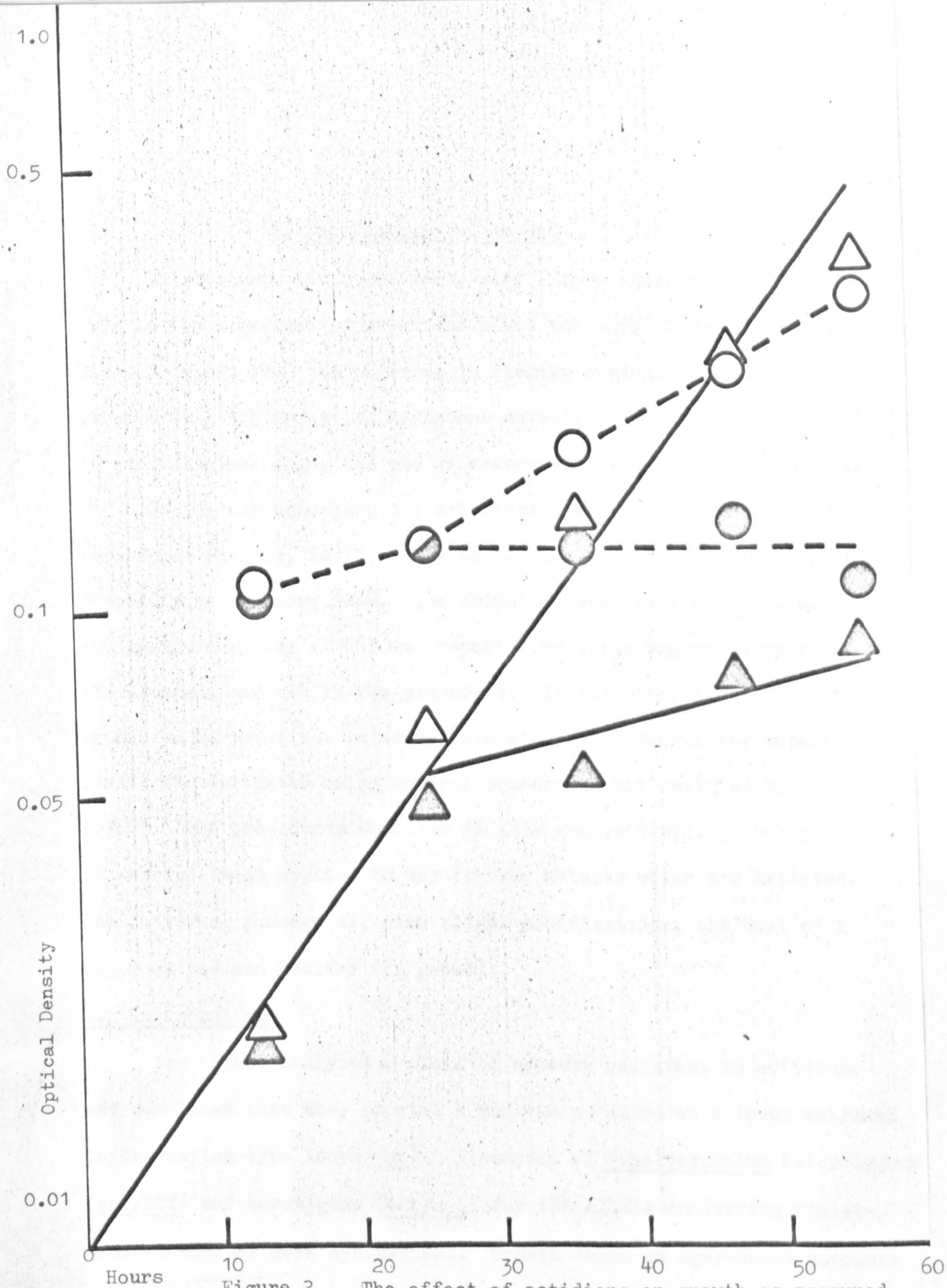


Figure 2

The effect of actidione on growth as measured by pigment(\circ), and protein(Δ), estimation.

5. The Analysis of Mutants

To summarise the above work, very little progress has been made in the selection of mutations since the work of Dee (1962, 1966a). However, apart from the progress in finding several drugs which may prove useful for selecting resistant mutants, the work has developed in two technical ways, the use of mutagenesis, and non-growing E.coli.

One of the necessary characteristics of mutants for use in senescence studies, is that they should not affect the viability of plasmodia which carry them. The actidione resistant mutant isolated and analysed by Dee (1966) was reported as being expressed only in the amoebae, and not in the plasmodia. It therefore seemed that this mutant would be of use in senescence studies. However the mutant was first reinvestigated using several procedures not employed by Dee (1966). The procedures employed in this analysis will probably be in essence those applied to any further mutants which are detected. The following passage is, with slight modifications, the text of a paper by Dee and Poulter (in press).

A. Introduction

Dee (1966) analysed a clone of amoebae resistant to actidione and concluded that they carried a nuclear mutation at a locus unlinked to the mating-type locus (mt). Plasmodia of P.polycephalum heterozygous (act/ACT) and homozygous (act/act) for the allele conferring resistance on amoebae were synthesised. Growth tests on agar-based nutrient

and non-nutrient media in the presence and absence of actidione indicated (Dee, 1966) that these plasmodia did not show resistance to actidione greater than that of homozygous sensitive strains (ACT/ACT). It was concluded that the actidione-resistant mutation selected in the haploid amoebae was not expressed in the diploid plasmodia, even when homozygous.

These conclusions were more precisely tested^{by} growing several strains of plasmodia of each genotype in shaken culture in liquid semi-defined medium in the presence and absence of actidione and measuring the rate of growth in the cultures by means of pigment and/or protein estimation.

The results confirmed that actidione resistance is recessive, but, unlike Dee's previous studies, indicated that plasmodia homozygous for the resistant allele do show resistance to actidione. On agar medium even in the absence of actidione, growth of homozygous resistant plasmodia is poor and their morphology is characteristically abnormal. It is concluded that the actidione resistant mutation selected in amoebae is expressed in homozygous plasmodia conferring on them both resistance to actidione and abnormal morphology. Both effects are recessive in heterozygous plasmodia. The fact that the mutant is expressed in the plasmodia resulting in rather poor viability, reduces the value of the mutant as a marker in senescence experiments.

B. Strains

All strains used in this investigation were derived from a cross between the actidione resistant strain, A7 (genotype act, mt₁) originally isolated by Dee (1966) on agar medium containing 4 µg/ml actidione and the sensitive strain, i (genotype ACT, mt₂). Resistant and sensitive progeny clones of amoebae of both mating types (mt₁ and mt₂) were isolated and crossed to give plasmodia of genotypes act/act, act/ACT, ACT/ACT. The progeny clones were assigned the numbers A7101 - A7150. The plasmodia are numbered according to the amoebal clones used (e.g. A7124 + A7120), the clone of mt₁ in each case being written first.

C. Culture of amoebae

Culture methods for amoebae are described in the Introduction (Chapter 1), non-growing Escherichia coli being used as food in the presence of the drug. Using this method, the concentration of actidione necessary for discrimination between resistant and sensitive strains of amoebae is lower than that used previously with E.coli growing on liver infusion agar (3 µg/ml instead of 4 µg/ml).

Methods for crossing amoebae, obtaining spores, and "progeny" amoebal clones are described in the Introduction (Chapter 1). Amoebal clones were tested for resistance to 3 µ actidione by adding a loop of the clone to a 2% water agar/3 mcg/ml actidione plate which had been spread with a lawn of non-growing E.coli. Clones were routinely

tested on a control plate containing no actidione. After 6 days incubation resistant clones could be easily differentiated, exhibiting vigorous growth. Sensitive strains did however eventually begin growing. The reasons for the sensitive clones commencing growth after a long lag-phase are unclear, but must presumably reflect some form of phenotypic adaptation.

D. Culture of plasmodia

Except for a few tests on SDM-agar, plasmodia were grown and tested in liquid SDM in 150 ml conical flasks on a rotary shaker. Routine tests for contamination were made by inoculating samples in appropriate broths. Most plasmodia were synthesized from amoebae shortly before tests were made, but stored microsclerotia were sometimes used.

E. Assays of plasmodial growth

Growth of microplasmodia in liquid culture was assayed by determination of protein by the Biuret reaction and of pigment by the method described by Daniel and Baldwin (1964). Pigment from a washed, centrifuged sample of microplasmodia was extracted in 4% TCA-acetone (8 ml 100% w/v TCA; 92 ml acetone; 100 ml water) and the optical density determined at 400 mμ in a Gilford Spectrophotometer. The amount of pigment determined by this method has been shown to be proportional to protein by Daniel and Baldwin (1964). In the present study, the relationship between assayed protein and pigment was

investigated in the presence and absence of actidione (see Results).

F. Results

(a) Relationship between Pigment and Protein

Determinations of pigment and protein in a culture of one homozygous sensitive (ACT/ACT) strain in the absence of actidione are shown in Fig.1. Such cultures grow exponentially with a doubling time of about 10 hours. After the first 24 hours increase in pigment is directly proportional to increase in protein. Fig.2 shows that when actidione was added to a culture of the same strain after 25 hours incubation, both protein and pigment production were strongly inhibited. Inhibition by actidione of protein synthesis in P.polycephalum plasmodia was reported by Cummins and Rusch (1966).

The high values for pigment during the first 24 hours are due to the presence of haematin. This has been shown by pigment assays on dilution series of microplasmodia. When dilutions are made in SDM, the relationship between pigment optical density and microplasmodial concentration is linear for optical densities down to 0.1, but at greater dilutions no O.D. values lower than this are obtained. When SDM lacking haematin is used, the relationship is linear down to the lowest measurable values. Optical densities of 0.1 or less for pigment assays on dilute cultures in SDM are therefore not accurate estimations of microplasmodial content but are elevated because of the presence of haematin. Since the absorption spectrum of haematin

is close to that of pigment with a maximum near 400 mμ, it is impossible to differentiate between them. This effect of haematin was not mentioned by Danile and Baldwin (1964) who showed protein and pigment to be proportional throughout the growth of a culture. In our experiments, the effect gave incorrect optical density values for pigment in the first 24 hours of growth only. This was not a source of error in growth tests since estimations were made after 2 or 3 days.

(b) Growth Tests of Different Genotypes in Liquid Culutre

In tests performed to compare the growth of strains of different genotype in the presence and absence of actidione, the basic method was as follows. A "starter" culture of each strain in liquid SDM was incubated for 24 hours and used to inoculate four experimental flasks, two of them containing 1 μg/ml actidione in SDM and the other two SDM alone. A 24-hour "starter" culture was used to ensure that the plasmodia were in the log. phase of growth at the beginning of the experiment. A large inoculum (5 ml of starter cultured added to 45 ml medium) was used because preliminary experiments using smaller inocula gave more variable results. Preliminary experiments using different concentrations of actidione showed that 1 μg/ml was the most suitable for discriminating between strains. The flasks were incubated for either 2 or 3 days (see below) and then sampled for protein and/or pigment estimations. Values obtained for duplicate flasks were always closely similar and were averaged. The average optical density value obtained

for cultures in SDM + actidione was then expressed as a percentage of the value for SDM alone, to give a measure of resistance to actidione. Two series of growth tests were performed; one in which protein estimations were done and another in which only pigment was estimated.

Growth Tests Using Protein Estimation

Results of three experiments in which protein was estimated are shown in Table 1. Samples were taken after 2 days.

Growth Tests Using Pigment Estimation

Seven experiments were done in which only pigment was estimated. Samples were taken after 3 days. In each experiment, strains of different genotype were compared, the total number of strains involved being five ACT/ACT, six ACT/act and five act/act. Results are summarized in Figure 3. They show that resistance to actidione was not significantly different in ACT/ACT and ACT/act but was greater in act/act plasmodia.

(c) Results on Agar Medium

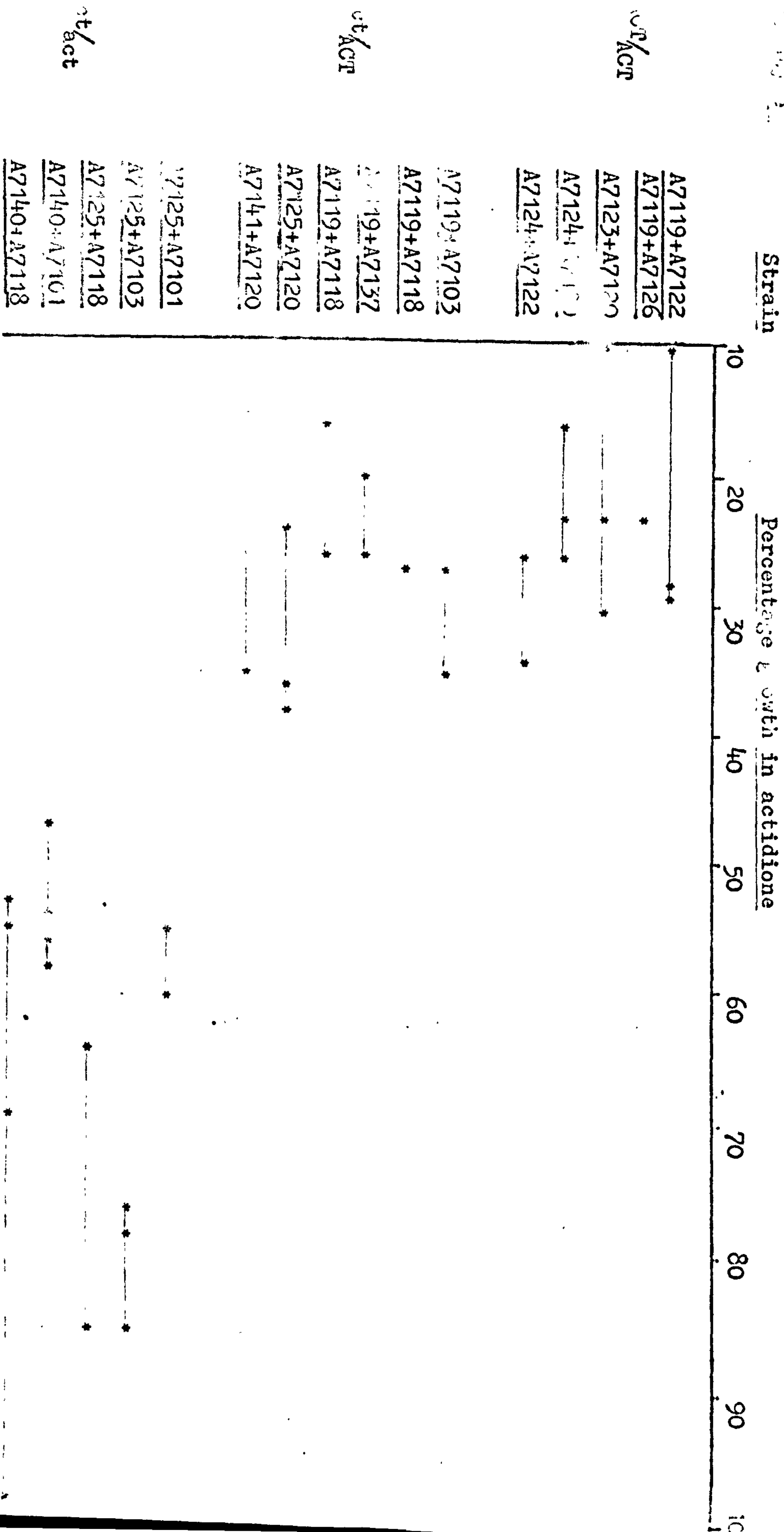
Two ACT/ACT and two act/act strains were inoculated on plates of SDM agar containing 0, 1, 2 and 3 µg/ml actidione. Both ACT/ACT strains showed the same amount of growth on 0, 1 and 2 µg/ml actidione but were partially inhibited by 3 µg/ml. The act/act plasmodia grew very poorly on all plates, including the controls on which they had

TABLE 17

Strain	Genotype	Expt	Protein (mg/ml)		% act growth in actidione (b/a %)
			SDM (a)*	SDM+act. (b)*	
<u>A7119+A7120</u>	<u>(ACT/ACT)</u>	I	0.576	0.036	6.25
		II	1.320	0.228	17.27
		III	1.254	0.138	11.00
<u>A7119+A7103</u>	<u>(ACT/act)</u>	I	0.327	0.084	25.69
		II	0.846	0.192	22.69
		III	0.798	0.147	18.42
<u>A7119+A7137</u>	<u>(ACT/act)</u>	III	1.194	0.123	10.30
<u>A7140+A7118</u>	<u>(act/act)</u>	I	0.297	0.147	49.49
		II	0.348	0.144	41.38
		III	0.558	0.219	39.24

* Average for two flasks of same medium.

Figure 3



reached a size of only a few square centimetres after 3 days incubation, when the ACT/ACT plasmodia had extended to the edges of the plates. The morphology of the act/act plasmodia was characteristically abnormal, even in the absence of actidione; the plasmodia becoming fragmented into many pieces which migrated over the plates increasing in area only slowly and leaving very slimy tracts (Plate 9). A closely similar morphology was later found in all act/act strains and it has not been observed in plasmodia of other genotype except as a stage in senescence (see Chapter 5).

G. Discussion

The experiments on plasmodia in liquid semi-defined medium show that growth of homozygous actidione-resistant plasmodia (act/act), measured in terms of assayed protein or pigment, is inhibited less by actidione than is growth of homozygous sensitive (ACT/ACT) or heterozygous plasmodia. Thus the actidione resistant mutation isolated in amoebae is recessive but confers resistance on homozygous plasmodia. The mutation has the additional effect, also recessive, of conferring abnormal morphology on plasmodia grown on agar in the presence or absence of actidione. These plasmodia grow so poorly that no clear resistance to actidione is shown in agar culture. This effect accounts for the failure of previous work (Dee, 1966) to detect resistance in homozygous plasmodia.

PLATE 9

A PLASMODIUM HOMOZYGOUS FOR ACTIDIONE RESISTANCE

The slime trails, and the breaking up of the plasmodium are shown clearly.



In yeast, both recessive and semidominant mutants resistant to actidione have been studied and experiments using cell-free systems (Cooper, Banthorpe and Wilkie, 1967) have shown that the resistance of recessive mutants resides in their ribosomes. Recessive resistance in P.polycephalum may also be a characteristic of the ribosomes. If this is so, the abnormal plasmodia morphology might be caused by malfunctioning of ribosomes in the absence of the drug, for example by an increased frequency of misreading of messenger RNA. It is known that some drug-resistant mutations in bacteria which involve changes in the ribosomes can alter the detailed translation of messenger RNA. (For review, see Weisblum and Davies, 1968.)

This is the first example in P.polycephalum of a mutation selected in amoebae being found to have a characteristic effect on plasmodial morphology.

Chapter 4

THE APPLICATION OF GENETIC MARKERS TO THE STUDY OF THE PLOIDY OF PLASMODIA

(The analysis of genes f, n and g is fully described in Chapter 2.)

The hypothesis that syngamy occurs when pairs of amoebae fuse, and that the resulting plasmodium is diploid, until the meiotic division associated with sporulation, is supported by two separate lines of investigation, the observation of syngamy to give zygotes (Ross, 1957) and the counts of chromosome number in amoebae and plasmodia of Physarum flavicomum (Ross, 1966).

Contradictory data has however been presented, involving both cytological observation and microdensitometry (Dee, 1966b). It was therefore considered worthwhile to confirm the ploidy of plasmodia by genetic means. If the plasmodia are diploid, then the fusion of two plasmodia carrying genetic markers will not lead to recombinant types occurring in the haploid spores. If, however, the plasmodia are haploid, karyogamy occurring just prior to sporulation, then the fusion of plasmodia carrying genetic markers might be followed by the fusion of haploid nuclei of the two marked types, recombinant spores being subsequently formed.

The experimental procedure used was to fuse two plasmodia of phenotype $\underline{f_2f_2n_2}$, one of which was heterozygous for the recessive allele $\underline{n_1}$, and the other of which was heterozygous for the recessive gene $\underline{g^-}$. (See Chapter 2 for the analysis by which these markers were detected.) By trial and error a pair of plasmodia were found which showed no killing reaction, a viable heterokaryon being established. The plasmodia used were 7141+i, $\underline{mt_1f_2n_2g^-}/\underline{mt_2f_2n_2g^+}$ and ai26+7118, $\underline{mt_1f_2n_2g^+}/\underline{mt_2f_2n_1g^+}$ (ai26 is a progeny clone of a+i). The heterokaryon was induced to spore and progeny clones isolated. These clones were crossed to four clones of amoebae, 7119 ($\underline{mt_1f_2n_1g^+}$), 7118 ($\underline{mt_2f_2n_1g^+}$), E55 ($\underline{mt_1f_1n_2g^-}$) and 7103 ($\underline{mt_2f_1n_2g^-}$). The resulting plasmodia were classified for \underline{g} type and fusion type (by testing with plasmodia representing fusion classes I-VI; I, a+3/a/E55i38; II, a+E55i38; III, 7132+5/a/E55i38; IV, a+i; V, 7119+7118; VI, 7141+i). From the fusion class, or \underline{g} type of the plasmodia the genotype of the (heterokaryon) progeny clones with respect to genes \underline{n} and \underline{g} was deduced. Table 19 describes this analysis, only those clones being included in which both genes \underline{n} and \underline{g} could be deduced.

Amongst the 27 clones analysed completely, no example of the $\underline{g^-n_1}$ type occurred. The data therefore supports the proposition that plasmodia are diploid, no recombination being detected between the nuclei of a heterokaryotic plasmodium.

TABLE 19

The Analysis of Progeny Clones from the Heterokaryon 7141+i/ai+7118

Where a cross produced a plasmodium, this is represented by a $\underline{q^-}$ or a Roman numeral representing the deduced fusion class of the plasmodium (see text).

Progeny clone of $\underline{7141+i/}$ $\underline{ai+7118}$ heterokaryon	Crossed with $\underline{7119}$ $(\underline{mt_1 f_2 n_1 q^+})$	Crossed with $\underline{7118}$ $(\underline{mt_2 f_2 n_1 q^+})$	Crossed with $\underline{E55}$ $(\underline{mt_1 f_1 n_2 q^-})$	Crossed with $\underline{7103}$ $(\underline{mt_2 f_1 n_1 q^-})$	Deduced Genotype of progeny clone
1	VI	-	IV	-	$\underline{mt_2 f_2 n_2 q^+}$
2	-	VI	-	IV	$\underline{mt_1 f_2 n_2 q^+}$
5	V	-	IV	-	$\underline{mt_2 f_2 n_1 q^+}$
6	VI	-	IV	-	$\underline{mt_2 f_2 n_2 q^+}$
8	V	-	IV	-	$\underline{mt_2 f_2 n_1 q^+}$
9	-	VI	-	$\underline{q^-}$	$\underline{mt_1 f_2 n_2 q^-}$
11	-	V	-		$\underline{mt_1 f_2 n_1 q^+}$
13	-	VI	-	IV	$\underline{mt_1 f_2 n_2 q^+}$
15	V	-	IV	-	$\underline{mt_2 f_2 n_1 q^+}$
16	VI	-	IV	-	$\underline{mt_2 f_2 n_2 q^+}$

18	VI	-	IV	-	$\overline{mt_2 f_2 n_2 q^+}$
24	V	-	IV	-	$\overline{mt_2 f_2 n_1 q^+}$
29	VI	-	\overline{q}	-	$\overline{mt_2 f_2 n_2 q^-}$
31	VI	-	\overline{q}	-	$\overline{mt_2 f_2 n_2 q^-}$
35	VI	-	IV	-	$\overline{mt_2 f_2 n_2 q^+}$
39	-	V	-	\overline{q}	$\overline{mt_1 f_2 n_1 q^-}$
40	-	VI	-	IV	$\overline{mt_1 f_2 n_2 q^+}$
44	-	VI	-	\overline{q}	$\overline{mt_1 f_2 n_2 q^-}$
46	VI	-	IV	-	$\overline{mt_2 f_2 n_2 q^+}$
48	VI	-	IV	-	$\overline{mt_2 f_2 n_2 q^+}$
49	-	V	-		$\overline{mt_1 f_2 n_1 q^+}$
50	VI	-	\overline{q}	-	$\overline{mt_2 f_2 n_2 q^-}$
53	-	VI	-	\overline{q}	$\overline{mt_1 f_2 n_2 q^-}$
54	-	VI	-	IV	$\overline{mt_1 f_2 n_2 q^+}$
57	-	VI	-	IV	$\overline{mt_1 f_2 n_2 q^+}$
60	V	-	IV	-	$\overline{mt_2 f_2 n_1 q^+}$
62	-	VI	-	IV	$\overline{mt_1 f_2 n_2 q^+}$

It would obviously be desirable to increase the numbers involved in this analysis, it being possible to analyse only 27 clones for the chosen markers, laborious and involved analysis being necessary. The use of amoebal, as distinct from plasmodial, markers would reduce the amount of work involved, and might enable the detection of rare recombination events between the diploid nuclei of a plasmodial heterokaryon. As was stated in the preceding chapter (Chapter 3) a vigorous search for stable amoebal markers has so far largely failed.

Chapter 5

SENESCENCE OF THE MYXOMYCETE, PHYSARUM POLYCEPHALUM

(SCHWEINITZ)

1. Introduction

(a) The phenomenon of senescence is of interest both for its own sake and because its study may provide insight into other, non-senescent degenerative phenomena, and even into the "perfect" functioning of the organism. The study of senescence has reached the stage of the careful definition of the phenomenon, and the proposition of hypotheses to explain its causation. However, the experimental testing of these hypotheses is at present hindered by certain features of the organisms which have been used in the study of senescence. The organisms habitually used are Drosophila, mice and men. The difficulty of conducting certain experiments with highly developed animals has led recently to increased interest in the study of senescence in simpler organisms, for example the filamentous fungi, the ciliates and certain protozoan amoebae. This Thesis reports the first observation of senescence in another group of organisms, the acellular slime-moulds or Myxomycetes. The Myxomycetes possess certain advantages as experimental organisms for senescence research.

(b) Definitions.

The terms "ageing" and "senescence" do not seem to have an agreed use, and have even been considered as synonyms. Strehler (1962) apparently considers the terms as synonyms and defines senescence "as the changes which occur generally in the postreproductive period and which result in a decreased survival capacity on the part of the organism". Medawar (1951) clearly does not consider the terms to be synonyms, and defines senescence "as that change of the bodily faculties and sensibilities and energies which accompanies ageing, and which renders the organism progressively more likely to die from accidental causes of random incidence. Strictly speaking, the word "accidental" is redundant, for all deaths are in some degree accidental. No death is wholly "natural"; no one dies merely of the burden of the years". Maynard Smith (1963) considers them as synonyms and defines ageing processes "as those which render individuals more susceptible as they grow older to the various factors, intrinsic or extrinsic, which may cause death". Frequently the terms are simply used without defining the manner in which they are being used.

In this Thesis the terms will not be considered as synonyms. The term ageing will be defined as all time correlated changes in an organism which cannot be attributed to changes in the environment.

