ANALYSIS OF THE AMOEBAL-PLASMODIAL TRANSITION IN *PHYSARUM POLYCEPHALUM*

by

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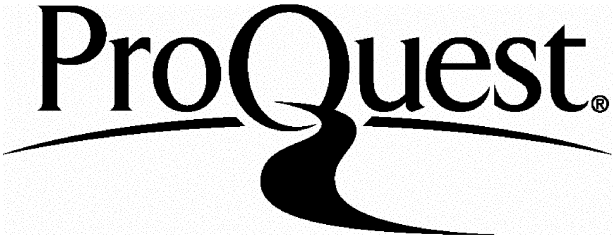

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GENERAL INTRODUCTION

CHAPTER I GENERAL INTRODUCTION

1.1 THE LIFE CYCLE OF PHYSARUM POLYCEPHALUM

Physarum polycephalum is a Myxomycete. The Myxomycetes form a discrete group distinguished by the inclusion of a true multinucleate plasmodium in their life cycle. They are frequently referred to as true slime moulds or acellular slime moulds, to distinguish them from the Acrasiales or cellular slime moulds. For general reviews of Myxomycete biology (including P. polycephalum) see Gray and Alexopoulos (1968) and Olive (1975).

The following account briefly summarises the life cycle of P. polycephalum. Further information may be obtained from Hutterman (1973). A complete account of the life cycle was first described by Howard (1931). The plasmodium is a macroscopic, yellow, motile, syncytial mass of protoplasm. It can be cultured axenically either in surface culture or as microplasmodia in shaken liquid culture. In surface culture vigorous cytoplasmic streaming occurs within thickened veins, which form a constantly changing network pattern. Streaming within veins reverses periodically. The nuclear membrane is retained during mitosis, which is synchronous throughout the plasmodium and is not followed by cell division. Genetically identical plasmodia coalesce when in contact, but non-identical plasmodia may or may not fuse. This process is under genetic control. In the absence of light, depletion of certain nutrients induces the conversion of plasmodia into sclerotia. These resistant resting structures are composed of many microscopic

spherules, each of which contains only a few nuclei. Sclerotia germinate to yield plasmodia when placed in suitably moist conditions. Nutrient depletion accompanied by a period of illumination induces sporulation. The entire plasmodium is converted into a number of characteristically lobed sporangia, each of which bears many brown spores about 10 μm in diameter. When spores are placed in moist conditions they germinate to yield uninucleate, colourless amoebae. These may be cultured axenically, but for most purposes they are grown with a bacterial food source on an agar surface. Mitosis is immediately followed by cell division. In adverse conditions, such as nutrient depletion, low temperature or drying, amoebae are reversibly converted into resting cysts. High moisture levels may stimulate amoebae to become flagellated. Drying reverses the process.

The life cycle is completed by the amoebal-plasmodial transition; that is, the formation of a multinucleate plasmodium from uninucleate amoebae. It is the analysis of the amoebal-plasmodial transition which forms the subject of this thesis.

The life cycle of P.polycephalum is illustrated in Figures 1 and 2.

1.2 PLASMODIUM FORMATION AND NUCLEAR CYCLES IN THE MYXOMYCETES

The life cycle of P. polycephalum is typical of the Myxomycetes. Thus, in reviewing the literature relevant to the amoebal-plasmodial transition, results of some work on Myxomycetes other than P. polycephalum have been included. In many cases there is good agreement between results on several species.

Reports of plasmodium formation in the Myxomycetes are generally consistent with one or more of the following types of behaviour (there is

Figure 1. The life cycle of *Physarum polycephalum*.