If defined in this manner, ageing can be sub-divided into two classes of phenomena, those which appear to be adaptively valuable to the organism or its progeny, and those which appear to be disadvantageous. It is to these apparently disadvantageous changes that the term senescence will be restricted in this Thesis.

An example of an adaptively valuable change with age would be the development and sexual maturation of a mammal. Some adaptively valuable changes, or developments, involve the partial degeneration of the organism, for example the extensive cell destruction occurring in the pupae of endopterygote insects, or degeneration of the flowering structures of an Angiosperm. More extreme examples involve the degeneration of the whole organism in the process of improving the fitness of the progeny. Examples of such extreme phenomena are the unavoidable death of nematodes at ovulation, and the death of annual plants which is involved with the diversion of energy into the production of fruit. These degenerative but adaptively valuable ageing phenomena have been not infrequently referred to as senescence phenomena. I consider that the mechanism underlying adaptively valuable ageing will probably be totally different from that underlying non-adaptive ageing, since selection will be acting to facilitate the former and hinder the latter. For this reason, the term senescence will be restricted to the functionally separate phenomenon of non-adaptive ageing.

The term senescence will now be defined. Strehler's definition (p.85) seems inadequate both through errors of inclusion and omission. It assumes as a separate phenomenon the post-reproductive period, while I consider the post-reproductive period should surely be viewed as part of senescence, and fails to distinguish between genetically adaptive and apparently non-adaptive degeneration of the organism. Medawar's definition (p.85) contains an assumption, that senescent death is a response to an environmental challenge which the organism in its weakened condition cannot withstand. I consider this to be neither necessarily true, nor useful as an experimental hypothesis. The assumption is possibly defensible within the context of senescence in a mammal, but is not useful when considering senescence in simpler organisms grown in a standardized environment. All three definitions contain two assumptions which should be made explicit, the environment must not undergo any significant change, and the environment must be benign.

In summary, senescence is defined "as the process which results eventually in the progressive loss of fitness of an organism per unit time, despite the maintenance of a constant and benign environment". Senescence is usually measured either as the mortality rate within a population of similar organisms, or at the individual level retrospectively after observing the organism's eventual death. In the definition the loss of fitness referred to is the apparent loss of genetic fitness.

(c) It is proposed in this section to review the current position of senescence research. The literature is obviously too considerable and too disparate to be reviewed thoroughly here, and recent reviews are in any case available (see Szilard, 1959; Strehler, 1962; Maynard Smith, 1963; S.E.B. Symposia, 1967). It is however necessary to give some outline of certain areas of the field so that this Thesis may be viewed in context. It is difficult to separate current hypotheses from the experimental systems in which they are founded and so this review will consider, under separate headings, the current hypotheses and the relevant experimental systems. A certain amount of overlap between sections is inevitable. The review will obviously be biased in the direction of those hypotheses to which this Thesis is relevant.

It should perhaps be said that senescence in complex organisms is unlikely to be a unitary phenomenon, and that in these organisms it is difficult to analyse cause and effect in senescence syndromes. Therefore it is possible that several, or indeed all, of the various hypotheses for senescence may be correct. The problem is whether particular processes occur to significant quantifiable extents.

(i) Senescence of Extracellular Components

The present state of knowledge on this subject has been reviewed by Strehler (1962), Hall (1967) and Houck et al (1967). The data is at present concentrated on qualitative changes in the two major

connective-tissue fibrous proteins of mammals, elastin and collagen. Experimentally it is frequently difficult to separate changes in these two components when considering a tissue. Factors contributing to this difficulty are, that collagen becomes degraded to pseudo-elastin which has many resemblances to elastin (Hall et al, 1955; Hall, 1956; Hall, 1957), and that the two fibres are similar in the composition of some of their subunits and are probably formed by a single cell type. Quantitative changes occur, particularly in the collagen content of tissues. However, in the space available, attention will be concentrated on qualitative changes.

Elastin undergoes two age correlated changes. The first process is largely complete at birth and involves the cross-linking of fibres by the formation of the complex amino-acids desmosine, isodesmosine and lysinonorleucine (Thomas et al, 1963; Franzblau et al, 1965). During post-natal life this cross-linking process proceeds, partly by accumulation of lysinonorleucine and partly it has been suggested (Banga, 1961) through the action of polysaccharide, lipid and coordinately bound calcium ions. It is difficult at present to know whether these changes in elastin should be considered solely as maturation, or to what extent they involve a certain loss of function, that is senescence.

It is apparent that collagen fibres also change with age, due

partially to cross-linking. Mature collagen has different solubility properties from newly synthesised collagen, and shows greater resistance to chemical or thermal denaturation. Denaturation manifests as the contraction of the fibres. In young fibres it is considered that the stabilizing bonds which have to be overcome to allow contraction are hydrogen bonds, while in mature collagen the stabilizing bonds are covalent. Veis and Anesey (1963) established that intermolecular links do occur between the macromolecules of tropocollagen of which collagen is composed. It is however still not certain what the exact nature of these bonds is. Milch and Murray (1962) have considered that aldehydes could be the most likely form of cross-linkage. Apart from cross-linkage another change in collagen is the accumulation of hexoses in the form of a cellulose-like carbohydrate (Hall and Sand, 1961).

The stabilisation of collagen by cross-linking is probably best considered as a maturation phenomenon, increasing the "stiffness" of the tissue. However the subsequent conversion of mature collagen into pseudo-elastin results in a loss of tone and is therefore degenerative. The mechanism of conversion to pseudo-elastin is still obscure, but it is possible that the occurrence of the cross-linking maturation process is necessary for the development of degeneration to pseudo-elastin, by enzymic or other means.

The fibrous proteins have attracted the attention of senescence research because, by means of labelling experiments, it has been shown that their rate of turnover is remarkably slow, for example the mature collagen of rat tissues has a half-life of as much as 300 days (Houck et al, 1967). These molecules therefore form a semi-permanent target at which damage may accumulate. As a generalization the fibrous proteins have long half-lives and the globular proteins show rather rapid turnover.

It might be mentioned here that radiation which shortens the life expectancy of various organisms (see below), does not produce any alteration in the maturation or degeneration of collagen (Sinese, 1960).

The molecular ageing, both maturation and senescence, of fibrous proteins has only been reviewed in brief since it is unlikely, for reasons which will be made clear below, that Myxomycetes will be affected by this type of phenomenon.

(ii) Another group of hypotheses involves the breakdown of inter-cellular relationships in multicellular organisms, the two systems of particular interest being the immune and the endocrine systems in mammals.

The immune theory of ageing has been recently reviewed by Walford (1962) who has neatly summarized the hypothesis: "It has been proposed that ageing is a consequence of increasing immunogenetic

diversification of the dividing cell populations of the body. The diversifying cells lose the ability to recognize "self" and a low-grade prolonged histo-incompatability reaction sets in, analogous to a chronic autoimmune state, and manifested as ageing. The theory in its present form is most conveniently regarded as an extension of the somatic mutation theory of ageing (see below) into the realm of pathogenesis. However, a direct connection between the two theories is not essential. Actual genetic change is not necessarily required for autoimmune reactions". However Walford also says: "Whether immune dysfunction is primary and causes ageing or secondary to other facets of ageing, is not established". The theory is probably irrelevant to ageing in Myxomycetes since the immune theory cannot be relevant to organisms possessing no immune system, in other words organisms other than vertebrates. However Walford says: "An immune theory might possibly encompass ageing in lower animals by reference to the phenomenon of contact inhibition or syngenic preference as quasi-immunological phenomena". (For a review of para-immune phenomena in non-vertebrates see Cushing, 1967)

The endocrine system of mammals is attracting less interest as a primary cause of senescence. It has been considered that the endocrine organs constitute a particularly vulnerable target, or alternatively that the inter-reactions of these organs is a possible area for malfunction. However the more cautious modern approach is

summarized by Bellamy (1967): "There are indication from parabiosis in rats that systemic factors are important in controlling the ageing process (Lunsford et al, 1963). Also some of the age-related changes in tissues that are target organs for hormones seem to be reversed when old tissue is transplanted into younger animals (Franks and Chesterman, 1964). But experiments involving the extirpation of endocrine glands and the injection of hormones indicate that hormones do not exert full control over the ageing process. Endocrine glands are involved in the manifestation of some signs of old age but hormones can only modify the rate of ageing in certain tissues". The hormonal control of the menopause in the female mammal has been used as support for theories of "programmed" or "adaptive" senescence. Alternative analysis is however possible.

The occurrence of malignancies has been viewed as a breakdown of the systemic control of cell division. This subject will be considered as part of the Section on somatic mutations (see below).

(iii) Somatic mutation theories of senescence

This field has received considerable attention, both conceptually and experimentally. However it has recently been doubted whether somatic mutation plays a significant role in senescence. In this section somatic mutation in the form of change in the presence or nature of nuclear genetic material will be considered. Other changes,

for example in control mechanisms, or in episomes will be considered under other sections.

The experimental situation is complicated by the fact that two distinct types of cell occur in higher animals, dividing cells and non-dividing (post-mitotic) cells. The consequences of a mutation occurring are different in the two types of target. In tissues composed of dividing cells selection between cells can operate, favouring those cell variants whose properties enable them to multiply rapidly, over those which perform efficiently the functions necessary for the survival of the organism. The model system for this process is the occurrence of malignant disease, but selection may result in less dramatic changes which nevertheless contribute to senescence. In post-mitotic cells, mutation is likely to be observed as resulting in cell death. It must also be borne in mind that the two cell types are different in sensitivity to certain mutagenic treatments. Dividing cells are especially sensitive to mutation through loss of genetic material following X-ray or UV induced breakage of the chromosome structure. The fragmentation of chromosomes is unlikely to be lethal for post-mitotic cells. Mitotic cells are also, on theoretical grounds, more likely to accumulate errors in DNA content, since errors are likely to occur more rapidly during synthesis of DNA than during the process of DNA damage and repair, or other forms of DNA turnover. Experimentally

it is however very difficult to measure the mutation rate of post-mitotic cells, since cytologically the chromosomes cannot be displayed, and direct observation of mutation is not easy at the single cell level. Finally it is obvious that a prerequisite for the malignant type of degeneration is the occurrence of dividing cells, preferably able to disseminate, and which are supplied by an efficient vascular system. Thus this type of phenomenon could not occur in adult Drosophila, which only show cell division in a few tissues.

The extensive work on cancer in mammals has been considered by some to be very relevant to senescence, but it is certainly not the whole story, and has even been considered as of dubious relevance to the main problems of senescence. Shock (1964) has stated: "The death of highly differentiated cells such as muscle or nerve cells which are incapable in the adult of undergoing mitosis, cells whose loss cannot be made good by division, is most closely allied to the physiological manifestations of ageing" (in the mammal). The loss of postmitotic cells during senescence has been recorded in numerous animals and tissues, for example by Andrew (1939) in the brain of the rat, and by Rockstein (1950) in the brain of the worker honey-bee. The somatic mutation theories of senescence considered below all seem to accept the view of Shock.

The first suggestion that senescence changes may have genetic damage as their source was the observation that ionising radiation, in sub-lethal doses, shortens the life expectancy of surviving animals, the effect being dose dependent. This effect was first observed by Henshaw, Riley and Stapleton (1947) and was formalized into a theory by Failla (1957) and later modified by Szilard (1959) and Maynard Smith (1963). Support for the theory has been obtained through the cytological observation of nuclear abnormalities, particularly in liver cells of mammals. Although there is no gross change in the ploidy of nuclei as measured by DNA content (Falgone et al, 1959), cytologically a time correlated increase in various types of aneuploidy is observed in unirradiated mammalian livers, figures as high as 40-60% abnormal being recorded in the Chinese hamster (Yerganian and Gagnon, 1958).

Curtis (1967) has shown that following exposure to X-rays, the frequency of aberrant anaphase figures is considerably increased. (The liver is induced to regenerate, and therefore display mitotic figures, by the destruction of a large proportion of the liver with a dose of carbon tetrachloride.) However this effect is not maintained; within 9-12 months after irradiation the level of abnormalities has fallen back to the control level.

The data from aneuploidy counts seems therefore to indicate, not that mutations are spontaneously accumulating in the liver, but

rather that the increased level of abnormal nuclei in the liver is a measure of a changing cell environment, which is unaffected by radiation. Alexander (1967) has presented a convincing critique of the somatic mutation theory. In particular he makes two points, the first of which is that the shortening of life expectancy following irradiation is caused predominantly by deaths due to malignancies, and that the particular forms of malignancy are not the normal terminal stage of senescence in the strains studied. In other words radiation is inducing a new class of malignancies, and does not therefore appear to be mimicking senescence. Alexander's second point is a quantitative one, which will be quoted in extenso:

"Direct experimental evidence that ionizing radiation causes somatic mutation in rats was provided by Russell and Major (1957). Mutations at specific loci in prospective pigment cells were detected by the formation of mosaic patches in the coat following 100 and 150 r of X-rays in 10-day-old embryos of a strain heterozygous at four coat-colour loci. The somatic mutation rates were $7.0 \times 10^{-7}/r/\text{locus}$ (95% confidence limits 2.5 - 11.3) as compared with a germ mutation rate at the same loci following irradiation of spermatogonia of $2.4 \times 10^{-7}/r/\text{locus}$. From this it can be deduced that the rate of induction of somatic mutations will be doubled by a dose of radiation in the range 10-100 r. This means that if the accumulation of somatic mutation is an important cause of ageing then irradiation

with doses in excess of 100 r should show an observable increase in the rate of ageing". It is Alexander's view that such an increase is not observed. It could be said, by way of criticism of Alexander's analysis, that both the gametic and somatic mutation rates in response to X-rays were measured on dividing cells. X-rays are particularly mutagenic to dividing cells and the failure to accelerate senescence of the adult might be due to many of the cells having become post-mitotic. In support of this criticism it is observed that mice show less liability to radiation-induced life-shortening as they get older. This problem of the quantitation of mutagenesis in post-mitotic cells cannot be easily resolved, but Alexander has attempted to satisfy the criticism by observing the effect on life-span of various mutagenic chemicals, in particular ethyl methane sulphonate. No shortening of life-span was observed with this highly mutagenic substance.

Another objection to the somatic mutation theory of senescence stems from the work of Clark and Rubin (1961). It is to be expected that many of the hypothetical mutations will be recessive. The wasp, Habrobracon, occurs in several forms, as diploid females, and as diploid and haploid males. All three forms have very similar life expectancies, but the haploid organism is more sensitive to high doses of radiation than the diploid. This suggests that

although radiation does induce mutations which are recessive in the diploid, and expressed in the haploid, that the phenomenon of somatic mutation is not significantly involved in the development of senescence. Attempts have been made to apply similar analysis to organisms showing XO or XY sex chromosome systems. However, the widespread acceptance of the Lyon hypothesis (Lyon, 1968) has, at least for mammals made such analysis redundant. It is perhaps regrettable that the attempts to find the target site of radiation damage have concentrated on dividing cells, particularly those of the liver. Data concerning the occurrence of cell death, especially in the brain and muscles, although difficult to collect, would be of considerable interest.

Failla (1957) proposed that ageing was due to dominant mutations killing or damaging somatic cells. It was proposed that the mortality rate is inversely proportional to "vitality", which is assumed to decay as a first-order decay process. The mutation rate was estimated after making the assumption that the variation observed in the occurrence of death in populations, that is the shape of the life table, can be used to calculate the development of senescence within individuals. This is however not necessarily true; the variation may be in part due to genotype or variations in environment. The theory acted as a considerably stimulus to research, but the above data do not seem to support any variation of the somatic

mutation hypothesis.

Szilard (1959) proposed a modification of the Failla theory. Using data from twin studies in man he argued that the variation in life-expectancy in human populations is genetically determined. Szilard hypothesized that the diploid organism carried with it a number of recessive cell lethal mutations, which were responsible for the variation in life expectancy. Senescence was caused by the occurrence of recessive mutational events, which either when the other homologue of the chromosome carried a genetic fault, or when a senescent mutation occurred in both homologues, resulted in the death of the cell. In order to reconcile data on the occurrence of recessive lethals in the population, it was necessary to assume that the senescence mutation involved the loss of a complete chromosome, or a large amount of it. (This is not unreasonable if dividing cells are assumed to be the target, but if they are then it is not explained why there is no selection for viable cells.) The organism was assumed to die when the surviving fraction of its cells had fallen to a critical value. Maynard Smith (1959) criticised this theory on the genetic grounds that, according to Szilard's theory, inbreeding, by eliminating deleterious recessives, should prolong life expectancy. It is however known both from Drosophila (Clarke and Maynard Smith, 1955) and from mice (Mühlbock, 1957) that inbreeding reduces life expectancy.

Szilard (1959) modified his theory suggesting that the level of senescent damage tolerated by inbred animals was less. However if this is accepted then it becomes uncertain how much of the variability of organisms with respect to life expectancy is due to recessive lethal genes and how much to the "tolerance level", which has to have some other explanation. The theory can be made to fit the observations on survival curves, but only at the expense of numerous experimentally unsupported ad hoc assumptions.

It should perhaps be mentioned here that the somatic mutation theory satisfies one requirement of a senescence theory; it explains why the physiological state of an offspring is unaffected by the age of the parent. Selection against mutant haploid nuclei would occur at the gametic level and later during the development of the diploid embryo.

To summarize, although the observation that cell death is one of the major factors of degeneration in a higher organism remains unchallenged, it does not seem likely that somatic mutation is the predominant cause of this cell death. Other possible causes of this cell death will now be considered.

(iv) Theories of failure in differentiation

Weismann (1891) considered that senescence was an inevitable consequence of the differentiated state of the metazoan soma. There appears to be a conceptual confusion in his theory between the post-

mitotic state making the organism inevitably mortal, and the actual causation of the process of degeneration. Minot (1907) considered that the slowing down of growth and eventual achieving of a predictable adult size, which is a characteristic of many animals, reflected a controlled "loss of vigour" of the cells of the organism. This loss of vigour, Minot theorised continued after the achievement of the adult form and led to senescence. Although of historical interest this theory does not appear to have any relevance in view of more modern data on the control of growth. Differentiation theories of senescence are at present underdeveloped because of underlying doubts about the actual mechanism of differentiation. Medvedev (1967) having considered that differentiation reflects the control of DNA transcription probably by a system of regulator genes, proposes that mutations in such regulator genes may have particularly widespread consequences and could be of significance in senescence. More generally, apart from mutations, it is theoretically conceivable that whatever substances are regulating transcription they may be damaged or caused to behave erroneously. Strehler (1967) considers that his model for differentiation, involving differentiation at the level of sRNA, may be relevant to the phenomenon of shortened life expectancy in inbred animals.

Hayflick (1965) has shown that some cell lines exhibit, during tissue culture, a limited division capacity (approximately 50

doublings). Strehler (1967) has said however that: "The relevance if any, of these in vitro findings to the cell biology of ageing in vivo has not yet been established It is more likely that the conditions of culture predispose these fibroblast-type cells to undergo 'terminal differentiation'." In summary, Strehler (1962) has said: "The slowness of the senescence process in long-lived mammals casts serious doubt on any theory of senescence which uses the intrinsic-determinant properties of cells as a direct cause of senescent change." This comment is pertinent to Minot's theory, and other theories involving the assumption that the differentiated cell cannot achieve a steady state. However an age-correlated change of post-mitotic cells, which may support the assertion that the apparent steady-state is in fact unstable, is the accumulation of intracellular waste products, which will be considered below.

(v) Theories involving the intracellular accumulation of waste products, especially "age pigments"

The accumulation of urates with advancing age is a unique storage phenomenon of many insect species. However the phenomenon seems restricted to the fat body (Metchinikoff, 1915). In mammals the occurrence of pigmented inclusion bodies, "age pigments", in such non-dividing cell lines as nerve and muscle has been known

since the turn of the century (Stübel, 1911). The properties of "age pigment" or "lipofusion" include a brilliant fluorescence, and incomplete solubility in aqueous or lipid solvents. The pigment consists of 30-40% lipid and substantial protein, which suggests a lipoprotein origin, probably from membranes (Hendley and Strehler, 1965). The origin of these pigment bodies is still in some doubt. Hess (1955) on the basis of electron microscope studies, considered them to be derived from degenerating mitochondria. Bondareff (1957) however reported that the pigment was derived from or related to the Golgi apparatus. Two histochemical studies, one by Gedigk and Bontke (1956,) and the second by Essner and Novikoff (1960) appeared to relate the pigments to lysosomes. However this last conclusion has been placed in doubt by the work of Hendley and Strehler (1965). Sulkin and Srivani (1960) have questioned the significance of age pigments as a cause of senescence, on the ground that the pigment accumulation is accelerated by various treatments, for example, cortisone, and no increase in senescence appears to result. The central unanswered question is whether the accumulation of these pigments ever impairs cellular function.

(vi) Theories of senescence involving mitochondria

This topic will be considered in some detail as it may be very relevant to senescence in the Myxomycetes. Mitochondria are responsible for aerobic respiration in Eukaryotes. Rafsky et al (1952)

studied the respiration of guinea pig tissue homogenates. Kidney homogenates showed some possibly significant decline in respiration rate with age. This is corroborated by a similar slight decline in succinoxidase activity in kidney and heart homogenates. Barrows et al (1960) have data which suggest that this reflects a decrease in the number of mitochondria rather than a change in the relative amount of the enzyme per mitochondrion. These results are complicated by the complex nature of the tissues, some cells of which are capable of division to replace cell death. Dempsey (1956) has attempted to show a correlation between the age of the animal and morphological changes in the mitochondria. However it is arguable that this could be a consequence rather than a cause of senescence, since mitochondria seem to be rather sensitive to the intracellular environment.

It has been mentioned above that Hess (1955) considered the lipofusion granules of post-mitotic cells to be derived from degenerating mitochondria. Rockstein (1967) has analysed in detail age-correlated changes in the mitochondrial and non-mitochondrial enzymes of the house-fly. It is however too soon to attempt a causative analysis of the changes. Fletcher and Sanadi (1961) have labelled mitochondria of rats by various procedures and have found that mitochondrial lipid, and all other constituents, have a half-life of about 12 days. This has been interpreted as meaning that the mitochondria themselves have a half-life of this order of

magnitude. Strehler (1962) has considered: " that this rapid turnover rate would preclude the type of deteriorative changes expected of unrenovable cell constituents it is of course possible that the frequency of errors in mitochondria newly synthesized by old animals is higher than that occurring in young ones." To evaluate whether this is a possibility it is necessary to consider briefly what has become known recently about the origin and function of mitochondria.

Gibar and Granick (1964) have reviewed the evidence that mitochondria possess a genetic system independent of the nuclear system. Perhaps the first clear evidence that mitochondria had a genetic role came from the aerobically incompetent "poky" mutants of the fungus Neurospora crassa (Mitchell and Mitchell, 1952) and the "cytoplasmic petites" of various facultative anaerobic yeasts, especially Saccharomyces cerevisiae (Roodyn and Wilkie, 1968). The cells of petite yeast cultures have lost their aerobic respiratory mechanism. The characteristic slow growth of the petite colonies is a consequence of this respiratory defect. The difference in rate of growth of normal and petite cells disappears under anaerobic conditions.

In Neurospora some aerobic respiration is necessary for survival. The respiration of the poky mutant is defective, but the oxygen uptake is still one-third normal. In Neurospora the poky character is inherited only through the female (protoperithecal) parent and

cannot be transmitted through the male parent. The male in Neurospora apparently contributes a negligible amount of cytoplasm when the nucleus is transmitted, which makes Neurospora particularly valuable for the study of cytoplasmic inheritance. In yeast the sexual cycle is initiated by the complete fusion of two haploid cells, which results in a zygote containing a mixture of the cytoplasms of both parents. The independent genetic system of mitochondria has been biochemically established by the finding of DNA in the organelle (Nais, 1965), and the demonstration of protein synthesis by isolated mitochondria from a number of organisms including mammals, yeast and Neurospora. The poky and petite cytoplasmic respiratory incompetence is established by enzyme analysis as almost certainly of mitochondrial origin. Poky and petite strains typically show the same change in spectrophotometrically observed cytochrome profiles, cytochromes a and b being absent in petite and reduced in poky, and cytochrome c being present in the normal amount (Haskins et al, 1953; Hardesty et al, 1963; Smith et al, 1969). The exact nature of the mitochondrial mutation is still not clear. In Neurospora it involves, in some strains, a point mutation leading to a single amino-acid substitution in a mitochondrial membrane structural protein (Woodward and Munkres, 1966). The connection between the change in structural protein and loss of cytochromes a and b is not clear, but these two enzymes are membrane bound, whereas cytochrome c is soluble. There is evidence that cyto-

chrome c is not synthesised in the mitochondria (Wagner, 1969), for example the amino-acid sequence of cytochrome c in yeast is demonstrably altered by nuclear mutations. It is possible that the altered membrane structure results in either the membrane bound enzymes being unstable, or in a pleiotropic failure in intramitochondrial protein synthesis. It is reported that some petite strains also show altered mitochondrial protein (Zuppy et al, 1968). However, Mounolou, Jakob and Slonimski (1967) have found in several petite strains that the M-DNA has been grossly altered, although not physically lost. Wilkie and Maroudas (1969) state that: "It is widely believed that the petite condition results from an effective loss of M-DNA as a functional genetic unit, a conclusion supported by the apparent irreversible nature of the mutation." The exact function of the mitochondrial DNA is still to be analysed, mitochondrial proteins having three possible origins, (i) extramitochondrial, (ii) coded for by M-DNA, or (iii) coded for by nuclear DNA but translated by the mitochondrial protein synthesising apparatus. A review of the current state of analysis is that of Roodyn and Wilkie (1968).

It has been assumed until recently that mitochondria can arise de novo within a cell lacking mitochondria. This was based in part on the observation that in yeast grown in anaerobic culture no mitochondria are evident, but that when there is a shift to aerobic respiration mitochondria appear. It now seems established however

that "promitochondria" persist in the anaerobic phase. A curious property of poky and petite mitochondria is that under certain circumstances they will dominate or eliminate wild type mitochondria if these are present in a common cytoplasm (Pittenger, 1956; Wagner, 1969). (A final point concerning this system: the occurrence of spontaneous mutations to the petite state is exceptionally high, of the order of 1% per cell generation, and can be accelerated even further by intercalating mutagens (acridine gives up to 100% induction, ethidium bromide is effective, and UV can result in 20% petite cells following irradiation) (Wilkie and Maroudas, 1969))

To summarize the aspects of mitochondria which may be relevant to senescence; mitochondria arise from preexisting mitochondria; mitochondria have their own DNA information content which is qualitatively and quantitatively different from nuclear DNA in its mutability; and a particular form of mitochondrial mutation frequently occurs which although non-functional replicates normally and is retained in the cell. It may therefore be said that it is in fact quite possible "that the frequency of errors in mitochondria newly synthesised by old animals is higher than that occurring in young ones" (see quotation from Strehler above).

If degeneration of mitochondria is a significant component of senescence, the following results should occur. For post-mitotic cells in a higher animal mitochondrial damage will accumulate as a

first-order decay process, leading eventually to cell death after a lag phase. Alternatively, in view of poky mitochondrial dominance over wild type mitochondria, cell death may occur directly as a first-order decay process. Senescence should show acceleration following the use of intercalating mutagens, of the acridine type; other forms of mutagenesis have not been investigated for their quantitative effect on mitochondria. An advantage of the model is that the spontaneous mutation rate of mitochondria is high. An interesting speculation is that the increased life expectancy displayed by outbred organisms could be ascribed to mitochondrial heterogeneity or even fusion. The work of Sarkissian and McDaniel (1967) on inbred and outbred maize, which shows just such an increased vigour resulting from mitochondrial heterogeneity and interaction, makes this speculation defensible. Finally, if it is possible to determine, the mitochondrial theory predicts that senescence will be found to be a cytoplasmic, not a nuclear phenomenon.

Two analyses will now briefly be considered, which could be interpreted as support for this hypothesis, or have alternatively been taken as support for the Orgel hypothesis (see vii below). Both systems involve the senescence of filamentous fungi; the first concerning Aspergillus glaucus occurs in all strains; the second involves a mutant of Neurospora crassa and is perhaps less relevant.

Both are of considerable interest because they involve the senescence of a growing mycelium, and may therefore involve the degeneration of synthetic systems rather than static ones, and secondly because in the filamentous fungi it is possible to determine, by contrasting sexual fusion with heterokaryosis, the roles of nuclei and cytoplasm separately.

Jinks (1959) was responsible for the analysis of "vegetative death" in Aspergillus glaucus. The irreversible cessation of growth, accompanied by the death of the growing tips and the appearance of an intense brown pigment is a regular feature of aged clones in this species, taking from five months to two years before occurring. The heterokaryon and related tests show that vegetative death is cytoplasmic in origin, while its segregation in the asexual progeny of mycelia with mixed cytoplasms and its infective spread via hyphal anastomoses suggest that it is some form of particle of mutational origin. The agent responsible for vegetative death suppresses its wild type counterpart in heterokaryons. Jinks does not speculate concerning the causative agent of vegetative death.

Sheng (1951) has analysed a mutant "natural death" or "nd", which was isolated following ultraviolet irradiation of conidia of Neurospora crassa. The mutant has been mapped and assigned to a chromosomal linkage group. The mutant mycelium shows an ever decreasing growth potential, eventually ceasing to grow. The dying

margin shows hyphae of irregular morphology which often burst; the cessation of growth is often associated with the production of a brown pigment. The nd gene is recessive to the wild type, balanced heterokaryons of normal form being achieved. Haploid nd vegetative asci derived from such heterokaryons show rejuvenation. Sheng interprets the phenomenon as being due to the mutant being deficient in excretion. This is the most probable explanation; however alternative analysis is possible (see vii below).

Kerr and Waxlax (1969) have recently described a mutant of the Myxomycete Didymium nigripes which shows "a limited life expectancy". This organism has a uninucleate haploid amoebal phase and a multinucleate diploid phase. One difficulty with the organism is that it apparently does not show a sexual cycle, haploid amoebae giving rise directly to diploid plasmodia. The "ageing" mutant was isolated in a strain which was already mutant for cycloheximide resistance. The mutant is characterized by a yellow pigment in the diploid plasmodium, and a restricted life expectancy for the plasmodium. Before death occurs the plasmodia lose the ability to fruit or form heterokaryons. However there is considerable variation in life expectancy between plasmodia which might be expected to be genetically identical, some plasmodia living only a few days and some being of apparently indeterminate life expectancy (in excess of 100 days). It is considered that the amoebal phase, which also accumulates the yellow pigment,

also shows ageing, which could presumably be antagonised by selection between amoebae. The yellow-ageing variant is rejuvenated after passage through a heterokaryon followed by fragmentation. The wild type plasmodium has been maintained in vegetative culture for periods in excess of one year without showing any age changes. The inheritance of the character, as far as it can be studied with the restricted system of heterokaryosis, suggests that the yellow-ageing character and the cycloheximide resistance remain together. This suggests that they are both located in the nucleus. However the development of the yellow character is variable, and the situation is further confused by the cycloheximide resistance character usually disappearing in heterokaryons, probably due to viability selection at the stage of spore formation.

It is difficult to interpret the data of Kerr and Waxlax because the paper does not mention the behaviour of genetically identical plasmodia maintained in parallel culture. Assuming that, because of homothallism, the clones isolated from spores by Kerr and Waxlax are effectively genetically identical, then from the small number of plasmodia reported, ~~i-g~~, it appears that the mortality rate is constant. In other words there is no increase in liability to death with the passage of time; the mutant organisms appear to be vulnerable to some environmental factor but are, by definition, showing impaired viability rather than senescence.

(vii) The Orgel hypothesis; the accumulation of errors in translation

Orgel (1963) has proposed an elegant hypothesis by which errors in protein synthesis, rather than being diluted out, become amplified. There is however at present little direct evidence concerning the accuracy of protein synthesis, either at the level of mistakes in transcribing mRNA or at the translation level. The attachment of each sRNA to its specific amino-acid is perhaps most liable to be a source of error. Orgel states: "Our lack of knowledge of the error-frequency in protein synthesis makes it impossible to say, a priori, whether or not the accumulation of errors of protein sequence is relevant to the processes of ageing in higher organisms, this point must be decided experimentally." Orgel's hypothesis states that if a small proportion of the protein molecules involved in intermediary metabolism were erroneous, then the reaction step would have a slightly less effective functioning. This effect would not be cumulative; once the faulty messenger RNA or protein had been degraded all consequences of the error would cease. However, if the erroneous protein molecule was involved in the process of "information handling", then the effect will be cumulative resulting in the exponential accumulation of errors and eventually cell death. Such a feedback is easily visualized in terms of a faulty sRNA activating enzyme causing erroneous sRNA-amino acid complexes, resulting in the production of a number of erroneous proteins, some of which will be

sRNA activating enzymes. Orgel proposed procedures for testing his hypothesis; he suggested applying to organisms pulses of amino-acid analogues which were known to become incorporated into proteins and cause their malfunctioning (e.g. parafluorophenylalanine or ethionine). Fluorouracil was suggested as a means of studying the consequences of errors in mRNA, as FU is incorporated in the place of uracil but behaves in coding in a manner analogous to cytosine.

Holliday (1969) has attempted to apply Orgel's suggestions, particularly to the filamentous fungi, and has also developed an original approach of his own. Harrison and Holliday (1967) showed that "non-toxic" concentrations of amino-acid analogues when fed to Drosophila larvae reduced the longevity of adults. However Maynard Smith (personal communication) has shown that the feeding of analogues to adults has no effect on life-expectancy. This suggests that the life-shortening effect of analogues operates through proteins synthesized during the larval or pupal period, and not during the adult period. If the life shortening effect is due only to a toxic effect of substandard non-renewable proteins, as seems apparent, it is clearly not an "Orgel phenomenon". It may be considered probable that the analogues are permanently present, and therefore the condition that the treatment must be present only as a pulse has not been met.

Harrison and Holliday (1967) performed some of their experiments with the senescence system of Podospora anserina (Rizet, 1959; Marcou, 1961), which is apparently closely analogous to the vegetative death phenomenon in Aspergillus glaucus. Marcou (1961) does not consider the Podospora degeneration to be explicable in terms of a particle, on the basis of two lines of evidence. Firstly it is possible to rejuvenate mycelia by a wide range of treatments, for example cold or dessication. However it has been suggested that these treatments result in the hyphae becoming discrete from each other and selection for vigour therefore becoming possible. Secondly and more curiously, the data of Marcou on pedigree analysis of senescence in a series of parallel cultures have been interpreted as meaning "that the probability of the event initiating senescence is directly correlated with the length of the incubation period (the period between initiation and death)." If the interpretation is sound this suggests that the phenomenon is not auto-catalytic, but both the primary "hit" and the subsequent accumulation of errors are due to the same process. Holliday has stated: "The explanation of senescence on the basis of Orgel's hypothesis would predict such a relationship (between initiation and incubation) since both the initial event and the incubation period are manifestations of the infidelity of protein synthesis." I disagree with this analysis. It appears to me that the initiation of senescence, on the Orgel hypothesis, is a manifestation of the

infidelity of protein synthesis, but the subsequent exponential increase in errors is due to the occurrence of mistakes in a system containing erroneous "information", a different process altogether.

Holliday (1969) treated Podospora mycelia with a variety of amino-acid analogues and acquired some rather unclear results. Since the analogues were incorporated in the medium, and were not therefore used as a pulse, the question of continuous toxicity confuses the analysis. Holliday has also experimented with Neurospora crassa. The leu-5 mutant (Printz and Gross, 1967) is believed to have an abnormal leucyl tRNA synthetase. The mutant is temperature sensitive, growth stopping at 37°C. Holliday interprets this as indicating that at the higher temperature the abnormal leucyl tRNA synthetase results in an Orgel phenomenon. However it could be considered that the erroneous proteins of the mutant are simply heat labile. It must also be considered that at 25°C the leu-5 mutant does not show senescence, which it perhaps might be expected to do if Holliday's analysis is correct. Finally, Holliday made the hypothesis that the natural death nd chromosomal mutation of N. crassa (Jinks, 1959) caused death through the induction of an Orgel phenomenon, the accumulation of an erroneous protein synthesising system. Holliday genetically combined the nd gene with a phenotypically revertible ad-3 mutation (revertible or suppressible by fluorouracil, which results in errors of protein synthesis). Holliday predicted that

if the nd gene was causing the production of an erroneous protein synthesising system, then, at least transiently, it would revert the ad-3 auxotroph. This prediction, transient reversion followed by death, was observed. This experiment provides very considerable support for the Orgel hypothesis. It would appear that only one other interpretation is tenable, that the ad-3 and nd mutants are interacting at some level other than the translation of ad-3. The necessary control experiment to eliminate this alternative analysis is the genetic combination of nd with another, non-revertible adenine requiring mutant. This control experiment has not been done.

It might be said by way of summary, that the senescence systems of filamentous fungi present unique advantages, but that they also have the disadvantage that, since they occur only at the fringe of a mycelium, it is difficult to confirm cytologically or biochemically any tentative hypothesis.

Maynard Smith (1963) considers as a working hypothesis that the senescence of Drosophila is possibly a failure in protein synthesis, "..... that the ageing process consists of an irreversible decay of the protein synthetic mechanisms of the animal, a decay which occurs at a rate approximately independent of the temperature."

(viii) The analysis of senescence in Protozoa and various lower animals

Strehler (1962) has stated "..... it does not seem likely that a great deal can be deduced about ageing of Metazoa from a study of

the process of clonal ageing in Protozoa." The justification for this attitude does not however appear clear. It seems to be based on the Metazoan distinction between germ line and soma, which is not reflected in the Protozoa. Weismann (1891) predicted on theoretical grounds that the Protozoa would not show senescence, since if they did so they would become extinct. However various lines of research, especially those of Jennings (1944), Sonneborn and Rofolko (1957), Sonneborn and Schneller (1960); Kimball (1964) and Siegel (1967) have established that in the ciliates senescence is a common, if not universal, phenomenon. The analysis of the causes of senescence in the ciliates is however at present made difficult by the incomplete state of knowledge concerning the interaction of micronuclei, macronuclei and "cytoplasm" (including the "cortex" and membranes). The senescence of Paramecium aurelia has received the most attention. If Paramecia are allowed, periodically, to undergo crossing or the similar process of autogamy, then the organism shows no degeneration. If however the organisms are prevented from undergoing these processes, then senescence and death occurs eventually. The essential feature of crossing and autogamy is the occurrence of nuclear reorganisation, and apparently it is the replacement of the prezygotic macronucleus by a newly formed macronucleus which is responsible for the maintenance of undiminished vigour. However in a partially senesced clone there occurs a sudden

deterioration of the micronuclei, as observed cytogenetically and by genetic analysis of the presence of recessive lethal mutations. Although this process is stable and irreversible it is apparently a response to a degenerating intracellular environment, caused primarily by macronuclear degeneration. In a cross between two *Paramecia* very little cytoplasm is exchanged when the micronuclear exchange occurs. This has enabled experiments to be performed which suggest that in the moderately senescent cell the cytoplasm has become altered and degenerate. The exact nature of the primary damage to the macronucleus is still uncertain and the further analysis of the system requires that the organisation and control of this structure must first be worked out. It has been suggested that the "secondary" genetic material of the macronucleus may be liable to erroneous sharing out during cell division, but there is little evidence to support this. The damage is probably not of a mutational nature, since no effect is apparent after 80,000 r of X-rays (Kimball and Gaither, 1954). It is of especial interest to know if any other organelle or system is replaced besides the macronucleus following autogamy or crossing.

"Life-spanning" has been observed in *Amoeba proteus* by Muggleton and Danielli (1968). *A. proteus* normally shows unlimited fission, but following a period of several weeks of poor food supply^P and subsequent return to optimal conditions the life of a clone is restricted to something of the order of six to nine months. It is apparent from

the division of the starved amoeba, and from micromanipulation experiments, that at least two processes are occurring. The first type of life-spanning (A) was characterised by the death of one product at successive cell fissions, eventually the "stem-line" cell dying. The second type of life-spanning (B) was characterised by the repeated fission of all cells of a clone, and then the death of all the cells. Muggleton and Danielli, on the basis of micromanipulation experiments, suggest that type "A" life-spanning has a nuclear and type "B" a cytoplasmic origin. The interaction of these two systems is however apparently impossible to understand at the present level of analysis. The authors consider the phenomenon to be a differentiation process, which is difficult to conceive of in a free-living protozoan, especially from the evolutionary point of view. They do however suggest that alternative hypotheses are the accumulation of waste products during the non-growing period, the faulty turnover of some organelle, or mutagenesis without the possibility of selection of viable amoebae.

Rotifers have been extensively employed in senescence research. The organisms show a short life cycle consisting of a period of growth lasting from a few days to a few months, depending on species, and a period of senescence characterized by a decrease in activity, degeneration of cells, deposition of pigment and finally death. Growth occurs by increase in cell size, the nuclear number remaining

constant. The system is chiefly of interest because of the "Lansing effect". It is found that if eggs are taken from old Rotifers, and this process is continued for some generations, the 'clone' shows degeneration and eventually death (Lansing, 1947). However in view of the established damaging effects of adverse external environment on the offspring of Rotifers (Lynch and Smith, 1934), it seems probable that the effect observed by Lansing is due to the cumulative effect on egg viability of a poor maternal environment. This probably has little relevance to senescence in other organisms. The effect has been looked for, and not detected, in Drosophila and mammals.

(ix) Theories of senescence based on mortality statistics

Strehler (1962) and others have considered that the study of the distribution and variation of the time of death in populations of mammals, particularly men, provides a uniquely valuable experimental approach to senescence. Of especial interest to such theories is the period during which most organisms die and mortality rate is increasing (the "Gompertz period"). Strehler has stated: "Recent theories of mortality have used the Gompertz function as one of the prime observations to be explained." However Strehler appears to lose sight of the fact that in populations, the Gompertz period is followed by a period characterised by non-logarithmic increase in mortality rate.

At present, it appears that the difficulties of separating the contribution of genotype, environment and individual variation in the development of senescence make the consideration of mortality statistics an exercise of doubtful merit. The whole subject is excellently reviewed in Strehler's book (Strehler, 1962).

(x) Theories based on the consideration of the evolutionary significance of senescence

There are three separate ways of viewing the evolutionary significance of senescence, that is, of considering why it is a characteristic of living things as distinct from what its actual mechanism is. These will be briefly considered as they are of some relevance to hypotheses concerning the mechanism.

(1) Weismann (1891) considered not only that senescence was inevitable in the metazoan, but that it was of actual selective value "to the species" by removing efficiently organisms which would anyway be partially impaired, and which would still be competing with their own species for food etc. It has frequently been pointed out that this theory is either circular or comes very close to it. It is also difficult to imagine a population structure occurring naturally in which a competitive advantage would be conferred on an organism's genes by that organism being removed from the population. The population would have to be very small, closed and isolated against competition from other groups. These conditions seem to rule out

senescence having a direct evolutionary value as a phenomenon in its own right. A modification of Weismann's theory is that the value of the organism's death is in terms of increasing the turnover rate of the population, and therefore the amount of variation available to the species over a prolonged period of time.

(2) The second possibility is that senescence is of no evolutionary importance, since it may occur only rarely in natural populations, "accidental" death eliminating most of the adults. The few organisms which survived long enough to develop degeneration would provide only a weak selection against senescence. As far as I am aware, the problem has not been considered quantitatively, and it is therefore impossible to decide whether this analysis is correct in particular circumstances.

The question of whether senescence does or does not play a significant part in ecology is an important one. Under special circumstances, the theory that senescence plays no part in death under natural conditions is probably correct, these circumstances being where the environment shows a predictable fluctuation which eliminates the adult forms of the species. In the case, for example, of annual plants or probably of fungi, evolution cannot have acted to maintain the organism's integrity for more than a single annual cycle. This may be relevant to the Myxomycetes which probably over-winter in the form of spores.

(3) The third possibility has been termed by Strehler "contradictions in design". The theory supposes, in its simplest form, that the maintenance of an organism's integrity requires an expenditure of energy and "information" proportional to the perfection of that maintenance. The organism is in competition with other members of its species, and other species, for survival, and at a certain level the expenditure of energy and information becomes limiting, the organism becoming uncompetitive compared with organisms relying on simpler systems combined with selection for viability at the gamete and zygote level. In other words the degeneration of the organism is due to the contradiction that it may have to sacrifice long-term maintenance in order to remain competitive.

The essential point is that theories (2) and (3) predict that senescence will have no specially selected mechanism, and that it will probably be manifest in numerous sites in the organism, whereas the less probable theory of Weismann (1) might predict the selection of special suicide mechanisms.

2. Senescence in P.polycephalum

A. Introduction

Kerr and Waxlax (1968) have reported what they describe as "ageing" in a mutagenised strain of Didymium nigripes plasmodia. From the reported data it appears that the occurrence of death within a population of similar plasmodia is random. The fusion behaviour, pigmentation and sporulation potential change during the vegetative culture of individual plasmodia, but the changes are both unpredictable and reversible. The "ageing" of D.nigripes is best viewed therefore not as senescence, but as lowered viability of mutational origin. It cannot at present be guessed whether the underlying cause of "ageing" in D.nigripes will be found to be in any way analogous to natural senescence. This Thesis reports the first observation of senescence in a Myxomycete, specifically in the plasmodia of Physarum polycephalum. The phenomenon is characterised by the occurrence of progressive changes in the morphology of plasmodia during prolonged vegetative culture, death eventually occurring. Populations of identical plasmodia maintained in separate vegetative culture undergo these changes synchronously, the plasmodia eventually dying approximately simultaneously.

B. The Occurrence of Death Following Prolonged Vegetative Propagation of Plasmodia

Plasmodia of P.polycephalum have been extensively used for biochemical and biophysical research for some years. It has been assumed, incorrectly, that plasmodia are of indeterminate life expectancy, and undergo no significant changes during prolonged vegetative culture. The reason for this incorrect assumption is probably the ease with which plasmodia can be induced to enter the resting stage or sclerotium, which has resulted in plasmodia being rarely maintained in active vegetative culture for prolonged periods, most workers preferring to resort frequently to stored sclerotia to initiate cultures.

This Thesis reports that plasmodia cultured on SDM agar or Oat agar show, after a period of vegetative growth during which no change is apparent, progressive morphological change, reduction of growth rate and eventual lysis and death. Although it is perhaps premature to generalise about all P.polycephalum plasmodia, this senescence has been observed in all plasmodia investigated. The plasmodia of the Indiana isolate take from one to several months to die in vegetative culture, plasmodia of the Wisconsin isolate taking a considerably longer time, of the order of 6 months to a year.

Certain feature of the culture procedure should be restated at this point. Plasmodia are sub-cultured on an inoculum agar block,

every third day when they have grown to cover the agar-nutrient plate. The plasmodia are inoculated onto fresh axenic agar-nutrient plates. Thus the plasmodia become degenerate and die despite the maintenance of an effectively constant benign external environment. The system is experimentally attractive for various reasons, which will be summarised in the discussion, but from the point of view of this introduction one point should be emphasised, the plasmodia develop senescence and die despite the maintenance, until very near death, of a doubling time of less than 12 hours. Therefore the senescence, which takes several months to a year to develop to the point of being lethal, is not a question of suboptimal nutrition or some other aspect of laboratory culture, the plasmodium having achieved a theoretical increase of at least $n \times 2^{120}$ by the time senescence occurs.

C. Survival Curves of Populations of Identical Plasmodia

To test whether the eventual death of plasmodia during prolonged vegetative culture was due to some extrinsic cause, for example chance infection, the survival of identical plasmodia maintained in parallel culture was studied.

(a) Indiana plasmodia

An "Indiana" plasmodium, B173+BB114, was produced by crossing amoebal clone B173 (mt₃f₄) with BB114 (mt₄f₄). (BB114 is a progeny clone derived from the plasmodium B173+B174.) Plasmodia appeared on the cross plate after five days, and were transferred to SDM agar

seven days after the cross was set up. The B173+BB114 was grown for several sub-cultures, and then divided into 50 separate cultures, each plasmodium being maintained by sub-culturing every three days until death occurred. Figure 4 represents the result of this experiment.

The 50 parallel cultures underwent no apparent change for the first 10 sub-cultures (30 days), but following this period the cultures underwent, approximately in synchrony, morphological changes. The first sign of developing senescence is the absence of the moist appearance characteristic of the surface of very vigorous plasmodia. The plasmodium illustrated in Plate 2 shows this moist appearance; Plate 9 shows a plasmodium in the first morphological stage of senescence. Plates 9-13 show the progressive development of senescence. The growing front of the plasmodium becomes progressively irregular. Gradually more and more of the plasmodium becomes represented by the veins (thickened channels), the organism taking on a lace-like appearance (Plate 12). The growth rate begins to fall at this time, the plasmodium no longer covering the SDM agar plate after 3 days growth. Within a few further subcultures the plasmodium fails to grow from the inoculum block and lysis occurs (Plate 14), the dead remnant appearing white due to the diffusion of the yellow pigment into the agar. (Alternatively lysis may occur at the "lace" stage, a curious feature being that lysis often occurs initially in a series of

PLATE 9

AN EARLY STAGE OF SENESCENCE

The plasmodium is morphologically almost identical
to the plasmodium shown in PLATE 2

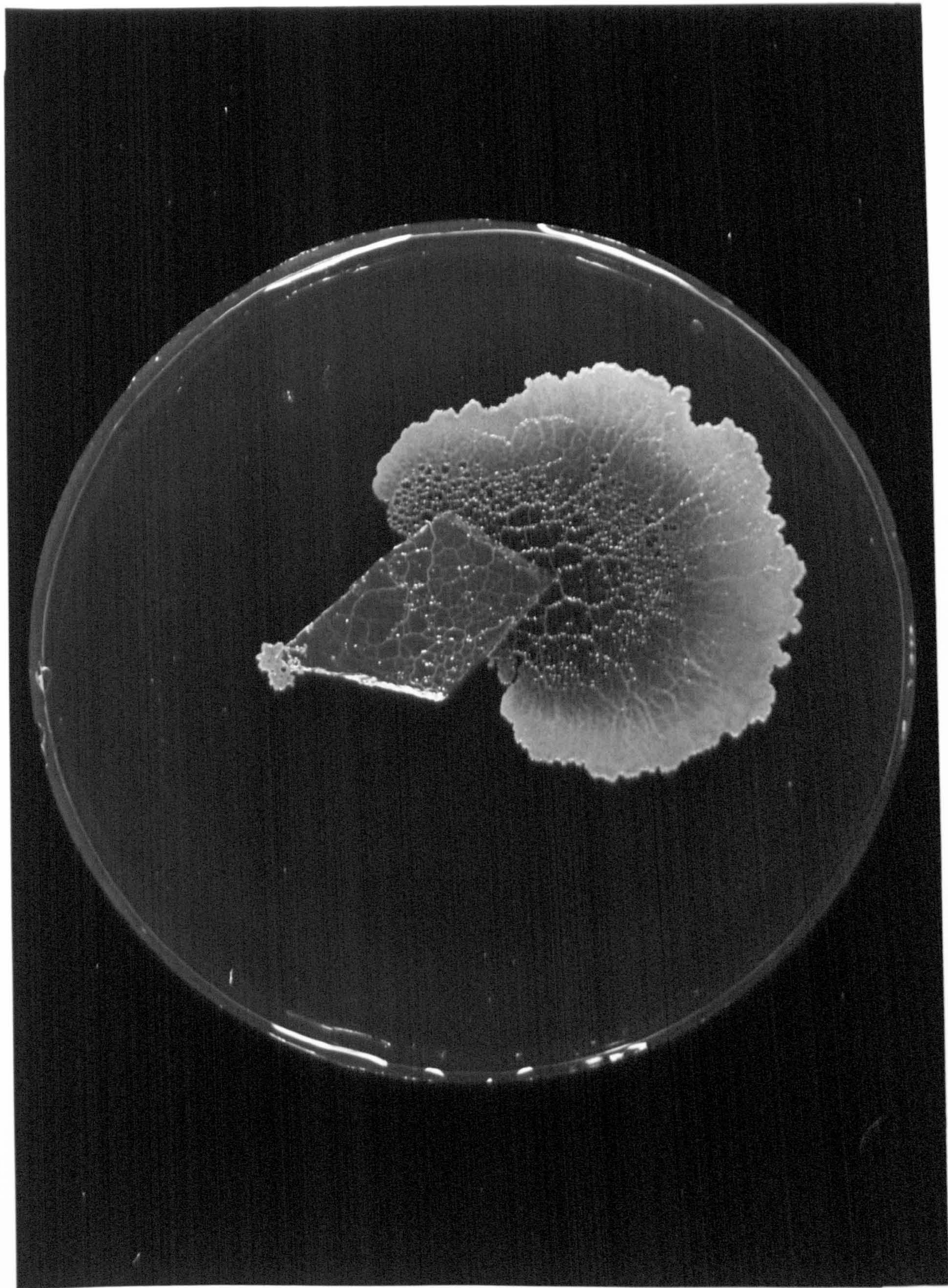


PLATE 10

A STAGE IN SENESCENCE

The growing edge of the plasmodium has
become slightly irregular

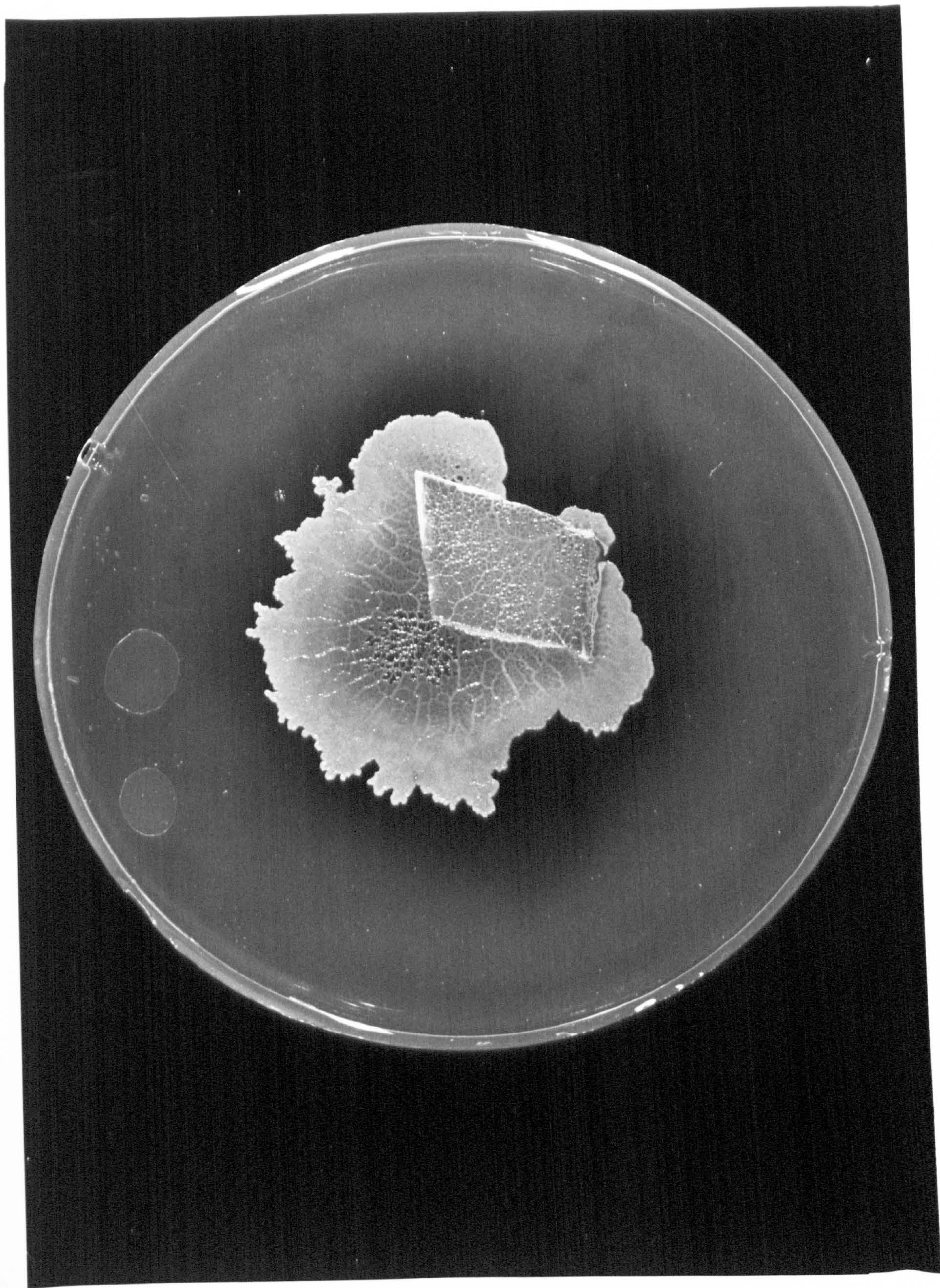


PLATE 11

A STAGE IN SENESCENCE

The growing edge of the plasmodium has become very irregular, and the cell has come to consist almost entirely of veins. The growth rate is impaired by this stage.