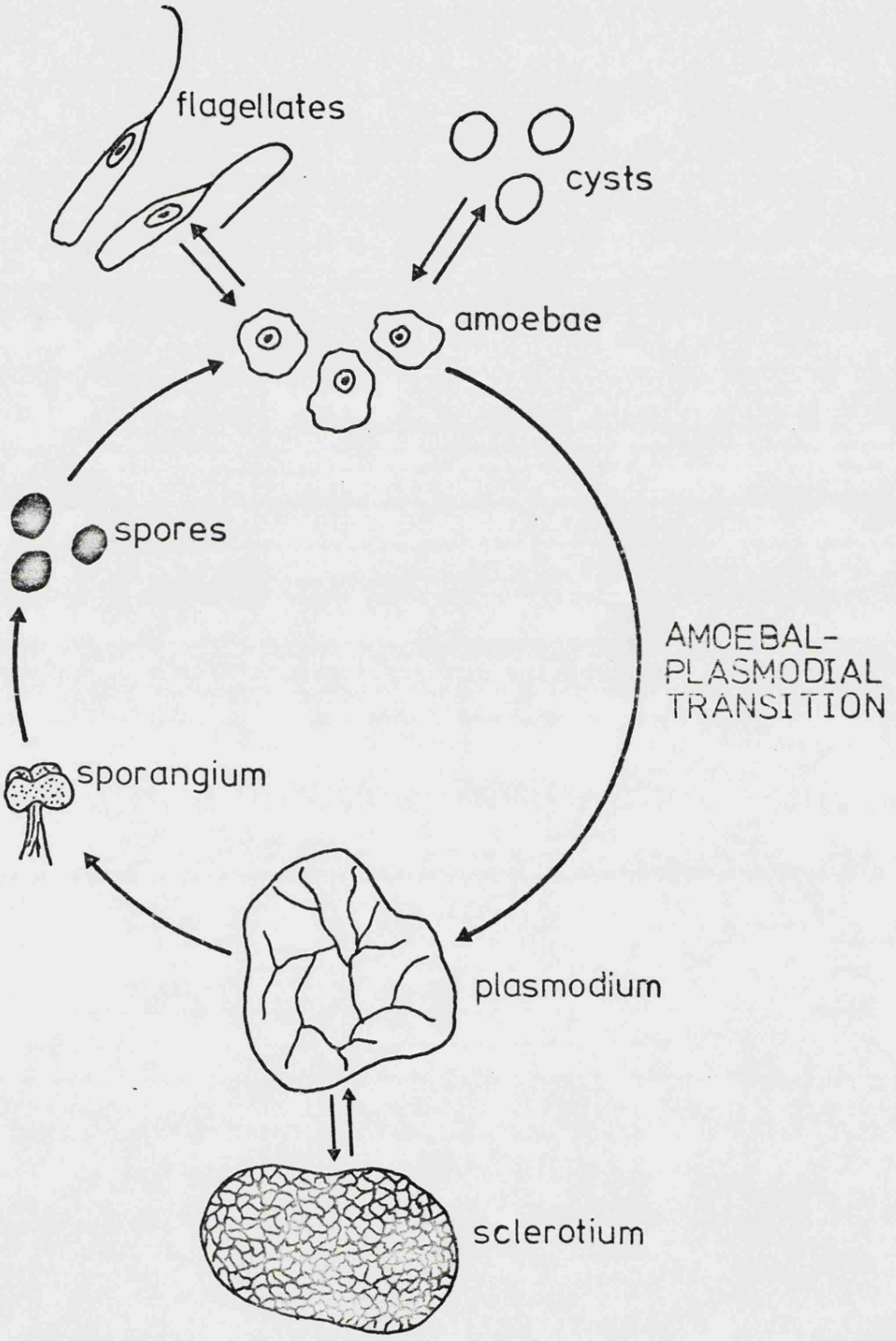

flagellates
cysts
amoebae
spores
AMOEBAL-
PLASMODIAL
TRANSITION
sporangium
plasmodium
sclerotium