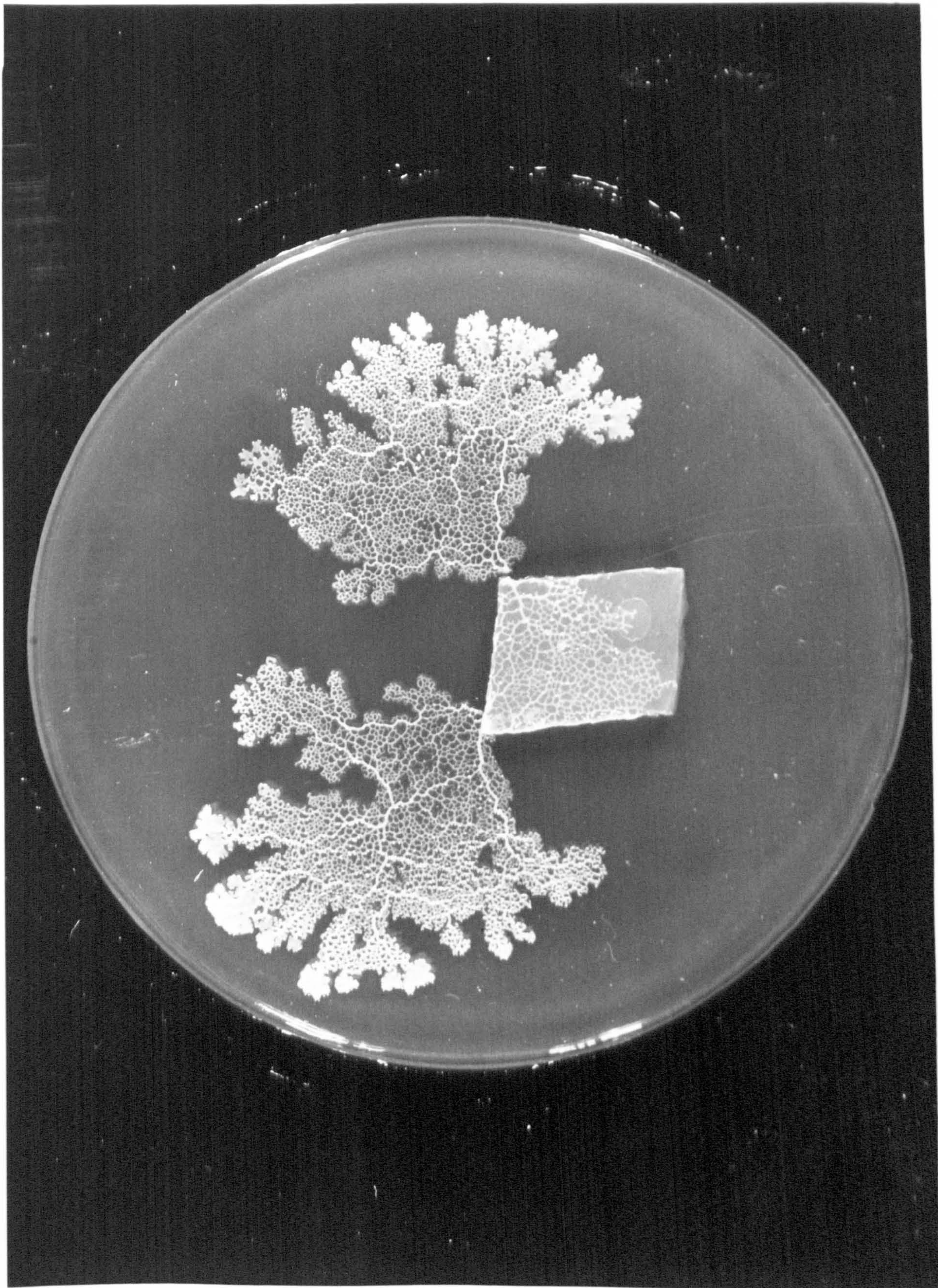


PLATE 12

A LATE STAGE IN SENESCENCE

The organism has taken on a lace like appearance

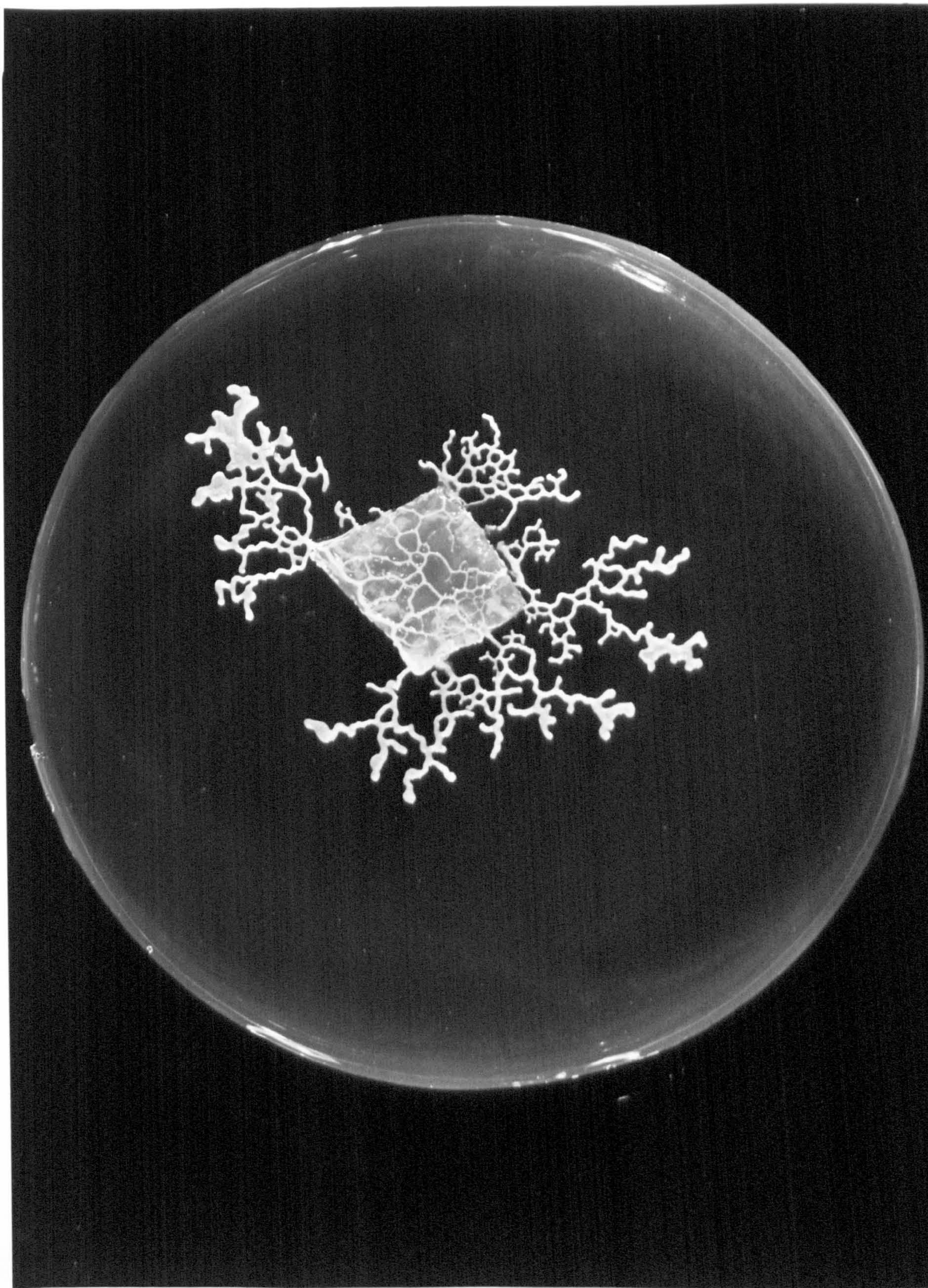


PLATE 13

A VERY LATE STAGE OF SENESCENCE

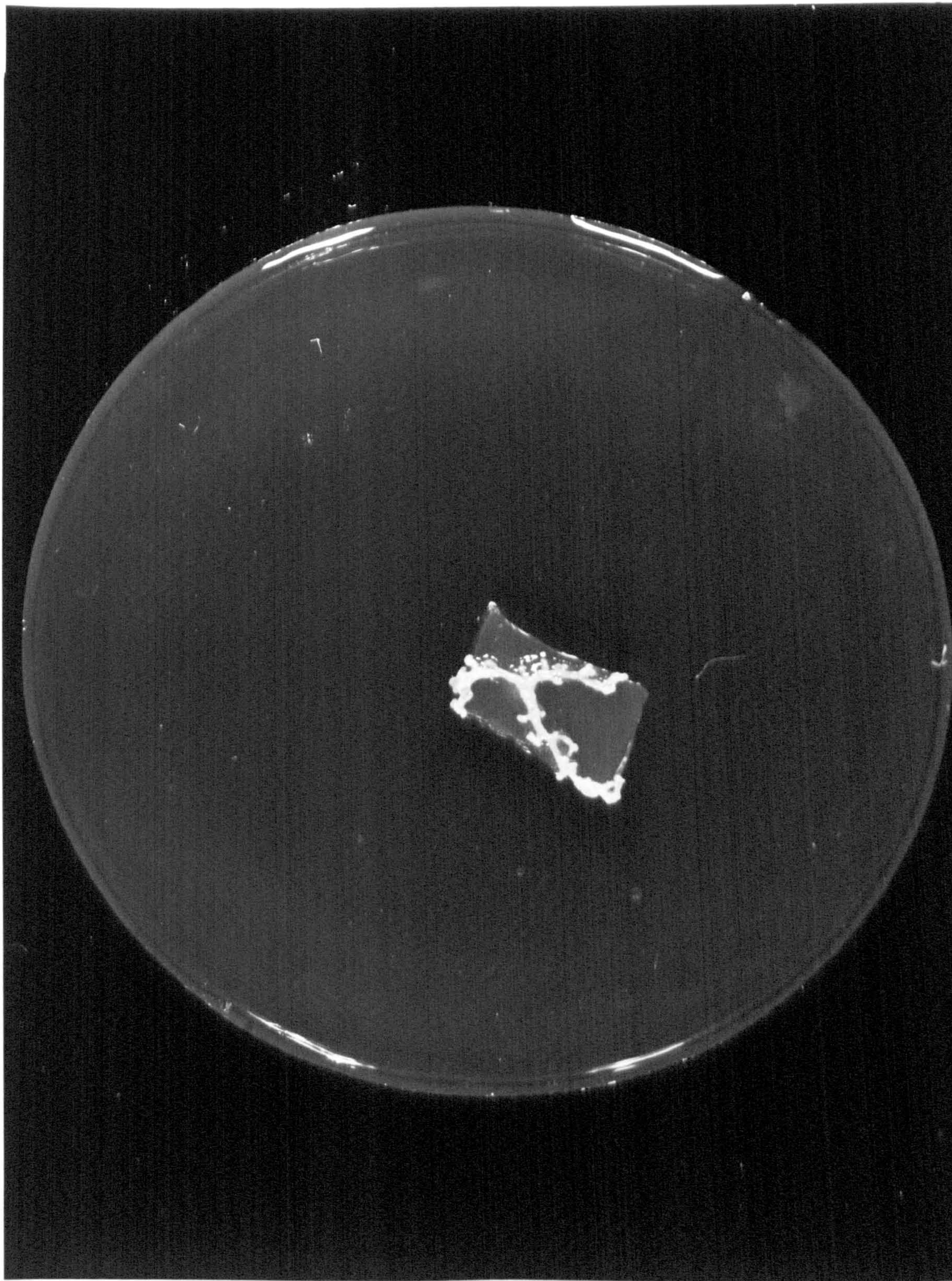
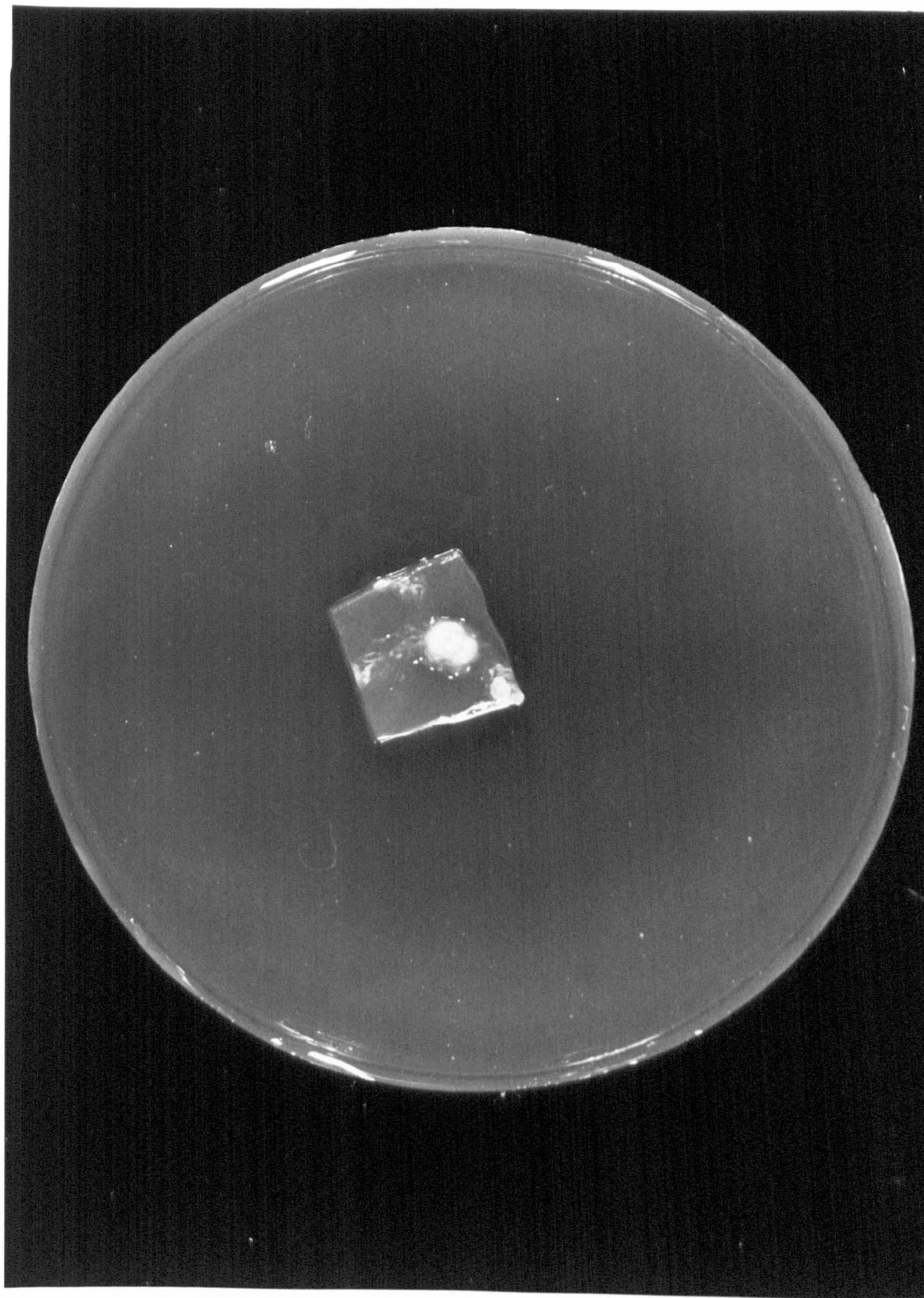


PLATE 14

SENESCENT DEATH

The organism has lysed



distinct areas, suggesting that the normally vigorous protoplasmic streaming is weak at this stage.)

The 50 "identical" parallel subcultures of B173+BB114 did not die exactly simultaneously, the first plasmodium dying 81 days (162 doublings) after the date the cross was set up, and the last subculture dying 114 days (228 doublings) after the cross date. ~~The distributions of deaths is described in Figure 4.~~ The average life expectancy was 97.2 days (194.4 doublings).

The life expectancy of the original B173+BB114 plasmodium from which the 50 subcultures were derived can be given in terms of the average life expectancy of its 50 subcultures, 97.2 days, and the standard error from this figure, 8.4. It is of course necessary to define the point from which life-expectancy is measured, in this case the date of the parental amoebal cross. The life expectancies reported in the subsequent data will be represented both in days and in terms of number of doublings, the survival in days being multiplied by 2 to give, approximately, the number of doublings (12 hour periods). The use of this natural time period enables the data to be more easily considered from a theoretical point of view.

It should be borne in mind that plasmodia were classified for death at 3 day intervals, when they would normally be subcultured. This discrepancy between the time intervals used to describe life

expectancy (days or 12 hour doubling), and the intervals used to actually record it (3 day periods), has statistical consequences which are not however relevant to the analysis developed in this Thesis.

This procedure was repeated to investigate the life expectancies of several other plasmodia derived from the Indiana isolate, with similar results (see Table 20). Fifty sub-clones of B173+B174 had an average life expectancy of 85.3 days (standard error 8.1) and fifty sub-clones of B174+BB128 had an average life expectancy of 81.6 days (standard error 8.6). The morphological changes undergone during the senescence of these three genetically different Indiana plasmodia showed some slight but constant differences (B173 mt₃f₄ and B174 mt₄f₃ are the "parental" Indiana amoebal clones, BB114 mt₄f₄ and BB128 mt₃f₃ are progeny clones from the plasmodium B173+B174. The three Indiana plasmodia used in these experiments were selected because of their lack of killing reaction on fusion, see p153 below.)

(b) Wisconsin plasmodia

Several plasmodia derived from the Wisconsin isolate were also investigated by this procedure. However these plasmodia showed markedly greater longevity. Maintaining parallel cultures at intervals of 3 days is laborious and therefore only one Wisconsin plasmodium, 7119+7122, was maintained for the necessary length of time to determine life expectancy. The two amoebal clones 7119 and 7122 were crossed,

and the resulting plasmodium 7119+7122 divided into 20 subcultures, which were maintained in parallel. The first death occurred 131 days later (262 doublings) and the last 194 days after crossing (388 doublings). The average life expectancy (\bar{x}) of the 20 subcultures was 160.2 days (320.4 doublings) with a standard error of 19.4 (day units). This experiment is described in Figure 3.4.

D. The Variation in Time of Death of Plasmodia Derived by Subdivision of a Single Plasmodium

It is apparent that there is considerably more variation in time of death amongst the twenty 7119+7122 plasmodia (\bar{x} 160.2 days, SE 19.4) than amongst the fifty B173+BB114 (\bar{x} 97.2 days, SE 8.4). The variation in time of death amongst plasmodia derived from a single plasmodium probably has three separate origins.

- (i) Slight variations in the SDM agar plates, or other environmental factors, which may cause some variation in time of death.
- (ii) Fluctuations in the intrinsic state of a plasmodium which may render certain plasmodia more vulnerable to death at a particular moment.
- (iii) Variation in the rate of development of senescence during the separate maintenance of the parallel subcultures.

The observation (see above) that the parallel subcultures of plasmodia with a longer life expectancy (7119+7122) show a greater standard error supports the belief that the variation in the rate

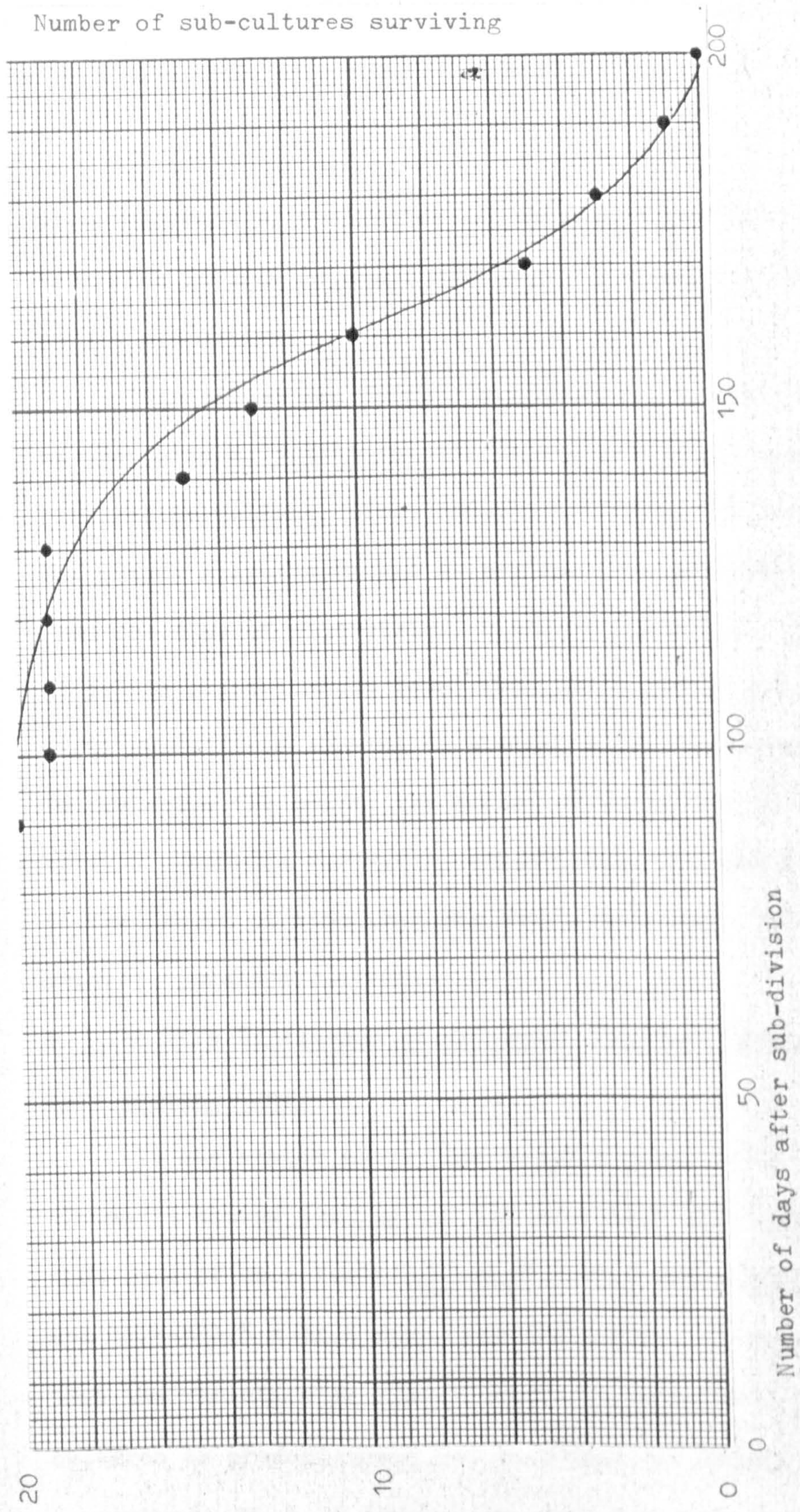


FIGURE 4
SURVIVAL CURVE OF 20 SUB-CULTURES OF A 7119+7122 PLASMODIUM

of development of senescence is the most significant cause of variation in time of death of plasmodia derived by subdivision of a single plasmodium.

This belief was further supported by the following result. One of the fifty B173+BB114 plasmodia was subdivided again into twenty parallel subcultures after being cultured on SDM agar for 60 days. The twenty subcultures had an average life expectancy of 41.8 days from the time of subdivision, standard error 5.9. The standard error of 5.9 is significantly lower than the standard error (8.4) of the fifty subcultures showing an average life expectancy of 97.2 days. To summarise the point, the amount of variation in time of death between plasmodia derived by subdivision of a plasmodium is proportional to the length of time that the subcultures have been maintained in separate parallel culture.

E. The Life Expectancy of Plasmodia Produced from Repeats of the Same Amoebal Cross

It was stated above that several genetically different "Indiana" plasmodia showed similar but not identical life expectancies (measured from date of crossing); B173+B174 \bar{x} 85.3 days, B173+BB114 \bar{x} 97.2 days and B174+BB128 \bar{x} 81.6 days (see Table 20). It was also stated above that the variation in time of death of originally identical subcultures is predominantly due to slight variations in the rate of senescence. To test whether the difference in life expectancy between

the three genetically different Indiana plasmodia was entirely due to the same cause, or whether it reflected some other cause, for example genetic predisposition to senescence, the following experiment was performed. The same three crosses were again set up, the plasmodia subdivided (into 25 or 50 subcultures) and the eventual occurrence of death recorded.

It was expected that, if the variation in life expectancy between the three genetically different plasmodia was partially genetically determined, then the difference in life expectancy between the three would be greater than the difference between plasmodia produced by the same amoebal cross.

Table 20 describes the result of this experiment. It is apparent that the difference in life expectancy of the B173+BB114 plasmodia (and to a lesser extent between the two B174+BB128 plasmodia) is very considerable, \bar{x} 97.2 (SE 8.4) and \bar{x} 75.7 (SE 5.8). The variation in life expectancy between different strains is apparently no greater than the variation between plasmodia produced by repeating the same cross, and there is therefore no reason to suggest that genetic differences contribute to the variation between different strains.

The difference between the two B173+BB114 plasmodia produced by separate crosses (Table 20) is obviously considerably greater than the variation occurring in life expectancy of plasmodia derived by

TABLE 20

The Life Expectancies of Plasmodia Produced from Repeats
of the Same Amoebal Cross

Genotype of plasmodium	No. of parallel subcultures	No. of days between crossing and transfer of plasmodium to SDM	Average life expectancy (in days after crossing) and standard error
<u>B173+B174</u> (a)	50	10	85.3 (8.1)
<u>B173+B174</u> (b)	25	11	84.0 (8.2)
<u>B173+BB114</u> (a)	50	7	97.2 (8.4)
<u>B173+BB114</u> (b)	50	15	75.7 (5.8)
<u>B174+BB128</u> (a)	50	12	81.6 (8.6)
<u>B174+BB128</u> (b)	25	10	73.7 (9.1)

subdivision of a plasmodium into parallel subcultures. Presumably this reflects some source of variation in the "crossing-procedure" (used in the broadest sense, see below). The variation in life expectancy due to "crossing procedure" could have one of three origins.

(i) It is possible that senescence is accelerated by variations in the micro-environment of the cross-plate. The crossing procedure (see Chapter 1) consists of mixing loopfuls of the two amoebal clones with a loopful of E.coli on a plate of liver-infusion agar. Despite the increase in numbers of amoebae up to many thousands and their intimate mixing, frequently only one or a few tiny plasmodia appear on the cross plate. The cross plate comes to exhibit a variety of microenvironments, fresh LIA agar, ungrazed E.coli colonies, areas of dense amoebal growth, areas of encysted amoebae etc. It is possible that a plasmodium may show accelerated senescence if it occurs in one of these microenvironments, due to lack of oxygen, starvation, presence of toxic substances etc.

(ii) It is possible that the plasmodia which result from amoebal fusion on a cross plate are particularly liable to show random variations (drift) in the rate of development of senescence, because of their initially very small size.

(iii) The third possibility is that the amoebal clones have become heterogeneous for some factor which affects the life expectancy of any plasmodia which they may form. This point will be returned to

in the discussion, but briefly, it should be borne in mind that although individual clones of amoebae have been maintained for more than 10 years, this does not rule out the possibility that certain "lines" of amoebae in the clonal culture may have become senescent, eventually dying out of the culture, the culture remaining vigorous because of selection. The amoebae involved in zygote production in repeated crosses of the same clones might by chance be derived from "sub-clones" showing different degrees of senescence, the resulting plasmodia having different life expectancies. This possibility is testable (see Discussion) but the necessary experiments have not yet been performed. The important consequence of this suggestion is that senescence may not be restricted to the diploid plasmodium; the same process may be occurring in the haploid amoebal stage. If it is established that the amoebal clones have become heterogenous for some "senescence factor", then the development of this variability is probably too rapid to be due to nuclear mutation. (The clones used in the above experiments had been recloned some weeks before the first cross was set up. During this procedure the clone is re-isolated from a plaque of amoebae derived from a single amoeba, see Chapter 1)

These experiments, establishing that repeated crossing of the same clones of amoebae did not yield plasmodia of the same life expectancy, meant that a difficulty arose in the planning of subsequent

experiments. Various experiments would be greatly facilitated by knowing at the beginning, the stage of senescence that a plasmodium had reached, although the life expectancy would of course be verified by observing death. This was not possible using plasmodia produced from crosses, since the life expectancy could not be predicted, only measured by time of death. Experiments could have been performed and analysed retrospectively, but this procedure would be wasteful of time, the experiments being already very time consuming. It was therefore decided to investigate the possibility of using sclerotia (the plasmodia resting stage) to initiate vegetative cultures for senescence experiments, in place of crossing amoebae. It was considered that a particular sclerotium (or collection of micro-sclerotia) might provide a convenient and stable source of plasmodia in a known stage of senescence. It was decided to use microsclerotia for this investigation, and therefore plasmodia were transferred to liquid culture (see Chapter 1).

F. The Effect of Liquid Culture on Plasmodial Life Expectancy

Two plasmodia, B173+BB114 and B174+BB128, were transferred from SDM agar to SDM solution by the procedure described in Chapter 1, the plasmodia also being maintained on the agar medium. The two different types of culture were maintained in parallel. The SDM agar cultures developed senescence and died, as expected, but the liquid cultures showed no apparent loss of growth rate, or change in microplasmodial

morphology etc. It thus appears that liquid cultures of microplasmodia do not develop senescence. This may be a partial explanation for senescence not having been previously reported, since much of the biochemical work with P.polycephalum is done in liquid culture.

The probable explanation of the failure of senescence to appear in liquid culture is that these cultures consist of numerous very small microplasmodia. During the initial breaking up of the SDM agar macroplasmodium and subsequent division of microplasmodia, slight variations may occur in the degree of senescence of individual microplasmodia. Selection will operate on these differences, preventing the whole culture from showing progressive senescence. It was considered probable, if this analysis is correct, that liquid culture would actually select against senescence, the plasmodia becoming progressively more vigorous (less senescent) during repeated liquid culture. To test this possibility the following experiment was performed.

A B174+BB128 plasmodium (on SDM agar) was divided into 50 parallel subcultures and the average life expectancy found by maintaining the cultures until death occurred. The original plasmodial culture was also transferred to SDM solution, and the resulting culture of microplasmodia was incubated for 5 days and then subcultured into a fresh flask of SDM liquid. This procedure was repeated until the plasmodia had been cultured in SDM solution for a total of 30 days.

A sample of the final culture was placed on an SDM agar plate, on which the microplasmodia coalesced to form a macroplasmodium. This macroplasmodium was divided into 50 parallel cultures, and the average life expectancy found by maintaining these cultures until death occurred. The final liquid culture (see above) after being sampled was incubated without any further subculturing until sclerotia were formed. A sample of these sclerotia was revitalised by placing them on an SDM agar plate, the remainder of the sclerotia being stored. The sample of sclerotia produced in a few days a macroplasmodium. This macroplasmodium was divided into 50 parallel subcultures, and the average life expectancy observed by the usual procedure. This experiment thus provided comparative data relating to the senescence of (i) plasmodia grown on SDM agar; (ii) plasmodia grown as microplasmodia in SDM solution and then cultured on SDM agar; and (iii) plasmodia grown in SDM solution, induced to form microsclerotia, hatched to form a macroplasmodium and then cultured on SDM agar. The average life expectancies are described in Table 21.

The difference in life expectancy between the plasmodia which had been grown in liquid, and those which had been grown on liquid and then sclerotized is probably not significant. The difference between these two and the plasmodia which had been grown only on SDM agar is however considerable. It is apparent that senescence is prevented by liquid

TABLE 21

The Effect of Liquid Culture and Sclerotum Formation on the Process of Senescence

	No. of parallel subcultures on SDM agar	Average life expectancy (and standard deviation) measured in days from the date of the original cross	Average life expectancy (and standard deviation) during culture on SDM agar
Plasmodia grown on SDM agar	50	32.4 (4.7)	32.4 (4.7)
Plasmodia grown in liquid culture, then SDM agar	20	72.1 (8.9)	42.1 (8.9)
Plasmodia grown in liquid culture, sclerotized, then grown on SDM agar	50	77.2 (9.3)	45.2 (9.3)

culture, the plasmodia which have passed through liquid culture lasting longer when returned to agar than the original agar culture, and having in all (including the period in liquid culture) a life expectancy more than twice that of the plasmodia grown only on SDM agar.

When the liquid culture was originally set up microplasmodia showed, at first, an extremely slow growth rate, growing into large hollow spheres of variable diameter (1 to several mm). During subsequent subcultures of these microplasmodia into SDM solution the growth rate increased to the normal, and the spheres were replaced by microplasmodia of normal morphology. This change in appearance of the liquid cultures probably reflects the selection against senescence proposed on the basis of the data of Table 21.

G. The Life Expectancy of Plasmodia Produced from Stored Microsclerotia

To test whether simultaneously taken samples of a stored microsclerotia preparation give rise to plasmodia showing the same life expectancy the following experiment was performed. At the same time as the sample of microsclerotia analysed in section (see above) was placed on SDM agar, another sample from the same microsclerotia preparation was set up in the same way. The second sample was also allowed to develop into a macroplasmodium, divided into 50 parallel subcultures and maintained in culture on SDM agar until the death of the 50 plasmodia was recorded, (see Table 22). Another preparation of microsclerotia of B174+BB128 was also sampled in duplicate, and

the same procedure was applied to a preparation of B173+BB114 microsclerotia (see Table 22).

The data of Table 22 indicates that if several samples of a microsclerotia preparation are revitalised simultaneously, the plasmodia produced will have closely similar life expectancies. To minimise the variation between repeated samples, large microsclerotia samples (.05 ml of sedimented microsclerotia) were used in all subsequent experiments, it being hoped that this would average any slight variations between the individual microsclerotia of the preparation.

As was stated above, the purpose of using microsclerotia was to enable plasmodial life expectancies to be predicted, rather than measured retrospectively. For this to be possible it was necessary to show that the life expectancy of plasmodia produced from microsclerotia did not change during the storage of the microsclerotia preparation. In other words it was necessary to show that the microsclerotia preparation was stable with respect to state of senescence, or if it was not, to be able to predict the change that would occur following periods of storage.

H. Investigation of the Stability of Microsclerotia Preparations

Microsclerotia preparations of two B173+BB114 and two B174+BB128 plasmodia were prepared (see above for method). The preparations were stored under refrigeration (5°-8°C). During storage the preparations underwent no obvious visible change. Samples of the microsclerotia

TABLE 22

The Repeatability of Life Expectancy of Plasmodia Produced
by Simultaneous Sampling of a Microsclerotia Preparation

Genotype of microsclerotia and reference no. of preparation	Average life expectancy (in days after revitalising) of two samples of each microsclerotia preparation. (The 3 macroplasmodia being divided into 50 parallel subcultures)	
<u>(B174+BB128)</u> ¹	45.2 (9.3)	42.7 (7.1)
<u>(B174+BB128)</u> ²	33.2 (4.7)	31.9 (1.5)
<u>(B173+BB114)</u> ¹	40.9 (6.5)	36.0 (6.2)

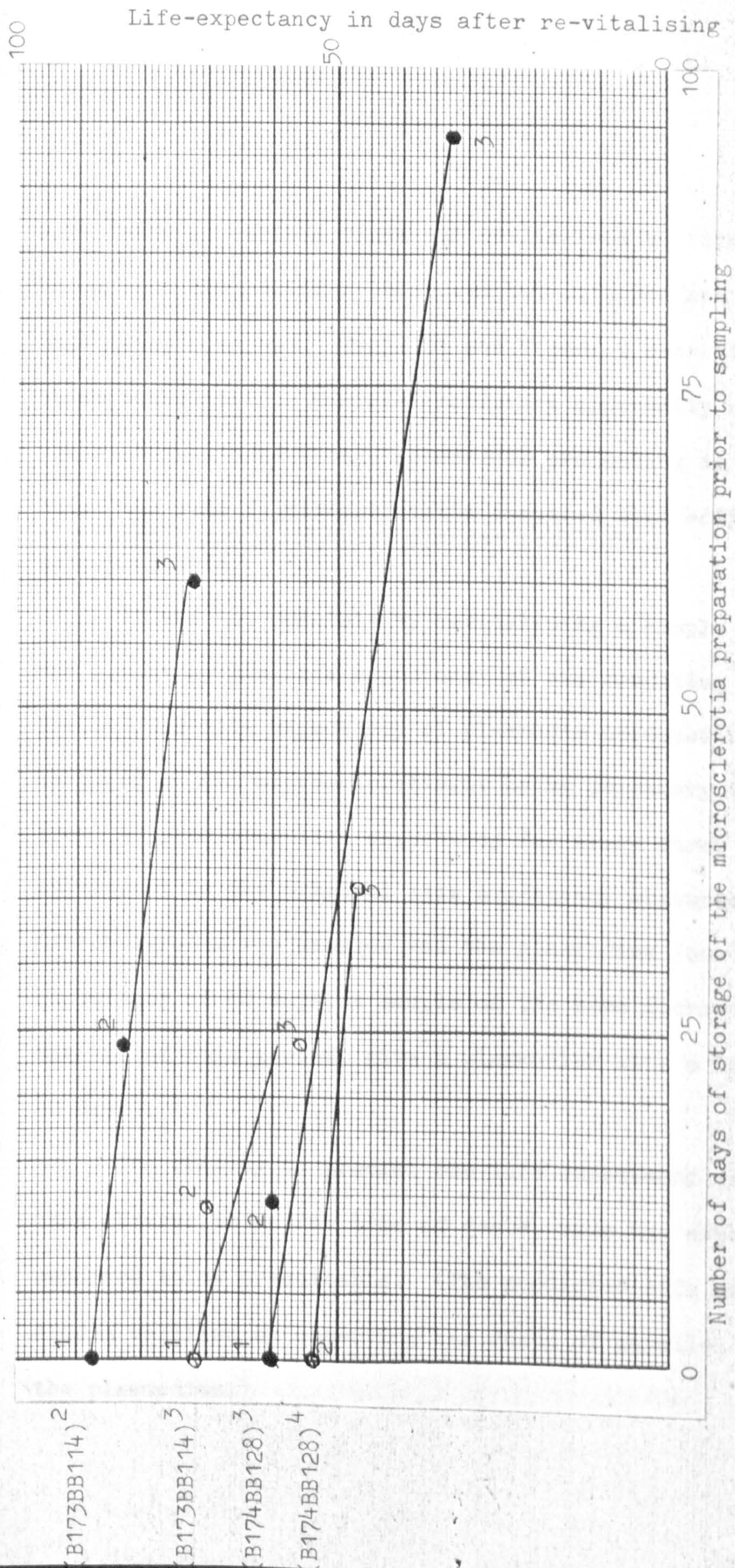


FIGURE 5

LIFE-EXPECTANCY OF PLASMODIA PRODUCED BY REPEATED SAMPLING OF VARIOUS MICROSCLELOTIA PREPARATIONS

were taken at various times and revitalised to form macroplasmodia which were then divided into parallel cultures and the average life expectancy observed. Table 23 and Figure 5 describe the results of these experiments. Microsclerotia are apparently not stable under these storage conditions, senescence proceeding at approximately one third the rate in microsclerotia compared with actively growing macroplasmodia.

It was now possible by revitalising a sample of microsclerotia and observing the life expectancy of the resulting macroplasmodium to predict the life expectancy of plasmodia produced by subsequent sampling of the microsclerotia, it being necessary to subtract 1 day from the "predicted" life expectancy for every three days of storage subsequent to the original life expectancy measurement (e.g. if a microsclerotium was sampled and the plasmodium found to have a life expectancy of 60 days, a sample of the same microsclerotia, would at the end of this period, give a plasmodium with a life expectancy of 40 days).

The ability to predict the life expectancy of plasmodia, rather than observe it at the time of death, made the experiments which are reported below practicable. (The predicted life expectancy was always verified by observing the death of parallel subcultures of the plasmodium.)

TABLE 23

The Instability of Microsclerotia Preparations

Genotype	Days elapsing since original measurement of (average) life expectancy of microsclerotia	No. of parallel subcultures	\bar{x} (average life expectancy and SE)
<u>(B174+BB128)</u> ³	0	10	59.9 (5.4)
	12	10	62.3 (4.2)
	94	20	33.7 (1.9)
<u>(B174+BB128)</u> ⁴	0	20	57.8 (1.3)
	36	20	48.0 (8.9)
<u>(B173+BB114)</u> ²	0	20	88.5 (0.6)
	23	20	81.2 (3.0)
	36	20	69.8 (5.7)
<u>(B173+BB114)</u> ³	0	10	70.7 (4.2)
	12	10	67.6 (1.8)
	24	10	53.4 (1.9)

I. Observations on the Rate of Development of Senescence, Making Use of Fusion of Plasmodia of Known (Predicted) Life Expectancy

The analysis of the rate of development of senescence is one approach to studying the cause of senescence.

- (i) If the rate of development of senescence is exponential this would suggest certain mechanisms, for example and "Orgel type" exponential accumulation of errors in protein synthesis (see Introduction) or the proliferation of mutant nuclei or episomes possessing a selective advantage over the normal type. (It should be stressed "selective advantage" is applied, in this context, to the mutant nucleus, the whole organism suffering from the accumulation of such deviant nuclei.)
- (ii) Alternatively if the development of senescence is linear, not exponential, this might be taken to suggest other mechanisms, for example the inactivation of nuclei or episomes, the mutant organelles being neither at a selective advantage nor disadvantage compared with the normal type. This type of degeneration would not in fact follow an exactly linear development, since although the fraction of normal organelles degenerating to the mutant type during each cell division is hypothesised to be a constant, the proportion (or absolute number) of normal organelles shows progressive reduction. Therefore the absolute number of normal organelles becoming mutant in each cell cycle becomes less as senescence develops (i.e. the curve is not

exactly linear, but shows a flattening). The curve for such a hypothesised degenerative process, in an organism which is growing, is described by the formula

$$x^n = (1-y)^t$$

where x^n = the proportion of normal organelles remaining at a particular stage. y = a constant, the proportion of normal organelles converted to mutant during each doubling. t = number of doublings occurring since the origin (x^n at origin equalling 1). For the purposes of calculation $(1-y)^t$ is considered as antilog. $[t \log(1-y)]$.

Figure 6 is a plot, on linear graph paper, of the curves for the accumulation of mutant organelles, assuming a simple linear hypothesis (A), and the corrected linear hypothesis, $x^n = (1-y)^t$ for various values of y . ($B_y = .1$, $C_y = .05$, $D_y = .02$, $E_y = .01$, $F_y = .005$). A line cuts the curve at the point where this represents 85% affected (senescent) targets. The point represents the hypothetical time of death used in subsequent calculations.

Figure 7 is a plot, on semi-log paper, of the curves of the accumulation of affected "targets" assuming the alternative hypothesis of the exponential accumulation of error. G is the graph for the accumulation of affected "targets", assuming an original (spontaneous) frequency of affected targets of 1 in 100,000, these affected targets doubling in number during each cell doubling. (The spontaneous error frequency, which is assumed to be constant is taken into account

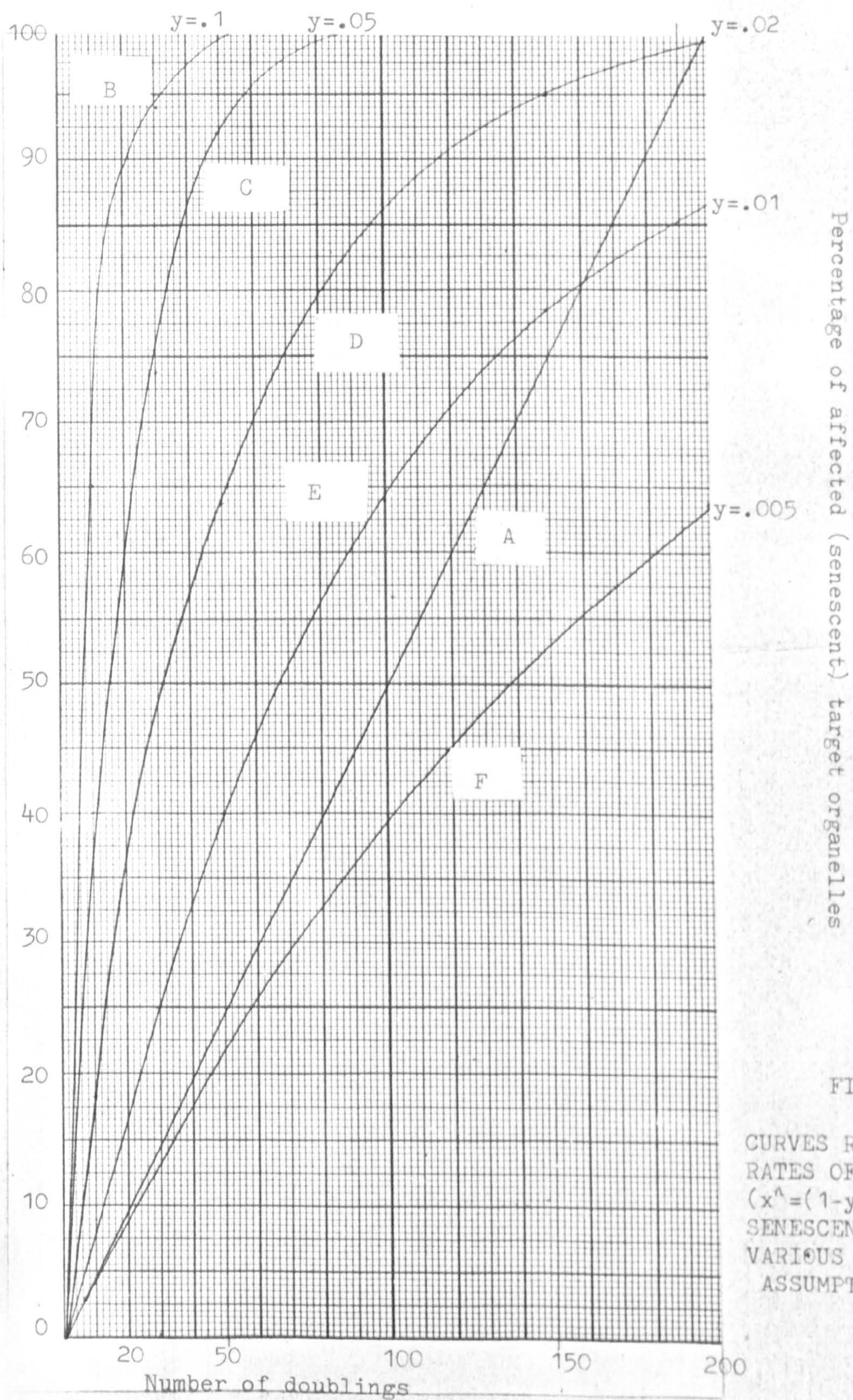
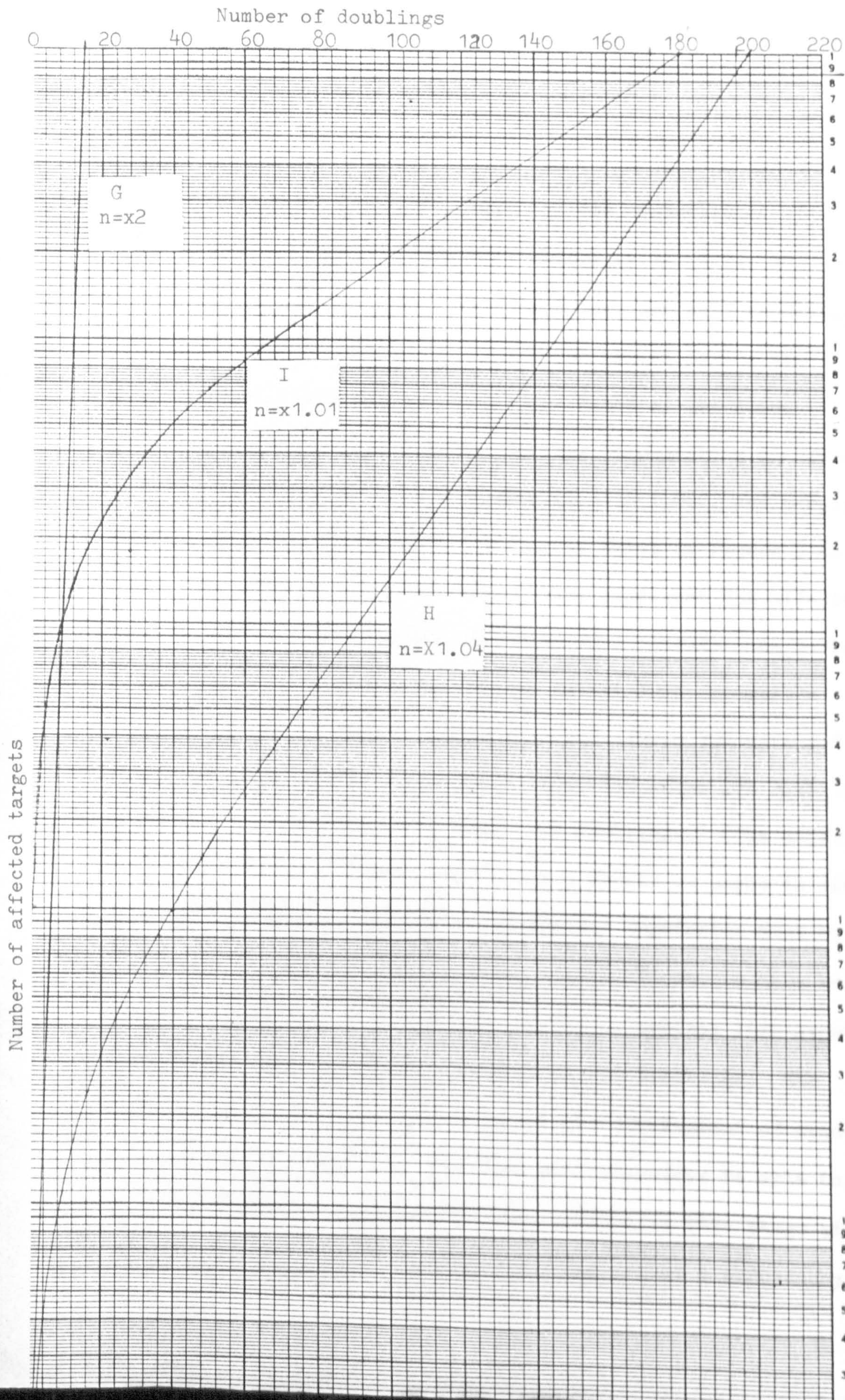


FIGURE 6

CURVES REPRESENTING
RATES OF DEVELOPMENT
($x^n = (1-y)^n$) OF
SENESCENCE, MAKING
VARIOUS HYPOTHETICAL
ASSUMPTIONS

FIGURE 7

CURVES REPRESENTING RATES OF EXPONENTIAL DEVELOPMENT
OF SENESCENCE DURING 200 DOUBLINGS, MAKING VARIOUS
HYPOTHETICAL ASSUMPTIONS



throughout the curve.) H is the graph for the accumulation of affected targets, assuming spontaneous frequency of .1 in 100,000, and an exponential rate of increase of $n \times 1.04$ per cell doubling. The contribution of accumulating spontaneous errors during the first 20 or so doublings is apparent in the slope of the curve during this period. I is the graph for the accumulation of affected targets assuming a spontaneous frequency of 1 in 1,000, and a rate of exponential increase of $n \times 1.01$. A line is drawn on Figure 7 to cut the three curves at the point where they represent 85% affected targets. This point is assumed, in subsequent calculations to be the lethal level. (Similar calculations result from assuming a wide range of lethal levels.) Graph G ($n \times 2$) shows 85% affected after 16 doublings; Graph I ($n \times 1.01$) shows 85% after 174 doublings; and Graph H ($n \times 1.04$) shows 85% after 194 doublings. (The different spontaneous error rates assumed for I and H should be borne in mind.)