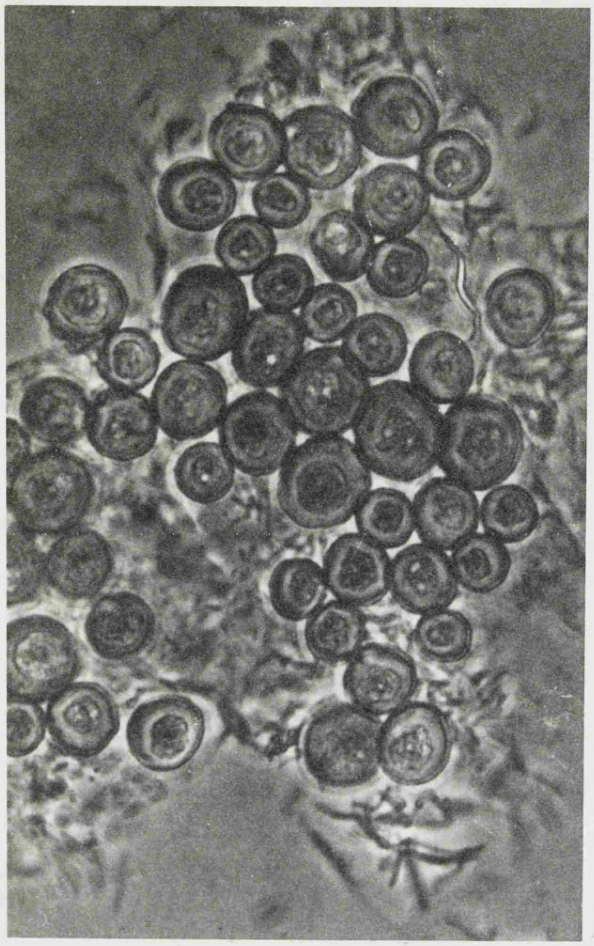
Figure 2. Stages in the life cycle of Physarum polycephalum.

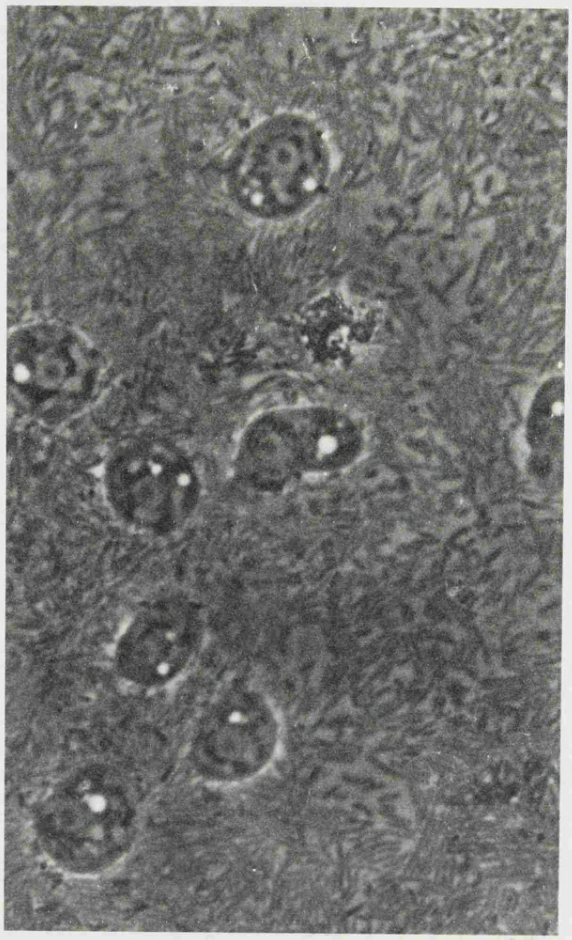
Top left: Spores. Among the spores can be seen sporangial fragments and bacteria. x 1000

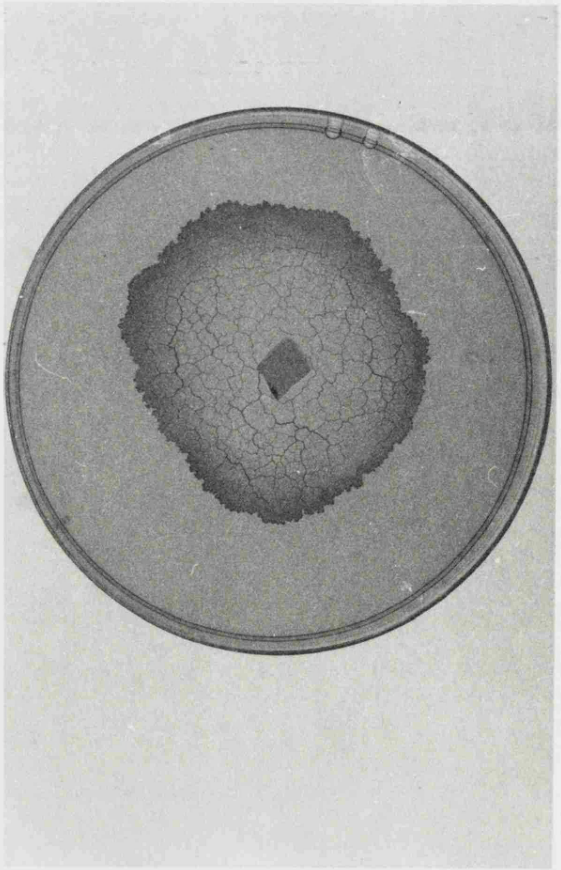
Top right: Amoebae among a population of E.coli. Nuclei can be seen as light circles containing darker areas (nucleoli). Contractile vacuoles appear white. x 1000

Bottom right: Large plasmodium in which the characteristic network pattern of veins can be seen. x 0.75

Bottom left: Three sporangia. x 50







some confusion in Myxomycete literature over the use of these terms; the following are the definitions used in this thesis):

Heterothallism: two genetically different haploid amoebae fuse to form a cell which differentiates to become a plasmodium. A large multinucleate plasmodium is formed by repeated nuclear divisions (without cell divisions) and/or fusions of small plasmodia. Nuclear fusion occurs, either at the time of amoebal fusion or later, and is followed by meiosis. Amoebal strains able to form plasmodia when mixed with one another are said to express different mating types; mixtures of strains expressing the same heterothallic mating type do not form plasmodia as described above.

Homothallism: as for heterothallism, except that pairs of genetically identical amoebae give rise to plasmodia.

Coalescence: plasmodia develop from fusion of two or more amoebae, but no nuclear fusion occurs.

Apogamy: plasmodia develop from amoebae without nuclear fusion or fusion of amoebae.

The following three sections review work on heterothallic plasmodium formation, clonal plasmodium formation in isolates also showing heterothallism, and clonal plasmodium formation in isolates within which there is no evidence of heterothallism.

1.2a Heterothallic plasmodium formation

There are many reports of the isolation of single Myxomycete spores by micromanipulation, followed by examination of the resulting amoebal cultures for plasmodium formation. In some isolates such cultures almost always failed to yield plasmodia when cultured alone, but

could be assigned to one or other of two heterothallic mating types by their ability to form plasmodia when mixed with certain other cultures. Apart from P. polycephalum, species for which such evidence of heterothallism exists include Didymium difforme (Skupienski, 1926, 1927, 1928), Didymium nigripes (von Stosch, 1935), Didymium iridis (Collins, 1961), Physarum pusillum (Collins, 1962), Comatricha laxa (Wollman, 1966), Physarum flavicomum (Henney, 1967) and Physarum rigidum (Henney and Henney, 1968).

Early observations on living material by de Bary (1854), Cienkowski (1863a,b) and Skupienski (1926, 1927) indicated that plasmodium formation involved the fusion of two or more amoebae. However, it is now generally accepted that plasmodium formation in heterothallic strains involves cell and nuclear fusion of pairs of amoebae to give diploid zygotes (Jahn, 1911; Ross, 1957; 1967a,c; Koevenig, 1964). There is little evidence to indicate whether fusion of amoebae of like mating type is possible, but Ross et al. (1973) reported that amoebal fusions did not occur within clones of D. iridis, under conditions in which fusions were detectable in mixed mating type cultures.