Certain of these graphically represented hypotheses must be considered improbable for the following reason. Plasmodia have been observed, in the experiments reported above, to exhibit life expectancies approaching, in some cases, 100 days or 200 doublings. The organisms show, during most the period of the development of senescence, no apparent loss in viability, and they must therefore be able to withstand the degree of degeneration they contain at that stage without phenotypic loss of vigour. This must reflect either a redundancy (or

a certain amount of excess capability) at the level of the affected "target", or alternatively reflect a capacity for compensatory regulation, the unaffected "targets" being induced to function more efficiently during the progressive development of degeneration. It is unlikely that an organism could withstand much more than 99% of a particular factor or process becoming degenerate in view of the probable selective disadvantage of maintaining excess capability, and conversely it is perhaps unlikely that an organism would die because of the loss of much less than 50% of a particular function, since this would result in a rather restricted range of tolerance of sub-optimal or adverse environments. The exponential graph G can therefore be considered to be improbable, since the organism is rendered totally degenerate in approximately 16 doublings.

This is an extremely interesting conclusion, since it establishes that, for P. polycephalum, hypothetical exponential senescence processes beginning at spontaneous levels of about 1 in 100,000, must show exponential increases of considerably less than $n \times 2$ per doubling. This consideration will be returned to in the Discussion, where its general relevance to senescence theory will be discussed. The $x^n = (1-y)^t$ graphs B and C are improbable for the same reasons. Graph F is the lowest value of y ($y = .005$) that is probable, lower values producing less than 50% degeneration in 200 doublings. It is therefore concluded that if the corrected linear hypothesis ($x^n = (1-y)^t$)

is correct, the rate of mutation of the affected organelle is probably between 5 per 1,000 and 2 per 100 for each doubling. This rate of mutation is obviously very high, which must be taken into account in subsequent consideration of this hypothesis (see Discussion).

These hypotheses have been described at this time in order to illustrate the purpose behind the following experiments. If two plasmodia in different stages of senescence are fused to give a single plasmodium, the observed life expectancy of the fused plasmodium enables a clear choice to be made between the exponential hypothesis and the $x^n = (1-y)^t$ (corrected linear) hypothesis of the rate of development of senescence.

Tables 24 and 25 illustrate the hypothetical predictions of the two hypotheses for a particular (theoretical) experimental situation, the fusion of two plasmodia, one of average life expectancy of 40 doublings and the other of 120 doublings. (The two plasmodia should be of equal size.)

Table 24 is assuming the $x^n = (1-y)^t$ (corrected linear) hypothesis for a particular value of y ($y = .01$, curve E in Figure 6). The number of doublings predicted before death will occur is translated by the curve, into hypothetical number of degenerate factors. This is done for the two plasmodia. (40 doublings before death = 77.5 affected targets, 120 doublings = .48 affected targets.) The number of affected targets in the plasmodium produced by fusing these plasmodia is the

average of their respective numbers of affected targets ($.775 + .48 = .6275$). This figure is translated, by the curve, into a predicted life expectancy of the fused plasmodium of 90 doublings. (Throughout death is assumed to occur at the 85% affected level, see above.)

Table 25 is the prediction assuming the hypothesis represented by graph H (Figure 7), exponential increase of affected targets ($n \times 1.04$) and a spontaneous (de novo) frequency of affected targets of 1 in 100,000. The plasmodium formed by fusion is predicted on this hypothesis to have a life expectancy of 56 doublings.

The essential point, which this illustration is intended to bring out, is that hypotheses of the $x^n = (1-y)^t$ type predict that if two plasmodia are fused, the resulting plasmodium will have a life expectancy close to, but greater than, the average life expectancy of the two fused plasmodia. The alternative exponential hypothesis predicts that the plasmodium resulting from the fusion will have a life expectancy closely similar to the more senescent of the fused pair. It should perhaps be stated that this conclusion applies to all the probable hypotheses of the $x^n = (1-y)^t$ and exponential type. The particular examples of these hypotheses which are used in these calculations (Graphs E and H, Figures 6 and 7) were selected for optimal fulfilment of such characters as life expectancy, and spontaneous error frequency.

TABLE 24

The Prediction of the $x^n = (1-y)^t$ hypothesis ($y = .01$, Graph E)
in a Particular Experimental Context

Genotype of plasmodium	Life expectancy in doublings	Frequency of mutant organelles	Predicted life expectancy
A	40	.775	
B	120	.48	
(A+B)		$\frac{.775}{2} + .48 = .628$	90

TABLE 25

The Prediction of the Exponential Hypothesis ($n \times 1.04$, Graph H)
in the Same Experimental Context as is Described in Table 24

Genotype of plasmodium	Life expectancy in doublings	Frequency of affected targets	Predicted life expectancy
A	40	.150	
B	120	.005	
(A+B)		$\frac{.150}{2} + .005 = .0775$	56

It may be helpful to appreciate the contrasting predictions of the two hypotheses if it is considered, intuitively, that if one of a pair of fused plasmodia is in an advanced state of exponential degeneration, the life expectancy of the plasmodium resulting from the fusion will be only marginally affected by the degree of degeneration of the other member of the pair.

The following experiment was therefore performed to obtain information on the rate of development of senescence. Microsclerotia of B173+BB114 and B174+BB128 were prepared, sampled, and the life expectancy of the resulting macroplasmodium observed. The average life expectancies from the time of revitalising were observed to be, B173+BB114 86.3 days (SE 3.7) and B174+BB128 27.0 days (SE 1.4) (20 parallel subcultures of each macroplasmodium being maintained). Eight days after the two microsclerotia preparations were revitalised one of the twenty subcultures of B173+BB114 was fused with one of the B174+BB128 subcultures. The plasmodia were trimmed just before fusion so that the fused plasmodia were of approximately equal size. (It was mentioned above that these two plasmodia fuse without killing, a viable heterokaryon being formed.) The heterokaryon B173+BB114/
B174+BB128 was left for 24 hours after fusion to allow complete mixing. The heterokaryon was then divided into 20 parallel subcultures which were maintained on SDM agar until death occurred. The average life expectancy of the heterokaryon subcultures was 54.7 days (SE 2.2) from

TABLE 26

The Observed Life Expectancy (\bar{x}) of (B173+BB114)⁴, (B174+BB128)⁵ and (B173+BB114)⁴/(B174+BB128)⁵ Plasmodia

Compared with the Predicted \bar{x} of the Heterokaryon Assuming the $x^n = (1-y)^t$ Hypothesis for the Development

of Senescence, or Alternatively the Exponential Hypothesis

	Genotype of plasmodium	No. of parallel subcultures	Observed average \bar{x} and SE in days after fusion of B173+BB114 and B174+BB128	Observed average \bar{x} in doublings	$\frac{\bar{x} \text{ B173+BB114} + \bar{x} \text{ B174+BB128}}{2}$	Predicted \bar{x} of heterokaryon on the $x^n = (1-y)^t$ hypothesis ($y = .01$)	Predicted \bar{x} of heterokaryon assuming the exponential hypothesis ($n = x1.04$)
A	(B173+BB114) ⁴	20	\bar{x} 78.3 (3.7)	156.6			
B	(B174+BB128) ⁵	20	\bar{x} 19.0 (1.4)	38.0			
A+B	(B173+BB114) ⁴ / (B174+BB128) ⁵	20	\bar{x} 54.7 (2.2)	109.4	93.3 doublings (46.6 days)	95.0 doublings (47.5 days)	52 doublings (26 days)

the time of fusion (see Table 26).

Table 26 also contrasts the observed life expectancy of the B173+BB114/B174+BB128 with the predicted life expectancy assuming the $x^n = (1-y)^t$ hypothesis (Graph E, Figure 6) and the predicted life expectancy assuming the exponential hypothesis for the rate of development of senescence (Graph H, Figure 7). (The number of doublings before death for the two fused plasmodia is converted, by reference to the graphs, to a number of affected targets. These two numbers are added together and the halved to give the proportion of affected targets in the heterokaryon. This number is then converted to the predicted life expectancy of the heterokaryon by reference to the graphs.)

It will be seen from Table 26 that the observed life expectancy of the heterokaryon is greater than the average of the two fused plasmodia, and that it corresponds well with the prediction of the $x^n = (1-y)^t$ hypothesis ($y = .01$).

The same experimental procedure, the observation of the life expectancy of heterokaryons formed by fusion of plasmodia in different stages of senescence, was applied in the more elaborate experiment reported below.

Two microglerotia preparations of B173+BB114 and B174+BB128 were sampled onto SDM agar, and the resulting macroplasmodia cultured on SDM agar. The microsclerotia preparations were sampled again 23

days later, and again after a further period of 36 days. The 6 macroplasmodia produced by this procedure were all maintained as 20 parallel subcultures. The original sample of B174+BB128 micro-sclerotia gave a plasmodium of \bar{x} 57.8 days from time of revitalising, and these 20 plasmodia were therefore lost before the third sample was revitalised.

The plasmodia derived from samples of microsclerotia taken at different times were assumed to be at different stages of senescence since they had spent different periods of time on SDM agar subsequent to revitalising. Plasmodia of each of the five surviving "types" were fused to form various heterokaryons 12 days after the third sample was taken. Plasmodial pairs were fused on SDM agar plates, care being taken that the size of the fusing plasmodia was approximately equal. The heterokaryons resulting from such fusions were left for 24 hours to allow complete mixing, and the plasmodium was then subdivided into 20 parallel subcultures. These were maintained in culture until death occurred. Table 27 describes the results of the experiment, the life expectancies of the original plasmodia and the heterokaryons derived from them, and also records the predicted life expectancies of the heterokaryons, assuming the $x^n = (1-y)^t$ hypothesis and the exponential hypothesis.

The data described in Table 27 agrees with the predictions of the corrected linear ($x^n = (1-y)^t$) hypothesis. The plasmodia fused

TABLE 27

The Observed Life Expectancy (\bar{x}) of Various Plasmodia and the Heterokaryons Formed Between Them, and the

Predicted Life Expectancy of the Heterokaryons

	Genotype	No. of parallel subcultures	Observed \bar{x} in days after date fusions were performed	Sum of \bar{x} (in days) of two fused plasmodia divided by 2	Predicted \bar{x} ($x^n = (1-y)^t$ ($y = .01$))	Predicted \bar{x} (exponential) ($n = 1.04$)
C	(B173+BB114) ² sample 1	10	12.5 (0.6)			
D	(B173+BB114) ² sample 2	10	33.2 (3.0)			
E	(B173+BB114) ² sample 3	20	57.8 (5.7)			
F	(B174+BB128) ⁴ sample 2	20	9.8 (1.3)			
G	(B174+BB128) ⁴ sample 3	20	36.0 (8.9)			
C+G	(B173+BB114) ² sample 1/ (B174+BB128) ⁴ sample 3	20	25.7 (2.9)	24.3	25.0	21.0
D+G	(B173+BB114) ² sample 2/ (B174+BB128) ⁴ sample 3	20	33.2 (5.1)	34.6	35.0	34.0
E+G	(B173+BB114) ² sample 3/ (B174+BB128) ⁴ sample 3	20	52.1 (2.6)	46.9	48.0	42.0
F+G	(B174+BB128) ⁴ sample 2/ (B173+BB128) ⁴ sample 3	20	29.5 (4.0)	22.9	24.0	17.0

to produce the data of Table 27 did not have such widely separated life expectancies as those described in Table 26, and therefore the observed life expectancies of the heterokaryons do not enable one to choose so surely between the $x^n = (1-y)^t$ hypothesis and the exponential hypothesis. Thus for the heterokaryon formed between (B174+BB114)² sample 1 (\bar{x} 12.5 days) and (B174+BB128)⁴ sample 3 (\bar{x} 36.0 days) is observed to have a life expectancy of 25.7 days, the two predictions being: $x^n = (1-y)^t$ \bar{x} 25.0; exponential \bar{x} 21.0.

The heterokaryon (B174+BB128)⁴ sample 2/(B174+BB128)⁴ sample 3 (termed F + G in the left hand column of Table 27) is of especial interest. This was formed by the fusion of two B174+BB128 plasmodia, that is plasmodia of the same genotype. Most of the heterokaryons, for reasons described below, were formed by plasmodia of different genotypes (B173+BB114 and B174+BB128). It was possible that the use of heterokaryons formed by plasmodia of different genotypes might produce difficulties for the analysis of rate of development of senescence, either because of heterosis or because of the nuclei interacting in the heterokaryon to produce a plasmodium of reduced viability. The observed life expectancy of (B174+BB128) sample 2/(B174+BB128)⁴ sample 3, and the relationship of this observation to the two predicted life expectancies, establishes that homokaryotic fusions behave in an essentially similar manner to the heterokaryotic fusions (B173+BB114 fused with B174+BB128).

It was considered desirable to repeat this experiment with plasmodia of more widely separate life expectancies, to confirm the suggested support for the corrected linear ($x^n = (1-y)^t$) hypothesis. The procedures used in this experiment (see Table 28) were of the same type as those used to produce the data of Table 27, and these procedures will therefore not be described again in detail.

The most significant of the heterokaryons described in Table 28 is that termed H+K in the left hand column. The plasmodia fused to form this heterokaryon had the greatest difference in life expectancy ($(\underline{B173+BB114})^5 \bar{x} 10.6$, $(\underline{B174+BB128})^3 \bar{x} 51.3$), and therefore the two predicted life expectancies show the widest separation (observed \bar{x} of heterokaryon 33.2, $x^n = (1-y)^t$ prediction 35.0, exponential prediction 19.0).

The data described in Tables 26, 27, 28 suggest that the observations support the $x^n = (1-y)^t$ hypothesis for the development of senescence, and not the exponential hypothesis. This conclusion will be returned to in the Discussion.

J. It was mentioned above that plasmodia of two genotypes were used in the heterokaryon experiments described in section I, these being B173+BB114 and B174+BB128. These two plasmodia were chosen for the following reasons, which enabled the heterokaryotic nature of the plasmodia in Tables 26, 27 and 28 to be confirmed.

TABLE 28

The Observed Life Expectancy (\bar{x}) of Various Plasmodia and the Heterokaryons Formed Between Them, and the

Predicted Life Expectancy of the Heterokaryons

	Genotype	No. of parallel subcultures	Observed \bar{x} in days after date fusions were performed	Sum of \bar{x} (in days) of two fused plasmodia divided by 2	Predicted \bar{x} ($x^n = (1-y)^t$ ($y = .01$))	Predicted \bar{x} (exponential) ($n = 1.04$)
H	(B173+BB114) ⁵	10	10.6 (3.6)			
I	(B173+BB114) ³ sample 1	10	23.7 (4.2)			
J	(B173+BB114) ³ sample 3	10	42.4 (1.9)			
K	(B174+BB128) ³	10	51.3 (4.2)			
H+K	(B173+BB114) ⁵ / ₃ (B174+BB128) ³	20	33.02 (2.9)	31.0	35.0	19.0
I+K	(B173+BB114) ³ sample 1/ (B174+BB128) ³	20	37.2 (4.0)	37.5	40.0	31.0
J+K	(B173+BB114) ³ sample 3/ (B174+BB128) ³	20	49.1 (3.9)	46.9	47.5	46.0
J+K	(B173+BB114) ³ sample 3/ (B174+BB128) ³	20	50.3 (3.8)	46.9	47.5	46.0

In Chapter 2 the analysis of the genetic control of plasmodial fusion was described. The Indiana isolate was found to carry two alleles, \underline{f}_3 and \underline{f}_4 of the gene \underline{f} . Plasmodia derived from this isolate could therefore be of three genotypes, $\underline{f}_3\underline{f}_3$, $\underline{f}_3\underline{f}_4$ and $\underline{f}_4\underline{f}_4$. $\underline{f}_3\underline{f}_4$ plasmodia would only fuse with plasmodia of the same fusion genotype. Plasmodia of the two homozygous genotypes, $\underline{f}_3\underline{f}_3$ and $\underline{f}_4\underline{f}_4$ would fuse with plasmodia of either homozygous type, i.e. $\underline{f}_3\underline{f}_3$ would fuse with $\underline{f}_3\underline{f}_3$ and $\underline{f}_4\underline{f}_4$. It was found that if heterokaryons were formed between $\underline{f}_3\underline{f}_3$ and $\underline{f}_4\underline{f}_4$ plasmodia the heterokaryon came, after a period of approximately 24 hours, to behave in the manner of an $\underline{f}_3\underline{f}_4$ heterozygote. This heterokaryon behaviour occurred if the proportions of the fused $\underline{f}_3\underline{f}_3$ and $\underline{f}_4\underline{f}_4$ plasmodia were 9:1 or more nearly equal. If the proportions were less equal than 9:1 the heterokaryon continued to behave as a homozygote. These properties of the \underline{f}_3 and \underline{f}_4 alleles were used in the analysis of senescence. The two strains used in section I were examples of the two homozygotes, B173+BB114 being $\underline{f}_4\underline{f}_4$ and B174+BB128 $\underline{f}_3\underline{f}_3$. These particular strains were used because these homozygotes were found, by trial and error, to be a non-killing pair, fusion occurring between them without subsequent killing, a heterokaryon becoming established.

Fusion behaviour was analysed during the experiments described in section I (above) to confirm that heterokaryons had been produced by fusing B173+BB114 with B174+BB128. The heterokaryons described in

Tables 26, 27 and 28 were routinely tested for fusion behaviour after being set up. A period in excess of 24 hours was allowed for the heterokaryons to develop heterozygous behaviour before the fusion tests were performed. The heterokaryons were then tested for fusion behaviour with a homozygote plasmodium (B173+BB114, $\underline{f}_4 \underline{f}_4$ or B174+BB128, $\underline{f}_3 \underline{f}_3$) and with a heterozygote (B173+B174, $\underline{f}_3 \underline{f}_4$) (See Chapter 2 for testing procedure.) In every case the heterokaryotic nature of the plasmodia was confirmed.

An alternative procedure for confirming the heterokaryotic nature of the plasmodia would have been to analyse the genotype of haploid spores derived from the plasmodium. Only two markers have been analysed in the Indiana isolate, mating type (alleles \underline{mt}_3 and \underline{mt}_4) and \underline{f} (\underline{f}_3 and \underline{f}_4). The mating type markers could not be used since all plasmodia of the Indiana isolate have to be formed by crossing a \underline{mt}_3 amoebal clone with a \underline{mt}_4 clone, and therefore all Indiana plasmodia are heterozygous for these alleles. The \underline{f}_3 and \underline{f}_4 alleles could have been analysed in the haploid spores of a heterokaryon, but this procedure was not attempted for the heterokaryons described in section I for the following reasons.

(i) The analysis of the fusion behaviour of the heterokaryons provided a convenient and convincing test of their heterokaryotic nature.

(ii) The analysis of the fusion behaviour of heterokaryons provides

a more direct assay of the proportions of the two nuclear types in the plasmodium. It was thought unwise to prejudge the issue and assume that nuclei derived from the senescent plasmodium (a relatively senescent and a vigorous plasmodium were fused to give the heterokaryons described in section I) would necessarily have "normal" viability after passing through meiosis and reaching the haploid amoebal stage. Therefore the proportions of genes detected in the haploid progeny of such a heterokaryon need not necessarily be the same as those present in the heterokaryon. The f gene provides a useful method of assaying the proportions of nuclear types present in a heterokaryon without recourse to progeny analysis. It would, of course, be desirable to perform the progeny analysis, and compare the proportions detected in this way with the proportions detected by f analysis, but this experiment was not attempted for the heterokaryons described in section I.

K. The Stability of Heterokaryons Formed from Senescent and Vigorous Plasmodia Prepared from Microsclerotia

Senescence has been considered to have at least two hypothetical rates of development, corrected linear ($x^n = (1-y)^t$) and exponential. One of the possible "targets" for senescence is the nuclei. If one combines these propositions it is conceivable that senescence could be a nuclear phenomenon, "degenerate" nuclei occurring spontaneously and then increasing exponentially due to some form of selective

advantage compared with normal nuclei. This proposition, if correct, predicts that a heterokaryon between a senescent and a non-senescent plasmodium will not be stable, the nuclei characteristic of the senescent plasmodium increasing exponentially within the heterokaryon. (The senescent plasmodium is considered hypothetically to contain two types of nuclei, normal and senescent, the senescent nuclei being the ones responsible for the proposed instability of any heterokaryon in which a senescent plasmodium is involved.)

This hypothesis, that senescence is a nuclear phenomenon showing exponential development, is testable by making use of the f gene. (The value of using fusion behaviour analysis rather than spore analysis has been discussed above.) The predicted observations is that the fusion behaviour of the $\frac{f_3f_3}{f_4f_4}$ heterokaryons formed from vigorous and senescent plasmodia will originally resemble the heterozygous ($\frac{f_3f_4}{f_4f_3}$) behaviour, but will be unstable, eventually reverting to the homozygous ($\frac{f_3f_3}{f_3f_3}$ or $\frac{f_4f_4}{f_4f_4}$) behaviour because of the selective advantage possessed by the senescent nuclei. It was mentioned above that "normal" $\frac{f_3f_3}{f_4f_4}$ heterokaryons show heterozygous behaviour when they consist of the two nuclear types in the ratio 9:1 or more nearly equal. A heterokaryon formed from equal quantities (1:1) of senescent and vigorous plasmodia would therefore have to show a very great shift in nuclear ratio for the heterozygous behaviour to change to homozygous behaviour. For this reason the heterokaryons produced to test

the stability of nuclear ratios in heterokaryons (formed from vigorous and senescent plasmodia) were not made up of equal quantities of the two plasmodia types. Instead two types of heterokaryon were produced for the senescent and vigorous plasmodia under consideration, one consisting of the ratio 9 parts senescent to 1 non-senescent, and the other 1 part senescent to 9 parts non-senescent. The senescent nuclei would only have to show a slight relative selective advantage (or disadvantage) for one of these two heterokaryons to become composed of a nuclear ratio less equal than 9:1 and therefore to show homozygous fusion behaviour.

The senescent and vigorous plasmodia described in Table 26 ((B173+BB114)⁴ and (B174+BB128)⁵) were fused in the proportions 9:1 and 1:9, this procedure being performed in quadruplicate. The resulting heterokaryons were maintained in vegetative culture until death occurred. Every third day of culture a block was subbed onto fresh SDM agar medium, and two further blocks were tested for fusion with "tester" plasmodia (B173+B174, f_3f_4 ; B173+BB114, f_4f_4). All 8 heterokaryons showed heterozygous behaviour during this test, no example of homozygous behaviour being recorded. The 8 heterokaryons were only maintained as single cultures and therefore no significance will be attached to the life expectancy. For a period just prior to death the heterokaryons failed to fuse with either tester plasmodium. During this period it was also difficult to induce the heterokaryons

to sporulate, any spore masses that were formed being abnormal. This experiment failed to detect any selective advantage of nuclei derived from the senescent or vigorous plasmodia, and therefore suggests that senescence of plasmodia derived from microsclerotia does not involve the exponential increase of "senescent" nuclei. (The same experiments were performed with the plasmodia described in Table 27, with the same result.)

L. Experiments were also performed, using the f markers, to test the hypothesis that senescence was associated with nuclear malfunction or inactivity. It should be said that if the exponentially increasing "senescent" nuclei hypothesised in section K (above) were inactive with respect to normal functioning (including the f gene) then their increase would not result in an alteration of the "active" proportions of f₃f₃ and f₄f₄ nuclei, and therefore the heterokaryon would not show instability with respect to fusion behaviour. Alternatively hypotheses could be proposed that involved inactivation of nuclei during senescence without the "senescent" nuclear type having any selective advantage or disadvantage. To test these hypotheses, which propose that senescent plasmodia contain a proportion of "inactive" nuclei the following experiment was performed.

It was mentioned above that if heterokaryons between f₃f₃ and f₄f₄ plasmodia are formed in the proportions of 9:1, or more nearly equal, they behave as heterozygotes. The hypotheses proposed above

predict that the critical ratio of $\underline{f_3f_3}$ to $\underline{f_4f_4}$ will be altered if one of the plasmodia is senescent (since this plasmodium will contain inactive nuclei). The senescent and vigorous plasmodia described in Table 26 were therefore tested to detect the critical ratio of fused plasmodia which would allow the heterokaryon to behave as a heterozygote. It was found, within the limits of accuracy imposed by the rather imprecise procedure used to regulate the proportions of plasmodia fused, that the critical ratio 9:1 was not affected by one of the plasmodia being senescent. The procedures used are probably sensitive enough to detect the number of inactive nuclei suggested by the second hypothesis, inactivation without selective advantage, and this hypothesis must therefore be considered unlikely in view of the observed result. The procedures are probably not, however, sensitive enough to detect the small proportion of inactive nuclei which might be present on the first hypothesis, inactivation followed by exponential increase, and these experiments cannot therefore be viewed as support or opposition to this hypothesis.

M. The various alternative theories of senescence predict that certain procedures will accelerate its rate of development. Thus for example any hypothesis which involves nuclear mutation predicts that mutagenic substances will accelerate senescence (assuming that mutagens are used which mimic spontaneous mutagenesis). Plasmodia were therefore exposed to a variety of procedures, returned to

normal culture conditions, subdivided and the average life expectancy compared with the life expectancy of untreated plasmodia. It should be stressed that the procedures were employed as a "pulse", and that subsequent to the treatment considerable growth occurred, therefore any alteration in time of death is reasonably assumed to be independent of the immediate toxic or otherwise adverse effect of the procedure.

The results of the various treatments are summarised in Table 29.

(i) A control plasmodium, (B173+BB114)⁴, was subdivided into 20 parallel subcultures, its observed life expectancy being found to be 65.0 days, standard error 5.1.