The ploidy of amoebae and plasmodia has been compared in a number of heterothallic Myxomycetes. Ross (1966) reported that plasmodia of P. flavicomum had twice the number of chromosomes per nucleus as amoebae. Therrien (1966a,b) carried out microdensitometry on Feulgen stained nuclei from amoebae and plasmodia of D. iridis, P. pusillum and P. flavicomum. He concluded that in each case plasmodia were diploid, with a 4C nuclear DNA content, while amoebae were haploid (2C).

There is some controversy over the timing of meiosis in different Myxomycete species, but there is general agreement that it is associated with sporulation (Ross, 1967b) and probably occurs typically

inside the spore about one day after spore cleavage (Aldrich and Mims, 1970; Aldrich and Carroll, 1971).

Genetic analysis in heterothallic strains has demonstrated the existence of multiple alleles at a single locus in D. iridis (Collins, 1961, 1963; Collins and Ling, 1964) and in P. pusillum (Collins, 1962; Collins et al., 1964). Mukherjee and Zabka (1964) presented inconclusive evidence for a two locus mating type system in D. iridis. Genetic proof of syngamy and meiosis in D. iridis was provided by Collins (1966) when he demonstrated recombination of genetic markers in a cross.

Early studies (Dee, 1960, 1966) on the genetics of plasmodium formation in P. polycephalum demonstrated the existence of a heterothallic mating type system; plasmodia normally arose only when amoebae from clones carrying different alleles at the mating type (mt) locus were mixed. Evidence on recombination of genetic markers (Dee, 1962) was consistent with fusion only of pairs of nuclei carrying different mating type alleles, followed by meiosis. This agreed with the cytological evidence of Ross (1957), who had observed plasmodium formation by cell and nuclear fusion of pairs of amoebae. Measurements of nuclear DNA content and chromosome number indicated that mixtures of haploid amoebal strains gave rise to diploid plasmodia (Mohberg and Rusch, 1971; Mohberg et al., 1973). Synaptonemal complexes, structures characteristically restricted to the nuclei of meiotic tissues (Moses, 1964), were observed in spores about 24 hours after spore cleavage (Aldrich, 1967; Arescaldino, 1971), indicating that meiosis occurred at this time.

Although cell fusion is a prerequisite for nuclear fusion in the formation of crossed plasmodia, no direct evidence has been presented to indicate whether fusion also occurs between P. polycephalum amoebae

carrying the same heterothallic mating type allele.

1.2b Clonal plasmodium formation in heterothallic isolates

Collins (1961) plated spores of D. iridis and isolated single amoebae by micromanipulation. Some amoebal cultures derived from these single cells (and thus presumably clones) gave rise to plasmodia. Single amoeba cultures derived from spores of these clonal plasmodia always segregated for two heterothallic mating types.

Dee (1966) crossed heterothallic strains of P. polycephalum amoebae, allowed the resulting plasmodia to sporulate and plated the spores. Each amoebal colony which arose from the spores was almost certainly derived from a single spore, yet some colonies gave rise to plasmodia, and progeny of these plasmodia segregated 1 : 1 for the heterothallic mating type alleles involved in the original crosses. Dee suggested that amoebae of both mating types could arise from a single spore. Plasmodia would then arise by mating within the mixed colony.

In the course of other work on P. polycephalum, Haugli (1971) crossed amoebae of a mt_2 strain with a mt_1 strain which was subsequently (Mohberg et al., 1973) shown to be polyploid. The resulting plasmodium had three times as much DNA per nucleus as normal crossed plasmodia. Forty out of a total of 94 amoebal progeny clones analysed formed plasmodia. The remaining clones were classified for their mating type alleles: 48 were mt_1 and 6 mt_2. Haugli suggested that the clones which gave plasmodia might carry the mt_2 allele, since the ratio mt_1 : mt_2 would then not be significantly different from 1 : 1.

During the course of the work described in this thesis, Adler and Holt (1975) presented further evidence on clonal plasmodium formation in P. polycephalum. They analysed a number of crosses of heterothallic

strains and recovered some progeny amoebal clones which gave rise to plasmodia. These amoebae had an apparently diploid nuclear DNA content and plasmodia formed clonally from them were also apparently diploid. Analysis of the clonally-formed plasmodia showed that, in each case, some progeny retained the ability to form plasmodia within clones and the remainder segregated for the two heterothallic mating type alleles in the cross. Adler and Holt tentatively concluded that plasmodium formation was promoted by heterozygosity at the mating type locus. Since many of the heterothallic strains used in the crosses contained more than the haploid nuclear DNA content (due to "aging"; Adler and Holt, 1974a), such heterozygous amoebae might have arisen through meiosis in polyploid nuclei. If clonal plasmodium formation is promoted by heterozygosity at the mating type locus, then it is possible that the plasmodia reported by Collins, Dee and Haugli (described above) were also derived from clones of heterozygous amoebae. Adler and Holt assumed that the diploid amoebae formed plasmodia by apogamy, though they pointed out that coalescence was not excluded.

The above accounts of clonal plasmodium formation described cases where each culture gave rise to many plasmodia. Other reports of clonal plasmodium formation in heterothallic strains refer to rare events, where only one or two plasmodia arose from a large amoebal population.

Collins (1965) analysed progeny of a plasmodium which arose in a clone of D. iridis of known mating type. The progeny segregated 1 : 1 for the original mating type and a previously unknown mating type. Collins suggested that the new mating type had arisen by mutation of the original allele, and that cells of the two mating types then crossed normally. There is no evidence of this sort for mutation at the mating

type locus in P. polycephalum.

Collins and Ling (1968), also working with D. iridis, mixed strains which carried the same mating type allele but differed at other loci. They analysed progeny of two plasmodia which arose in such "common-mating type crosses" and found only parental combinations of markers. They concluded that the plasmodia had arisen through apogamy. However, since one of the plasmodia gave rise to progeny of both the amoebal parental types, the evidence was also consistent with coalescence. Collins and Ling suggested that clonal plasmodium formation in D. iridis was probably due to the "production of occasional aneuploids (i.e. hyperhaploids)", but they also suggested the possibility of cytoplasmic inheritance.

Yemma and Therrien (1972) investigated nuclear DNA contents of amoebae and clonally formed plasmodia of D. iridis, using microdensitometry of Feulgen stained preparations. They concluded that amoebae and plasmodia had the same nuclear DNA content, which was half that of crossed plasmodia. However, Yemma and Therrien also investigated the nuclear DNA content of sporangial nuclei of the selfed plasmodia and found this to be the same as in the nuclei of crossed plasmodia. They concluded that the selfed plasmodia were diploid, but in an extended G1 phase; haploid G2 amoebal nuclei and diploid G1 plasmodial nuclei contained the 2C amount of DNA while diploid G2 sporangial nuclei contained the 4C amount. If clonally formed plasmodia in D. iridis are diploid, they could arise homothallically by cell and nuclear fusion, or by nuclear fusion without cell fusion, or by spontaneous diploidisation.

Adler and Holt (1975), working with P. polycephalum, also showed that clones of apparently haploid amoebae carrying a single heterothallic

mating type allele were occasionally able to give rise to a plasmodium. They measured nuclear DNA contents of plasmodia known to be in G2 phase and showed that they were haploid rather than diploid. These plasmodia were formed very rarely, from large cultures which had been incubated for prolonged periods. Only one or two plasmodia arose from any culture, and most cultures showed none. In crosses, or clonal plasmodial formation by amoebae heterozygous at the mating type locus, many plasmodia arose in every culture. Plasmodia arising from heterothallic clones usually yielded amoebal progeny of the original mating type.

Yemma et al. (1974) suggested that clonal plasmodium formation ("selfing") in D. iridis was due to a cytoplasmic factor which they assumed induced cell and nuclear fusion between cells of the same mating type. However, their evidence was far from convincing; although their conclusions were apparently based on a "heterokaryon test", examination of their results does not reveal the existence of any heterokaryons. They merely showed that the capacity to self was transmitted from one mating type to another in the meiotic segregants of heterozygous plasmodia. This is clearly consistent with recombination of nuclear genes.