(ii) To test whether senescence was due to the presence of toxic waste products accumulating during growth on SDM agar a subculture derived from the original plasmodium used in section (i) above was incubated on an SDM agar plate for three days at which time the normal procedure would have been to subculture it, the plasmodium having grown to cover the medium. The plasmodium was, however, left to grow on this plate for a further three days, during which time any toxic waste products could increase in quantity. The plasmodium was then subbed onto SDM agar, incubated for three days and subdivided into 20 parallel subcultures. The observed life expectancy from the start of the experiment was 64.9 days, SE 5.9. Thus this procedure had no effect on the life expectancy of the plasmodia.

TABLE 29

Life Expectancy of Plasmodia Exposed to Various Procedures(Measured from End of Pulse)

Treatment	\bar{x} (in days)	SE
Untreated (control)	65.0	5.1
Starvation on exhausted SDM agar	64.9	5.9
Starvation on 3% water agar	66.4	6.6
Plasmodia grown to large size	64.1	4.5
Damage by cutting	67.5	5.8
Elevated temperature (43°C)	68.1	6.1
UV	68.7	4.2
NMG	69.8	3.6
DL-ethionine	67.4	6.1
Phenethyl alcohol	66.5	3.3
Streptomycin	66.3	8.0
Acridine	85.3	4.1
Control	29.25	4.8
DL-parafluorophenylalanine	27.4	5.8

* N.B. 4 cultures died almost immediately after the pulse

(iii) A subculture of (B173+BB114)⁴ was placed onto 3% water agar and incubated for three days under these starvation conditions.

The plasmodium migrated around the plate the organism consisting of thin veins with no "feeding areas" between them. The plasmodium was then subbed onto SDM agar, and subsequently subdivided into 20 cultures. The observed \bar{x} was 66.4 days, SE 6.6. Thus this procedure had no effect on life expectancy.

(iv) It is known that mitotic synchrony breaks down in plasmodia larger than about 7 cm in diameter, and to test whether this might be relevant to senescence plasmodia were grown on 12 cm diameter petri dishes on SDM agar. (This procedure might also be expected to result in the build up of abnormally high levels of toxic waste products.) The subculture of (B173+BB114)⁴ took 6 days to cover the 12 cm diameter plate, at which time it was subcultured and maintained by the normal procedure after being subdivided into 20 parallel subcultures. The observed \bar{x} was 64.1 days, SE 4.5. This procedure therefore had no effect upon senescence.

(v) The normal procedure for maintaining plasmodia in vegetative culture involves subculturing them onto fresh SDM agar plates every three days. This involves cutting the plasmodium with a spatula and inoculating the resulting agar block onto the fresh plate. To test whether senescence might be due to this periodic physical damage, a

subculture of (B173+BB114)⁴ was sliced with a spatula into extremely small pieces (of the order of 1 mm square). The tiny plasmodia were then allowed to fuse with each other, and the life expectancy of the resulting plasmodium observed by the normal procedure. (The plasmodium being subdivided into 20 parallel subcultures). The observed \bar{x} was 67.5 days, SE 5.8. Thus this procedure did not affect senescence.

(vi) Several subcultures of (B173+BB114)⁴ were incubated at 37°C instead of 25°C. After 3 days incubation, during which streaming of the cytoplasm continued but no growth was apparent, the plates were transferred to a 43°C incubator for various lengths of time. Cultures can be left at 43°C for periods up to 10 minutes without the procedure proving lethal. At 10 minutes streaming has ceased, the veins being very dark in appearance and apparently blocked with some form of debris, perhaps gelled cytoplasm. However if removed at this time, the plasmodium recovers, streaming recommencing throughout the plasmodium after a period of some hours. A plasmodium which had been subjected to this procedure, 37°C for 3 days then 43°C for 10 minutes, was allowed to recover, subcultured onto SDM agar and subsequently subdivided into 20 parallel subcultures. The observed \bar{x} was 68.1 days, SE 6.1. Thus this procedure had no effect upon senescence.

(vii) To test whether senescence involved the occurrence of genetic mutations (nuclear or episomal) a subculture of (B173+BB114)⁴ was exposed to UV irradiation, at the intensity of 20 ergs per square m.m.

per second for various periods of time. The organism is apparently very insensitive to UV irradiation, exposure for up to 20 minutes having no apparent effect. Exposure for 30 minutes produced some obvious effect, small areas of the plasmodium showing lysis. A plasmodia treated for 30 minutes was subcultured onto SDM agar, and subsequently subdivided (x 20). The observed \bar{x} was 68.7 days, SE 4.2. This procedure thus had no apparent effect on senescence.

(viii) An alternative mutagenic procedure was employed, using N-methyl-N-nitro-N-nitrosoguanidine (NMG). NMG was incorporated in SDM agar plates at various concentrations, and subcultures of (B173+BB114)⁴ inoculated onto the plates. NMG was lethal in twelve hours at the concentration of 100 mcg/ml, a concentration of 10 mcg/ml inhibiting growth completely, but not being lethal. A plasmodium exposed to SDM agar + 10 mcg/ml of NMG for three days was subcultured onto SDM agar and subsequently subdivided (x 20). The observed \bar{x} was 69.8 days, SE 3.6. This procedure therefore had no effect upon senescence.

This procedure used for NMG, the finding of a lethal level and the use of a concentration that was just sublethal, was employed for all the various substances used in the experiments described below. The procedure allows one to assume that the drug involved has been assimilated.

(ix) To test whether errors in protein synthesis (see Introduction, the Orgel hypothesis) were responsible for senescence, DL-ethionine was incorporated in SDM agar at various concentrations. This substance, an analogue of the amino acid methionine, has been found in bacteria to become incorporated in place of methionine during protein synthesis, and to produce abnormal proteins (*ref*). This substance was considered both by Orgel (1963) and Holliday (1969) to be a useful method of testing the relevance of the Orgel hypothesis to a particular senescence phenomenon. SDM agar + 10^{-2} M ethionine was lethal to plasmodia in 48 hours. SDM agar + 10^{-3} M ethionine allowed some slight growth. A subculture of (B173+BB114)⁴ was grown on this medium for three days, subcultured onto SDM agar and subsequently subdivided (x 20). The observed \bar{x} was 67.4 days, SE 6.1. This procedure therefore had no effect upon senescence.

(x) Phenethyl alcohol has been reported (Wilkie and Maroudas, 1968) to be an inducer of mitochondrial petite mutations in Saccharomyces cerevisiae, although this effect has been considered to be possibly a side effect of the production of an anaerobic state (Terenzi and Stork, 1968). SDM agar + 1% phenethyl alcohol is lethal to plasmodia in twelve hours. SDM agar + 1% phenethyl alcohol completely inhibits growth. A subculture of (B173+BB114)⁴ exposed to this medium (SDM + 1% PEA) for 3 days was subcultured onto SDM agar and subsequently subdivided (x 20). The observed \bar{x} was 66.5 days, SE 3.3. This

procedure therefor had no effect upon senescence.

(xi) Streptomycin has been reported as affecting chloroplasts in several organisms, for example Euglena gracilis and the drug has also been reported to inhibit flagella formation in myxamoebae of Didymium nigripes (Kerr, 1965). One of the proposed modes of action of streptomycin in bacteria is that it produces misreading during protein synthesis. For this reason it was considered to be worthwhile to investigate the effect of streptomycin on the rate of senescence, the mitochondrial protein synthesising system being the hypothetical target site. (Streptomycin would not be expected to affect the cytoplasmic protein synthesising system.) The organism is very resistant to streptomycin, the highest concentration of drug tested, 10 mg/ml having no apparent effect on growth rate or morphology. It seems probable that the organism is completely insensitive to this antibiotic. A subculture of (B173+BB114)⁴ was grown on SDM agar + 10 mg/ml streptomycin for 3 days, subbed onto SDM agar and subsequently subdivided (x 20). The observed \bar{x} was 66.3 days, SE 8.0. This procedure therefore had no effect upon senescence.

(xii) Using another plasmodium, (B173+BB114)⁵, the effect of DL-parafluorophenylalanine on senescence was investigated. This substance, an analogue of ^{phenyl}alanine, acts in a similar way to ethionine (see ix above). SDM agar + 10^{-2} M parafluorophenylalanine was found to be lethal to the plasmodia. A plasmodium was grown on SDM agar +

10^{-3} M parafluorophenylalanine for 3 days, subcultured onto SDM agar and subdivided (x 20). The \bar{x} of the control plasmodium was 29.25 days (SE 4.8), the \bar{x} of the treated plasmodium 27.4 days (SE 5.8). This procedure therefore had no effect on senescence.

(xiii) Acridine dyes are extremely effective in inducing mitochondrial "petite" mutants in the facultatively aerobic yeast Saccharomyces cerevisiae (Roodyn and Willie, 1968). These mutant mitochondria are reported to contain altered DNA (Mounolou, Jakob and Slonimski, 1968). The mutant yeasts are unable to perform oxidative respiration, their mitochondria lacking cytochromes a, a_3 , b and c_1 (cytochrome c is present). The acridine dye acriflavine has been reported (Hill, Winston and Anderson, 1969) to effect the mitochondria-kinetoplast complex of the trypanosome, Crithidia fasciculata, reducing the quantity of the membrane bound cytochromes and producing "dyskinetoplastic" cells (showing when studied by electronmicroscopy a variety of abnormalities). It is unclear whether the acridine dye is acting primarily as a mutagen, but it seems more likely that in this case the primary effect of the dye is to inhibit transcription of the M-DNA template. The effects of acridine on several other trypanosomes have been reported. Of considerable interest is the recent report of Kellerman, Biggs and Linane (1969) who observed the effect of euflavine on the obligate aerobic yeast Candida parapsilosis.

(This yeast has two respiratory systems, one being cyanide insensitive and the other being the cyanide sensitive mitochondrial cytochrome system.) It has been clearly established that euflavin reduces the quantities of the membrane bound cytochromes of this organism in a reversible manner, the dye not acting as a mutagen.

(xiii) (1) Plasmodia ((B173+BB114)⁴) were exposed to SDM agar containing various concentrations of the dye acridine orange. At a concentration of 1 mg/ml the drug caused some inhibition of growth and a rather irregular morphology. A subculture of (B173+BB114)⁴ was cultured on SDM agar + 1 mg/ml acridine orange for three days and then subcultured onto SDM agar. After three days growth on this plate the plasmodium was subdivided into 20 parallel subcultures which were inoculated onto fresh SDM agar plates. Four of these parallel subcultures died almost immediately showing lysis resembling senescent death. The 16 remaining subcultures showed normal morphology, and were maintained in culture until death eventually occurred. The observed \bar{x} was 85.3 days, SE 4.1 (see Table 29). Thus acridine orange, used as a "pulse", affects the life expectancy of plasmodia in a complex manner, some parallel subcultures showing death soon after return to SDM agar, and other subcultures showing a life expectancy significantly greater than the control (untreated) plasmodia.

(xiii) (2) To investigate this effect further the experiment was repeated using a subculture of (B173+BB114)⁶. The plasmodium was in a moderately advanced state of senescence, \bar{x} 15.7 days (SE 3.8) measured from the time of the experiment's start.

Subcultures of the plasmodium were exposed to various concentrations of acridine orange. It was found that, for this moderately senescent plasmodium, SDM agar + 1 mg/ml acridine orange was lethal in 48 hours. (It was stated above that vigorous plasmodia (\bar{x} 65.0) showed some growth on this concentration.) Subcultures of (B173+BB114)⁶ were exposed to lower, sublethal concentrations of acridine orange, SDM agar + 500 mcg/ml, and SDM agar + 125 mcg/ml. Subcultures were grown on these two concentrations for three days, subcultured onto SDM agar and subsequently subdivided (\times 20). The life expectancies, measured from the time of return to SDM agar after exposure, were: control SDM agar \bar{x} 12.7 days (SE 3.8); SDM agar + 125 mcg/ml acridine orange \bar{x} 11.15 days (SE 3.6); SDM agar + 500 mcg/ml acridine orange \bar{x} 6 days (SE 0) (see Table 30). Thus for a moderately senescent plasmodium (\bar{x} 15.7 at the beginning of the experiment) acridine orange accelerated senescence, apparently in proportion to its concentration.

The above experiments with acridine orange clearly implicate mitochondria in the phenomenon of senescence. The results will be discussed more fully, but it should be mentioned here that the

TABLE 30

Life Expectancy of Plasmodia Exposed to Various Concentrations
of Acridine Orange for Three Days (see also Table 29) Measured
from End of Pulse

Concentration of acridine	Life expectancy	Standard error
Control (untreated)	12.7	3.8
.125 mg/ml	11.15	3.6
.5 mg/ml	6.0	0
1 mg/ml	Lethal	

implication of mitochondria in senescence does not establish that they are the primary target or site of degeneration. It is possible that mitochondria, or the process of respiration itself is particularly sensitive to degenerative changes in another site, for example cytoplasmic protein synthesis.

N. To investigate further the suggested involvement of mitochondria in senescence, it was considered desirable to investigate the cytochrome profiles of vigorous and senescent plasmodia, and also to investigate the respiratory functioning of the organelles by other procedures.

The reports in the literature are scanty concerning the respiratory system of P.polycephalum (see I_ntroduction). The only cytochrome detected spectroscopically is a soluble c cytochrome (Yamanaka et al, 1962). Plasmodia were harvested from SDM agar plates and homogenised in a "Teflon" Dounce homogeniser, using Ca/Mg buffer, and a difference spectrum prepared with hydrogen peroxide and sodium dithionite, using a Unicam SP800 recording spectrophotometer. No cytochrome profiles were apparent, the possible explanation being interference due to the yellow pigment which shows changes in absorption with change of pH and oxidation/reduction. An alternative explanation is that due to the considerable quantities of carbohydrate stored by the plasmodia the preparation was not converted to an oxidised state by the quantities of peroxide added.

Centrifugation of homogenised preparations was used in an attempt to reduce the interference caused by the yellow pigment. The homogenate was spun at 500 g for 10 minutes to remove debris, and then spun at 10,000 g for 10 minutes to produce a pellet containing the mitochondria (see Rusch, 1968). The pigment granules are however of similar size to the mitochondria, and separation of the two proved difficult. Using centrifugation prepared mitochondrial suspensions it was still impossible to detect a cytochrome difference spectrum, using either a Unicam SP800 recording spectrophotometer or the more sensitive Shamadzu MPS-50L. Time prevented a more methodical approach to the investigation of the properties of mitochondrial preparations derived from senescent and vigorous plasmodia, but it is hoped that subsequent work will result in some data becoming available concerning this subject.

Discussion

The data reported in section I (see above) indicating that a heterokaryon formed by fusion of equal quantities of two plasmodia has a life expectancy intermediate between the life expectancies of the two fused plasmodia establishes that the development of senescence is approximately linear (which would agree with the corrected linear hypothesis, $x^n = (1-y)^t$, see Figure 6). This evidence suggests that the Orgel hypothesis, which specifies exponential accumulation of errors in protein synthesis, is not applicable to senescence in P.polycephalum.

The hypothesis that the development of senescence shows a linear form suggests that senescence involves discrete targets vulnerable to inactivation and that the proportion of inactivated/unaffected targets increases linearly during senescence. Assuming this, it is possible to make certain deductions concerning the targets. Since the plasmodium is showing continued growth (doubling every 12 hours) during the development of senescence, a majority of the macromolecules present in a senescent plasmodium have been recently synthesised. Assuming the linear hypothesis, it is necessary to assume that the newly synthesised targets reflect, or reproduce, the senescent damage sustained during the total period of the development of senescence.

(i) The simplest hypothesis to explain this is that the affected target is copied during synthesis at approximately the same rate as

the normal (unaffected) target.

Alternatives to this hypothesis are considered less likely for a variety of reasons but two will be briefly considered (iia and b).

(ii) . It could be assumed that the affected targets do not reproduce themselves. Two hypotheses ((iia) and (iib)) can be constructed involving this assumption. Hypothesis (iia) does not predict senescence, hypothesis (iib) predicts senescence showing the $x^n = (1-y)^t$ rate of development and is therefore a possible alternative to (i).

(iia) It might be assumed that the rate of increase of other macromolecules and organelles could be adjusted to correspond to the rate of synthesis of unaffected targets (which would equal the number of targets synthesised minus the number of affected by damage, per unit time.) This would result in the actual growth rate of the organism being less than the rate which would have occurred without damage. The hypothesis is not however adequate to explain the accumulation of damage (senescence), since the prediction of this hypothesis is that a stable state will occur. Therefore this model does not predict senescence.

(iib) Alternatively it might be assumed that the rate of increase of other macromolecules and organelles was not adjusted to compensate for the loss of affected targets. This would predict that disbalanced growth would occur and would lead eventually to the death of the organism

since the "unaffected" targets would become numerically inadequate to perform their function in the cell. This hypothesis therefore predicts that senescence will occur, and develop in the corrected linear manner ($x^n = (1-y)^t$) and it is therefore compatible with the data of the heterokaryon experiments, which establish that the development of senescence shows this form. The hypothesis is however considered less attractive than hypothesis (i) (see above) because it requires the assumption that the rate of synthesis of the targets is fixed, and cannot be adjusted to compensate for loss. The hypothesis which is preferred((i), see above) has the advantage that the proportion of affected to unaffected organelles will remain constant despite any attempts at compensatory adjustment. The proportion of affected targets will therefore increase progressively despite any compensatory adjustment in the absolute number of targets per unit cytoplasm.

All three of the hypotheses considered have in common the suggestion that the unaffected targets are responsible for their own synthesis. This suggestion appears to be an inevitable deduction, if senescence is presumed to develop in a linear manner.

If the targets are responsible for their own synthesis it may be assumed that they contain genetic information. This suggests that the targets might be either the nuclei or the mitochondria. Some form of mutation could occur in either of these organelles resulting

in the production of stable "defective" or mutant nuclei or mitochondria. Assuming the linear hypothesis for the development of senescence and considering the observed life expectancy of plasmodia (approximately 90 days, 180 doublings) it can be deduced that the rate of "mutation" (i.e. the rate at which newly affected targets spontaneously occur, "y" in Figure 6) must be between 1 in 20 and 1 in 200 per doubling. This rate fits well with the frequency of spontaneous "petite" mitochondrial mutations in Saccharomyces cerevisiae, but it is difficult to imagine nuclear mutations occurring spontaneously at this rate.

The results of section L (see above) can be interpreted to suggest that the organelle involved in the development of senescence is not the nucleus.

The various procedures described in section M (see above) support the hypothesis that senescence is due to the accumulation of defective mitochondria. The observation that the amino acid analogues para-fluorophenylalanine and ethionine fail to accelerate senescence suggests that the Orgel hypothesis is not relevant to the senescence of P.polycephalum. The failure of UV irradiation and the mutagen NMG to accelerate senescence suggests that senescence is not due to chromosomal deletions, point mutations etc.

The effect of acridine orange:

The complex effect of acridine orange (M,xiii above) is not easy to interpret, but the data support the belief that mitochondria

and respiration are intimately involved in senescence.

Taken in isolation, the results of treating moderately senescent plasmodia (M,xiii(2), Table 30) with the dye suggest that acridine orange accelerates senescence in proportion to its concentration. The time of death of the moderately senescent plasmodia, on all concentrations of acridine orange, was however relatively soon after the "pulse". The purpose of using a "pulse" for the procedures used in section M was to ensure that any effect upon time of death was due to a stable effect (assumed to be the acceleration of senescence) and was not due to the short term toxic effect of the procedure. For the immediate toxic effect of a procedure to be prevented from confusing the results it is necessary that a fairly long period of growth should occur subsequent to the exposure of a plasmodium to a "pulse" treatment. This condition, the occurrence of prolonged growth after the "pulse", was not fulfilled in the experiments involving the treatment of moderately senescent plasmodia with acridine. It must therefore be borne in mind that the earlier time of death observed in these experiments (M, xiii(2)) may be due not to the acceleration of senescence but to a residual toxic effect being still present ("carried over") after the pulse of acridine. This toxic "carry over" effect could result in the plasmodia becoming liable to death at a stage of senescence at which they would normally be viable.

This "carry over" effect should be envisaged in one of two ways. (It is assumed as a working hypothesis, based on the data of section I and L, that senescence is due to the accumulation of defective mitochondria resulting in respiratory inadequacy.)

(i) It is possible that the acridine remained within the plasmodia for some time after the exposure to a pulse of acridine. The dye is known to form very stable complexes with DNA. During a period subsequent to the pulse the retention of acridine by the plasmodium would result in impaired respiration. The continued development of senescence during this "toxic carry over" period might result in the respiration of the organism becoming inadequate, due to the effect of residual acridine combined with the accumulation of defective mitochondria.

(ii) Alternatively it is possible that acridine is removed from the plasmodium quite quickly following a pulse of exposure, but that it takes some time for the organism to recover from the immediate, toxic effect of the dye. (Acridine inhibits the transcription of M-DNA, and it might be visualised that a recovery period would be necessary before this inhibition is made up.) During this recovery period the cell would show impaired respiration, which might combine with the developing senescent respiratory inadequacy to produce an acceleration of rate of death.

Consideration of the effect of acridine on vigorous plasmodia (M, xiii(1)) supports the belief that the dye is not accelerating senescence, but that during a period subsequent to the pulse the drug produces a "toxic carry-over" effect which can combine with developing senescence to produce premature death.

Of the 20 subcultures, derived from the vigorous plasmodium exposed to acridine, 4 died soon after their return to SDM agar. It is proposed that these deaths, as well as the deaths of the plasmodia described in M,xiii(2), occurred during the period of the "toxic carry over", death being due to the carry over effect combined with the natural progression of senescence. The remaining 16 subcultures of the treated vigorous plasmodium survived the period immediately subsequent to the pulse. The continued growth of these 16 subcultures would allow recovery from the toxic carry over, either through dilution of mitochondria containing stable M-DNA acridine complexes, or through synthesis making up for the inhibitory effect of acridine on M-DNA transcription. The reason for 4 of the subcultures dying soon after the pulse, and the remaining 16 surviving must be presumed to be due to slight variation in amount of acridine incorporated, or other slight variations.

The most difficult part of the data to interpret is the life expectancy of the 16 subcultures which survived the period immediately

subsequent to the pulse. It might have been predicted that having survived the "toxic carry over" period the subcultures would die at the same time as the control, untreated, plasmodia, the cause of death being the progressive development of senescence, unaffected by the acridine pulse. However, contrary to this prediction, the 16 surviving subcultures showed a life expectancy significantly longer than the untreated control plasmodia. There is no obvious explanation of this phenomenon. One tenable hypothesis is that the treatment selectively affects defective mitochondria, causing them to be at a selective disadvantage compared with normal mitochondria. Various other hypotheses are tenable, but this curious phenomenon clearly requires further experimental investigation, using plasmodia in various stages of senescence. To summarise the conclusions derived from the experiments described in section M:

- (i) Of all the procedures investigated, only acridine affects the time of death of plasmodia (acridine is mutagenic for mitochondria in Saccharomyces cerevisiae, and inhibits mitochondrial protein synthesis in Candida parapsilosis).
- (ii) It appears that the "lethal" effect of acridine is not due to the acceleration of senescence, but rather to a temporary residual effect of the pulse. Plasmodia may die prematurely during the period immediately subsequent to the pulse. If they survive the period immediately subsequent to the pulse they do not show premature death.

Summary

To summarise the results of all the experiments concerning senescence, the life expectancy of heterokaryons has established that senescence shows a linear development. Various considerations of the rate of senescence lead to the belief that the affected target must be responsible, in part, for its own synthesis. The results of experiments with f markers (L) suggest that the affected target is not the nucleus, and this hypothesis is supported by conclusions concerning the hypothetical rate of "mutation". The use of various drugs, and other procedures, especially treatment with acridine orange, suggest that mitochondria are the most satisfactory hypothetical target for senescence. It is proposed that senescence in P. polyccephalum is due to the mutation of mitochondria, mutant mitochondria reproducing themselves at the same rate as normal mitochondria. The mutant mitochondria are envisaged as having similarities with the "petite" mitochondria of yeast. The "linear" (corrected linear, $x^n = (1-y)^t$) accumulation of mutant mitochondria results in progressive respiratory inadequacy. This development does not affect the plasmodium for some time but eventually the growth rate is decreased. Shortly after this, the plasmodium dies as a result of respiratory inadequacy.

Consideration of the Orgel Hypothesis

It should be stated that the observation that a particular senescence phenomenon is due predominantly to some cause other than any Orgel phenomenon does not illustrate that no Orgel phenomenon occurred, merely that another degeneration process occurred at such a rate that no Orgel phenomenon could be detected.

Concerning a theoretical consideration of the Orgel hypothesis; the exponential accumulation of errors in protein synthesis, it has been argued that the phenomenon is inevitable. (The occurrence of spontaneous errors in "information" molecules should lead inevitably to the exponential increase of errors in such molecules.) I consider that this proposition is not valid.

If it is assumed that protein synthesis continues without degradation then it appears that the Orgel hypothesis is logically inevitable. If however the occurrence of protein degradation is taken into account then it appears that if the rate of accumulation of "derivative" erroneous molecules (erroneous molecules produced because of the malfunctioning of already existing "spontaneous" erroneous molecules) is less than the rate of accumulation of "spontaneous" erroneous molecules during the degradation cycle, then the accumulation of errors is not exponential. On the contrary, the frequency of errors becomes stabilised at a level related to the frequency of spontaneous and derivative errors. It will be seen from a consideration of Figure 7 that the exponential accumulation of errors must

be assumed to show a very shallow slope (n , the rate of increase = $x1.04$) for the hypothesis to give an organism with a life expectancy of as much as 200 doublings. It is difficult to apply this deduction to multicellular organisms containing non-dividing cells because there is no parameter comparable to doubling time. The data from P.polycephalum indicates that if an Orgel phenomenon is occurring in this organism its rate of increase (n) is so low that the phenomenon is not detected by the procedures employed. The rate of increase (n) must therefore be less than $x1.04$. If the organism can perform protein synthesis with this degree of precision (n = less than $x1.04$) it is possible that the precision is such that the rate of occurrence of derivative errors is less than that of spontaneous errors in which case it appears that an Orgel phenomenon will not occur. If it is accepted that P.polycephalum can perform protein synthesis with sufficient precision to prevent the exponential accumulation of errors, it is possible that other organisms can do the same.

Possible Future Experiments

The experiments concerning senescence described in this Thesis obviously suggest further experiments, and it is hoped to develop the analysis further. Some of the experiments which need to be performed before the proposed hypothesis can be accepted without reservation are listed below.