The selfing strains used in the crosses initially formed plasmodia in 10-20% of clonal cultures. Progeny of the crosses showed selfing frequencies of 1-7%. Progeny clones carrying the mating type allele which was previously not associated with the ability to self were allowed to go through two generations of selfing. It was found that the selfing frequency had now risen to 100%, and Yemma et al. proposed that this was further evidence of cytoplasmic inheritance. While their interpretation is possible, it is equally possible to assume that picking selfed plasmodia at each generation selected for strains carrying nuclear gene mutations which enhanced the ability to self.

Recent work has caused Yemma and Therrien to alter their earlier (1972) conclusion, that clonally formed plasmodia in D. iridis were diploid but in an extended G1 phase. Therrien and Yemma (1975) report that clonally formed plasmodia in heterothallic isolates of D. iridis are haploid, and thus exclude the possibility that nuclear fusion is involved in their formation. This observation suggests that the mechanismsof clonal plasmodium formation in P. polycephalum and D. iridis may, after all, be very similar.

1.2c Clonal plasmodium formation in non-heterothallic isolates

Amoebal cultures derived from single spores of most Myxomycetes tested give rise to plasmodia, and many workers have deduced from this that most species do not show heterothallism. However, there are numerous reports of more than one amoeba hatching from a single spore (de Bary, 1864; Smith, 1929; Ross, 1957) and it is reasonable to assume that these might be of more than one mating type.

In order to exclude the possibility of heterothallism,some workers have investigated the possibility of plasmodia arising from clones derived from single amoebae. Plasmodium formation in amoebal clones has been reported in Didymium squamosum (Jahn, 1911), Physarella oblonga (Gehenio and Luyet, 1950), Fuligo cinerea (Collins, 1961), Didymium nigripes (Kerr, 1961) and others.

Luyet (1950) regarded the ability to form plasmodia within clones as showing apogamy, while Collins (1961) considered that it implied homothallism. In the absence of other evidence it can be considered only as a demonstration that heterothallism is probably not involved; even then, heterothallism can only be satisfactorily excluded by clonal plasmodium formation over several generations. Isolates of D. nigripes and

P. polycephalum satisfy this criterion, and have been extensively studied, as described below.

Comparative chromosome counts on amoebae and plasmodia of D. nigripes have been reported by von Stosch et al.(1964) and S. Kerr (1968). Both studies showed that amoebal and plasmodial nuclei contained the same number of chromosomes, suggesting that this isolate might lack a sexual cycle. Kerr reported that nuclei of several ploidy levels were present in both amoebae and plasmodia, but that there was no overall difference between them. S. Kerr (1970) also measured nuclear sizes and concluded that amoebal and plasmodial nuclei did not differ significantly.

Therrien (1966a, b) carried out microdensitometry of Feulgen stained nuclei of amoebae and clonally formed plasmodia of D. nigripes. He concluded that plasmodial nuclei were diploid, with a 4C DNA content, while amoebae were haploid (2C). He showed two size classes in his results for amoebae, one which he identified as haploid (2C) amoebae and one which he identified as diploid (4C) zygotes. S. Kerr (1968) compared her chromosome counts with Therrien's data, suggesting that "he, too, found nuclei with amounts of DNA intermediate to the two ploidy classes and greater than the 4C class.... it is curious that Therrien chose to recognise only one size of [plasmodial] nuclei in his preparations for examination of his graph suggests that several size classes could have been recognised".

S. Kerr (1967) observed living cultures of D. nigripes amoebae, using high power phase-contrast microscopy. She studied mitosis in amoebae and in plasmodia which formed in the cultures, noting the different characteristics of these divisions. The nuclear membrane was retained during mitosis in plasmodial nuclei, but not in amoebae. She used this and other differences to distinguish between amoebae and "uninucleate

plasmodia". S. Kerr concluded that "the impression gained from observations of differentiating populations is that uninucleate plasmodia can be formed without fusion of cells, simply by growth and differentiation of single amoebae".