- (1) It is necessary to understand the origin of the difference of life expectancy of plasmodia produced from repeats of the same amoebal cross. It has already been mentioned that it is possible that the amoebae may themselves develop senescence, cultures of amoebae having indefinite life expectancy because of selection between senescent and vigorous amoebae.
- (2) The effect of chloramphenicol on plasmodial senescence should be investigated. This substance selectively inhibits mitochondrial protein synthesis in Eukaryotes.
- (3) The cytochrome profiles of senescent and vigorous plasmodia should be obtained and compared. The respiratory activity of plasmodia and mitochondrial preparations should also be investigated.
- (4) Electronmicroscopy of senescent and vigorous plasmodia, with particular reference to the structure of mitochondria, should be performed.
- (5) Confirmation that the mitochondria are the primary target of senescence can be obtained most directly by microinjection into recipient plasmodia of the separated fractions of plasmodial homogenotes.
- (6) The direct assay of the fidelity of protein synthesis is desirable, both for proteins synthesised by the cytoplasmic protein synthesising system and for those produced within the mitochondrion. (The possibility of an intra-mitochondrial Orgel phenomenon being borne in mind.)

Relevance of the Analysis to the General Phenomenon of Senescence

Finally the relevance of the analysis of senescence in P.polycephalum to the study of senescence as a general phenomenon will be briefly considered. The plasmodia of myxomycetes have several advantages for the purpose of senescence research, but the main one is the ease with which one can prepare bulk homogeneous preparations of cells or cell fractions derived from plasmodia in a known state of senescence. This advantage should result in the senescence phenomena of P.polycephalum being eventually conclusively analysed. Such an analysis might result in worthwhile analogies being drawn with the senescence (particularly the cell death) of mammals and insects and perhaps also with the senescence phenomena reported for the filamentous fungi.

REFERENCES

- ADELBERG, E.A., MANDEL, M. AND CHEN, G.C. (1965) Optimal conditions for mutagenesis by NMG in E.coli K12. Biochem. Biophys. Res. comm., 18, 788.
- ALDRICH, H.C. (1967) The ultrastructure of meiosis in three species of Physarum. Mycologia, 59, 127.
- ALDRICH, H.C. (1968) The development of flagella in swarm cells of a myxomycete Physarum flavicomum. J. gen. Microbiol., 50, 217.
- ALEXANDER, P. (1967) The role of DNA lesions in the processes leading to ageing in mice. S.E.B. Symposia XXI
- ALEXOPOULOS, C.J. (1963) The Myxomycetes II. Botanical Review, Vol.29 No.1.
- ANDERSON, N. AND POLLOCK, B.M. (1952) A comparison between the cytoplasmic components in Physarum polycephalum and Chaos chaos. Compt. Rend. Lab.Carlberg. Ser.Chim., 28, 247.
- ANDREW, W. (1939) The Golgi apparatus in the nerve cells of the mouse from youth to senility. J. Comp. Neurol., 70, 413.
- BANGA, I. (1961) Abstract, 5th Internat. Congr. Biochem, Moscow, p.99.
- BARROWS, C.H., YIENGST, M.J. AND SHOCK, N.W. (1960) Senescence and the metabolism of various tissues of rats. J. Gerontol. 15, 130.

- BELLAMY, D. (1967) Hormonal Effects in Relation to Ageing in Mammals. S.E.B. Symposia, XXI.
- BONDAREFF, W. (1957) Genesis of intracellular pigment on the spinal ganglia of senile rats. An electron microscope study. J. Gerontol., 12, 364.
- BRANDZA, M. (1927) Sur la fusion des plasmodium des myxomycètes. Compt. Rend. Acad. Sci., Paris, 185, 1072.
- BRAUN, R., MITTERMAYER, C. AND RUSCH, H.P. (1965) Sequential temporal replication of DNA in Physarum polycephalum. Proc. Natl. Acad. Sci., U.S.A. 53, 924.
- BRAUN, R., MITTERMAYER, C. AND RUSCH, H.P. (1966) Ribonucleic acid synthesis in vivo in the synchronously dividing Physarum polycephalum. Biochim. Biophys. Acta, 114, 527.
- BREWER, E.N. AND RUSCH, H.P. (1966) DNA synthesis by isolated nuclei of Physarum polycephalum. Biochem. Biophys. Res. Comm., 21, 235.
- CARLILE, M.J. AND DEE, J. (1967) Plasmodial fusion and lethal interaction between strains in a myxomycete. Nature, Lond. 215, 832.
- CATEN, C.E. AND JINKS, J.L. (1966) Heterokaryosis: its significance in wild homothallic Ascomycetes and Fungi Imperfecti. Trans. Br. mycol. Soc., 49, 81.

- CLARK, A.M. AND RUBIN, M.A. (1961) The modification by X-rays of the life span of haploids and diploids of the wasp, *Habrobracon* species. Radiat. Res., 15, 244.
- CLARKE, J.M. AND MAYNARD SMITH, J. (1955) The genetics and cytology of *Drosophila subobscura*. XI Hybrid vigor and longevity. J. Genet., 53, 172.
- COHEN, A.L. AND SOBELS, J. (1952) Mineral requirements of some myxomycetes with possible bearing on their ecology. Georgia Academy of Science, X No.3.
- COHEN, A.L. (1959) An electron microscope study of flagellation in myxomycete swarm cells. Abstract, IX Internat. Bot. Congr., 2, 77.
- COLLINS, O.R. (1961) Heterothallism and homothallism in two myxomycetes. Amer. J. Bot., 48, 674.
- COLLINS, O.R. (1966) Plasmodial compatibility in heterothallic and homothallic isolates of *Didymium iridis*. Mycologia, 58, 362.
- COLLINS, O.R. AND CLARK, . . (1968) Genetics of plasmodial compatibility and heterokaryosis in *Didymium iridis*. Mycologia, 60, 90.
- COOPER, D., BANTHORPE, D.U. AND WILKIE, D. (1967) Modified ribosomes conferring resistance to cyclohexamide in mutants of *Saccharomyces cerevisiae*. J. Mol. Biol., 26, 347.
- CUMMINS, J.E. AND RUSCH, H.P. (1966) Limited DNA synthesis in the absence of protein synthesis in *Physarum polycephalum*. J. Cell Biol., 31, 577.

- CURTIS, H.J. (1967) Radiation and ageing. S.E.B. Symposia, XXI.
- CUSHING, J.E. (1967) Invertebrates, immunology and evolution. Fed. Proc., 26, 1666.
- DANIEL, J.W. AND BALDWIN, H.H. (1964) Methods of culture for plasmodia/Myxomycetes. Methods in Cell Physiology (D.M. Prescott, ed.) Vol.1, p.9. New York: Academic Press.
- DANIEL, J.W. (1966) Light induced synchronous sporulation of a myxomycete. In Cameron, I.L. and Padilla, G.M. eds. Cell Synchrony. New York: Academic Press.
- DAVIES, J., JONES, D.S. AND KHORANA, H.G. (1966) A further study of misreading of codons induced by streptomycin and neomycin using ribopolynucleotides containing two nucleotides in alternating sequence as templates. J. Mol. Biol., 18, 48.
- DAVIES, J.E. (1964) Studies on the ribosomes of streptomycin-sensitive and resistant strains of Escherichia coli. Proc. Natl. Acad. Sci., U.S. 51, 659.
- DEE, J. (1960a) A mating-type system in an acellular slime-mould. Nature (London), 185, 780.
- DEE, J. (1960b) Genetic analysis of a myxomycete. Ph.D Thesis (University of Glasgow).
- DEE, J. (1962) Recombination in a Myxomycete. Genet. Res., Camb. 3, 11.

- DEE, J. (1966a) Multiple alleles and other factors affecting plasmodium formation in the true slime-mould, Physarum polycephalum. J. Protozool., 13, 610.
- DEE, J. (1966b) Genetic analysis of actidione-resistant mutants in the Myxomycete, Physarum polycephalum, Schw. Genet. Res., Camb. 8, 101.
- DEMPSEY, E.W. (1956) Mitochondrial changes in different physiological states. CIBA Colloquia on Ageing, 2.
- ELLIOT, E.W. (1949) The swarm cells of Myxomycetes. Mycologia, 41, 141.
- ESSNER, E. AND NOVIKOFF, A. (1960) Human hepatocellular pigments and lysosomes. J. Ultrastructure Research, 3, 374.
- FAILLA, G. (1957) Radiology, 69, 23.
- FALZONE, J.A., BARROWS, C.H. AND SHOCK, N.W. (1959) Age and ploidy of rat liver nuclei as measured by volume and DNA content. J. Gerontol., 14, 2.
- FLETCHER, M.J. AND SANADI, D.R. (1961) Turnover of rat liver mitochondria. Biochim. Biophys. Acta, 51, 356.
- FRANKS, L.M. AND CHESTERMAN, F.C. (1964) Irreversible cellular changes in old mice. Nature, (London) 202, 821.
- FRANZBLAU, C., SINEX, M., FARIS, B. AND LAMPIDIS, R. (1965) Identification of a new crosslinking amino acid in elastin. Biochem. Biophys. Res. Comm., 21, 575.

- GEDIGK, P. AND BONTKE, E. (1956) Über dem Nachweis von hydro-
lytischen enzyemen in Lipopigmenten. Z. Zellforsch., 44, 495.
- GEHENIO, P.M. AND LUYET, B.J. (1950) Complete development of a
mycetozoon from a single spore, a single myxamoeba. Biodyn.
7, 11.
- GIBAR, A. AND GRANICK, S. (1964) Plastids and mitochondria:
Inheritable systems. Science, 145, 890.
- GOODMAN, E.M. (1967) A cytological and physiological study of
synchronous mitosis in the slime-mould Physarum polycephalum.
Ph.D. dissertation, State University of New York, Buffalo.
- GOODWIN, D. (1961) Morphogenesis of the sporagium of Comatricha.
Amer. J. Bot., 48, 148.
- GRAY, W.D. (1938) The effect of light on the fruiting of Myxomycetes.
Amer. J. Bot., 25, 511.
- GRAY, W.D. (1945) The existence of physiological strains of
Physarum polycephalum. Amer. J. Bot., 32, 157.
- GUTTES, E., GUTTES, S. AND RUSCH, H.P. (1961) Morphological observa-
tions on growth and differentiation of Physarum polycephalum
grown in pure culture. Develop. Biol., 3, 588.
- GUTTES, E. AND GUTTES, S. (1962) Cell growth and mitosis in Physarum
polycephalum. Exp. Cell Res., 26, 205.
- GUTTES, E. AND GUTTES, S. (1964) Mitotic synchrony in the plasmodia
of Physarum polycephalum and mitotic synchronisation by
coalescence of microplasmodia. In Prescott, D.M. ed. Methods

- in Cell Physiology, Vol.1, p.43. New York: Academic Press.
- GUTTES, E. AND GUTTES, S. (1967) Transplantation of nuclei and mitochondria of Physarum polycephalum by plasmodia coalescence. Experientia, 23, 713.
- HALL, D.A., KEECH, M.K., REED, R., SAXLE, H., TUNBRIDGE, R.E. AND WOOD, M.J. (1955) J. Gerontol., 10, 388.
- HALL, D.A. (1956) Experientia, 4, 19.
- HALL, D.A. (1957) Gerontologia, 1, 347.
- HALL, D.A. AND SARD, H. (1961) Studies of human and tunicate cellulose and of their relations to reticulin. Proc. Roy. Soc., B, 155, 202.
- HALL, D.A. (1967) The ageing of connective tissue. S.E.B. Symposia, XXI.
- HARDESTY, B.A. AND MITCHELL, H.K. (1963) The accumulation of free fatty acids in poky, a maternally inherited mutant of Neurospora crassa. Arch. Biochem. Biophys., 100, 330.
- HARRISON, B.J. AND HOLLIDAY, R. (1967) Senescence and the fidelity of protein synthesis in Drosophila. Nature, 213, 990.
- HASKINS, F.A., TISSIERES, A., MITCHELL, H.K. AND MITCHELL, M.B. Cytochromes and the succinic acid oxidase system of poky strains of Neurospora. J. Biol. Chem., 220, 819.
- HAYFLICK, L. (1965) The limited in vitro lifetime of human diploid cell strains. Expl. Cell Res., 37, 614.
- HENDLEY, D.D., STREHLER, B.L. (1965) Enzymic activities of lipo-fusion age pigments: comparative histochemical and biochemical

- studies. Biochim. Biophys. Acta, 99, 406.
- HENSHAW, P.S., RILEY, E.R. AND STAPLETON, G.E. (1947) Radiology,
49, 349.
- HESS, A. (1955) The fine structure of young and old spinal ganglia.
Anat. Record, 123, 399.
- HIGNETT, R.C. (1966) Interference of 5-fluorouracil in the bio-synthesis
of ribosomes in Staphylococcus aureus (strain Duncan). Biochim.
Biophys. Acta, 114, 559.
- HOLLIDAY, R. (1969) Errors in protein synthesis and clonal senescence
in Fungi. Nature, 221, 1224.
- HOLTER, H. AND POLLOCK, B.M. (1952) Distribution of some enzymes in
the cytoplasm of the myxomycete Physarum polycephalum. Compt.
Rend. Trav. Lab. Carlsberg, Ser. Chim. 28, 221.
- HOUCK, J.C., DE HESSE, C. AND JACOB, R. (1967) The effect of ageing
upon collagen catabolism. S.E.B. Symposia, XXI.
- JENNINGS, H.S. (1944) Paramecium bursaria: Life history: III.
Repeated conjugations in the same stock at different ages, with
and without inbreeding, in relation to mortality at conjugation.
J. Exp. Zool., 96, 243.
- JINKS, J.L. (1959) Lethal suppressive cytoplasms in aged clones of
Aspergillus glaucus. J. gen. Microbiol., 21, 397.

- JUMP, J.A. (1954) Studies on sclerotisation in Physarum polycephalum.
Amer. J. Bot., 41, 561.
- KAMIYA, N. (1959) Protoplasmic streaming. Protoplasmatologia, 8 (3a).
- KELLERMAN, G.M., BIGGS, D.R., AND LINNANE, A.W. (1969) Biogenesis of mitochondria. XI. A comparison of the effects of growth limiting oxygen tension, intercalating agents and antibiotics in the obligate aerobe Candida parapsilosis. J. Cell Biol., 42, 378.
- KERR, N.S. (1960) Flagella formation by myxamoebae of the true slime-mould Didymium nigripes. J. Protozool., 7, 103.
- KERR, N.S. (1961) A study of plasmodium formation by the true slime-mould Didymium nigripes. Exp. Cell Res., 23, 603.
- KERR, N.S. AND WAXLAX, J.N. (1969) A yellow variant of the Eumycetozoon Didymium nigripes which exhibits ageing. J. Exp. Zool., 168, 351.
- KIMBALL, R.F. AND GAITHER, N. (1954) Lack of an effect of a high dose of X rays on ageing in Paramecium aurelia, variety 1. Genetics, 39, 977.
- KIMBALL, R.F. (1964) In Hutner, ed. Biochemistry and Physiology of Protozoa, III. New York: Academic Press.
- KOEVENIG, J.L. (1964) Studies on the life cycle of Physarum gyrosum and other myxomycetes. Mycologia, 56, 170.

- KUDO, R.R. (1954) In Thomas, ed. Protozoology, 4th edition, p.427, Springfield, Ill.
- LANSING, A.I. (1947) A transmissible, cumulative, and reversible factor in ageing. J. Gerontol., 2, 228.
- LARK, K.G. AND LARK, C. (1966) Regulation of chromosome replication in E.coli: A comparison of the effects of phemethyl alcohol treatment with those of amino acid starvation. J. Mol. Biol., 20, 9.
- LEWIS, D. (1964) A protein dimer hypothesis of incompatibility. In Genetics Today, vol. III (ed. Geerts, S.J.) Proc.XI Internat. Congr. Genetics. Pergamon Press.
- LOEWY, A.G. (1952) An actomyosin-like substance from the plasmodium of a myxomycete. J. Cell. Comp. Physiol., 35, 151.
- LUNSFORD, W.R., McCAY, C.M., LUPIEN, P.J., POPE, F.E., AND SPERLING, G. Gerontologia, 7, 1.
- LYNCH, R.S. AND SMITH, H.B. (1934) A study of the effects of modifications of the culture medium upon length of life and fecundity in a rotifer, Proales sordida, with special relation to their heritability. Biol. Bull., Woods Hole, 60, 30.
- LYON, M.F. (1968) Chromosomal and subchromosomal inactivation. Ann. Rev. Genet., 31.
- MARCO^U~~LLI~~, D. (1961) Ann. Sci. Nat. Bot., 12, 653.

- MARTIN, G.W. (1961) The systematic position of the myxomycetes.
Mycologia, 52, 119.
- MAYNARD SMITH, J. (1959) A theory of ageing. Nature, London, 184,
956.
- MAYNARD SMITH, J. (1962) The causes of ageing. Proc. Roy. Soc, B,
157, 115.
- MEDAWAR, P.B. (1951) An unsolved problem of biology. Lewis, London.
- MEDVEDEV, Z.H.A. (1967) Molecular Aspects of Ageing. S.E.B. Symposia,
XXI.
- METCHNIKOFF, E. (1915) Ann. Inst. Pasteur, Paris.
- MILCH, R.A. AND MURRAY, R.A. (1962) Proc. Soc. Exp. Biol. Med., 111,
551.
- MINOT, C.S. (1907) The problem of age, growth and death. Popular
Sci., Monthly, 71.
- MITCHELL, H.K. AND MITCHELL, M.B. (1952) The case of "maternal"
inheritance in Neurospora crassa. Proc. Natl. Acad. Sci., U.S.
38, 442.
- MITTERMAYER, C. AND RUSCH, H.P. (1964) RNA synthesis in the mitotic
cycle of Physarum polycephalum. Biochim. Biophys. Acta, 91, 399.
- MOHBERG, J. AND RUSCH, H.P. (1964) Isolation of nuclei and histones
from the plasmodium of Physarum polycephalum. J. Coll. Biol.,
23, 61.

- MOUNOLOU, J.C. (1967) The control of nuclear activity. New Jersey: Prentice-Hall.
- MUGGLETON, A. AND DANIELLI, J.F. (1968) Inheritance of the "life-spanning" phenomenon in Amoeba proteus. Exp. Cell Res., 49, 116.
- MUHLBOCK, O. (1957) CIBA Colloquia on Ageing, 3, 115.
- NAKAJEMA, H. AND HATANO, S. (1962) Acetylcholine esterase in the plasmodium of the myxomycete Physarum polycephalum. J. Cell. Comp. Physiol., 59, 259.
- NASS, M.M.K., NASS, S. AND AFZELIUS, B.A. (1965) The general occurrence of mitochondrial DNA. Exp. Cell. Res., 37, 516.
- NYGAARD, O.F. AND RUSCH, H.P. (1960) Nucleic acid metabolism in a slime mould with synchronous mitosis. Biochim. Biophys. Acta, 38, 298.
- OLIVE, L. (1961) Echinostelium minutum. Mycologia, 52, 159.
- ORGEL, L.E. (1963) The maintenance of the accuracy of protein synthesis and its relevance to ageing. Proc. Natl. Acad. Sci., U.S. 49, 517.
- PITTENGER, T.H. (1956) Synergism of two cytoplasmically inherited mutants in Neurospora crassa. Proc. Natl. Acad. Sci., Wash. 42, 747.
- PLAGEMANN, P.G.W. (1968) Phenethyl alcohol: reversible inhibition of synthesis of macromolecules and disaggregation of polysomes in rat hepatoma cells. Biochim. Biophys. Acta, 155, 202.

- POULTER, R.T.M. AND DEE, J. (1968) Segregation of factors controlling fusion between plasmodia of the true slime mould Physarum polycephalum. Genet. Res., Camb., 12, 71.
- PRINTZ, P.B. AND GROSS, S.R. (1967) An apparent relationship between mistranslation and an altered leucyl-tRNA synthetase in a conditional lethal mutant of Neurospora crassa. Genetics, 55, 451.
- RAFSKY, H.A., NEWMAN, B. AND HORONICK, A. (1952) Age differences in respiration of guinea pig tissues. J. Gerontol., 7, 38.
- RIZET, G. (1953) Sur l'impossibilité d'obtenir la multiplication végétative ininterrompue et illimitée de l'Ascomycete Podospora anserina. C.R. Acad. Sci., 237, 838.
- ROBINSON, (1948) Structure and biogenesis of emetine. Nature, London, 162, 524.
- ROCKSTEIN, M. (1950) The relation of cholinesterase activity to change in cell number with age in the brain of the adult worker honey bee. J. Cell. Comp. Physiol., 35, 11.
- ROCKSTEIN, M. (1967) Cellular age changes in insects. S.E.B. Symposia, XXI.
- ROODYN, D.B. AND WILKIE, D. (1968) The Biogenesis of Mitochondria. London: Methuen.
- ROSS, I.K. (1957) Syngamy and plasmodium formation in the Myxogastres. Amer. J. Bot., 44, 843.

- ROSS, I.K. (1961) Further studies on meiosis in the myxomycetes.
Amer. J. Bot., 48, 244.
- ROSS, I.K. (1962) Sporangial development in Lamproderma arcyronema
Mycologia, 52, 621.
- ROSS, I.K. (1966) Chromosome numbers in pure and gross cultures of
myxomycetes. Amer. J. Bot., 53, 712.
- RUSSELL, L.B. AND MAJOR, M.H. (1957) Radiation induced presumed
somatic mutations in the house mouse. Genetics, 42, 161.
- SACHSENMAIER, W. AND IVES, D.H. (1965) Periodische "Änderungen der
Thymidinkinase Aktivität in Synchronen Mitosecyclus von Physarum
polycephalum. Biochem. Z., 343, 399.
- SARKISSIAN, I.V. AND McDANIEL, R.G. (1967) Mitochondrial polymorphism
in Maize. De novo origin of hybrid specific mitochondria. Proc.
Natl. Acad. Sci., U.S. 57, 1262.
- SAUER, H. AND BABCOCK, K. Unpublished data quoted in Rusch, H.P.
Some biochemical events in the life-cycle of Physarum polycephalum.
In Prescott, D.M. ed. Advances in Cell Biology, vol.I
Appleton-Century-Crofts.
- S.E.B. Symposia XXI (1967) Aspects of the Biology of Ageing.
- SHENG, T.C. (1951) The gene that causes natural death in Neurospora
crassa, Genetics, 36, 199.
- SHOCK, N.W. (1964) Age with a Future. Copenhagen: Munksgaard.

- SIEGEL, R.W. (1967) Genetics of ageing and the life cycle in ciliates. S.E.B. Symposia XXI.
- SINEX, F.M. (1960) The Biology of Ageing, p.268. Publ. No. 6 Amer. Inst. Biol. Sci., Washington.
- SKUPIENSKI, F.X. (1937) Sur l'existence des races physiologiques chez les myxomycètes. Ann. Protistologie, 4, 121.
- SMITH, D.G., MARCHANT, R., AND MAROUDAS, N.G. (1969) A comparative study of the mitochondrial structure of petite strains of Saccharomyces cerevisiae. J. gen. Microbiol., 56, 47.
- SONNEBORN, T.M. AND ROFOLKO, M. (1957) Ageing in the P.aurelia multimicronucleatum complex. J. Protozool., 4, 21.
- SONNEBORN, T.M. AND SCHNELLER, M. (1960) The Biology of Ageing. Publ. No.6, Amer. Inst. Biol. Sci., Washington.
- STREHLER, B.L. (1962) Time, Cells and Ageing, p.11. New York & London: Academic Press.
- STREHLER, B.L. (1967) The nature of cellular age changes. S.E.B. Symposia XXI.
- STÜBEL, H. (1911) Die Fluoreszenz tierischer Gewebe in ultravioletten Licht. Pflügers Arch. Ges. Physiol., 142.
- SULKIN, N.M. AND SRIVANIJ, P. (1960) The experimental production of senile pigments in the nerve cells of young rats. J. Gerontol., 15, 2.

- SZILARD, L. (1959a) A theory of ageing. Nature, London, 184, 956.
- SZILARD, L. (1959b) On the nature of the ageing process. Proc. Natl. Acad. Sci., U.S., 45, 30.
- THOMAS, J., ELSDEN, D.F. AND PARTRIDGE, S.M. (1963) Constitution of the cross-linkages in elastin. Nature, London, 197, 1297.
- TOWEND, C. (1939) Races physiologiques chez les myxomycètes Didymium squamulosum. Compt. Rend. Soc. Biol., 131, 355.
- TUPPY, H., SWETLY, P. AND WOLFF, I. (1968) Structural protein of wild type (P^+) and respiration-deficient (P^-) yeast mitochondria: A comparative study using electrophoretic and immunological methods. Europ. J. Biochem., 5, 339.
- ULRICH, R. (1943) Les constituents de la membrane chez les champignons. Rev. Mycol. (Paris) Mém. Hors-Sér. 3, 1.
- VEIS, A. AND ANESEY, R. (1963) Modes of intermolecular cross-linking in mature insoluble collagen. J. Biol. Chem., 240, 3899.
- WAGNER, R.P. (1969) Genetics and phenogenetics of mitochondria. Science, 163, 1026.
- WALFORD, R.L. (1962) J. Gerontol., 17, 281.
- WARD, J.M. (1956) Comparative cytochrome oxidase and atypical ascorbic acid oxidase activities in the plasmodia and spores of Physarum polycephalum. Plant Physiol., 31 (supl.) XXIX (Abs.)

- WARD, J.M. (1958) Shift of oxidases with morphogenesis in the slime-mould, Physarum polycephalum. Science, 127, 596.
- WEISBLUM, B. AND DAVIES, J. (1968) Antibiotic inhibitors of the bacterial ribosome. Bacteriol. Rev., 32, 493.
- WEISMANN, A. (1891) "Essays upon Heredity" Oxford University Press (Clarendon).
- WILKIE, D. AND MAROUDAS, N.G. (1969) Induction of cytoplasmic respiratory deficiency in yeast by phenethylalcohol. Genet. Res., Camb., 13, 107.
- WILSON, C.M. AND ROSS, I.K. (1955) Meiosis in the myxomycetes. Amer. J. Bot., 42, 743.
- WOHLFORTH-BOTTERMAN, K.E. (1964) Differentiations of the ground cytoplasm and the generation of the motive force for amoeboid movement. In Allen, R.D. & Kamiya, N. ed. Primitive Motile Systems in Cell Biology, p.79. New York: Academic Press.
- WOODWARD, D.O. AND MUNKRES, K.D. (1966) Alterations of a maternally inherited mitochondrial structural protein in respiratory-deficient strains of Neurospora. Proc.Natl. Acad. Sci., U.S., 55, 872.
- YAMANAKA, T., NAKAJIMA, H. AND OKUNUKI, K. (1962) Purification and some properties of a c-type cytochrome from a slime-mould Physarum polycephalum. Biochim.Biophys. Acta, 63, 510.

YERGANIAN, G. AND GAGNON, H. (1958) Chromosomes of an adenocarcinoma
in the Chinese hamster. Proc. Amer. Assoc. Cancer Res., 2, 358.
ZUREIFACH, BLACK, AND SHOR (1951) Proc. Soc. Expt. Biol. Med., 76
446.

This is to certify that the thesis I have submitted in fulfilment of the requirements governing candidates for the degree of Doctor of Philosophy in the University of Leicester, entitled "Senescence in the Myxomycete Physarum polycephalum" is the result of work done mainly by me during the period of registration for the above degree.



(R.T.M. POULTER)