N. Kerr (1967) carried out an analysis of plasmodium formation in the mutant S-3 strain of D. nigripes, using time-lapse microcinematography. He showed that plasmodium formation occurred without cell or nuclear fusion. Since this strain was one in which S. Kerr had found no ploidy difference between amoebae and plasmodia, this was strong evidence for apogamic development of plasmodia.

Von Stosch et al. (1964) also reported an isolate ("Colonia") of P. polycephalum in which amoebal clones gave rise to plasmodia. They noted that meiosis in this strain was replaced by a "nuclear division of a curious abortive character", but did not give detailed evidence. Their observation suggested that nuclear fusion might be lacking in the life cycle of this isolate.

Wheals (1970) presented convincing evidence that the ability of amoebae of the Colonia isolate to form plasmodia within clones was conferred by an allele (mt_h) of the mating type locus. He crossed Colonia amoebae with amoebae carrying the four heterothallic mating types then known. In each cross the amoebal strains also differed at a locus unlinked to the mating type locus. Analysis of amoebal progeny of a crossed plasmodium showed recombination of alleles at the two unlinked loci and the ability to form plasmodia within clones segregated 1 : 1 from the heterothallic mating type.

Wheals (1971) obtained inconclusive evidence which suggested that cell and nuclear fusion were involved in the formation of plasmodia within clones of mt_h amoebae, that is that they were homothallic. He carried out

preliminary tests of nuclear DNA content, using scanning microdensitometry of Feulgen stained amoebal and plasmodial preparations, and suggested that clonally formed $\underline{mt}_h$ plasmodia were diploid, arising from haploid amoebae. Wheals also showed (1970, 1971) that mixtures of $\underline{mt}_h$ amoebae carrying different plasmodial fusion alleles gave rise to plasmodia of hybrid fusion behaviour, and regarded this as additional evidence for homothallism.

Cooke and Dee (1974) demonstrated conclusively that $\underline{mt}_h$ strains were not homothallic. They carried out scanning microdensitometry on Feulgen stained preparations of nuclei isolated from amoebae and plasmodia in G2 phase. Amoebae and plasmodia of $\underline{mt}_h$ strains had the same nuclear DNA content. This was about half the value for plasmodia produced by crossing heterothallic and $\underline{mt}_h$ amoebae, or amoebae of two different heterothallic strains, but the same as for heterothallic amoebae. Thus they concluded that clonally formed $\underline{mt}_h$ plasmodia were haploid and that nuclear fusion was not involved in their formation. However, the question of whether cell fusion occurred was left open.

Wheals (1970) showed that plasmodia derived from clones of $\underline{mt}_h$ amoebae were able to sporulate and that the spores germinated to yield amoebae. It is not clear how viable spores are produced by these strains, since normal meiosis is clearly impossible in haploid nuclei. Lacorre-Arescaldino (personal communication) has observed synaptonemal complexes in spores of $\underline{mt}_h$ plasmodia but this does not necessarily imply the presence of diploid nuclei; synaptonemal complexes have also been observed in haploid plants (Menzel and Price, 1966; Ting, 1969), in tissues where meiosis would normally take place in diploids. Haploid parthenosporophytes of the alga Ulva mutabilis undergo a "haploid meiosis", which yields viable spores, at the same time in the life cycle as meiosis occurs in heterozygous diploid sporophytes (Hoxmark and Nordby, 1974). Viability of spores from

mt_h plasmodia is generally lower than from crossed plasmodia (Mohberg, 1976) and there is some evidence that viable amoebae may arise from meiosis in the few diploid nuclei which are sometimes found in these plasmodia (Laffler and Dove, 1976).

1.2d Summary

P. polycephalum shows several different nuclear cycles (see Figures 3-6). Diploid plasmodia are normally formed by sexual fusion of haploid amoebae carrying different mating type alleles. Meiosis occurs during sporulation and the spores yield haploid amoebae of the two original mating types. Haploid mt_h amoebae give rise to haploid plasmodia within clones or diploid plasmodia when crossed with amoebae of other mating types. Clonally formed mt_h plasmodia yield haploid mt_h progeny. Diploid amoebae heterozygous at the mating type locus give rise to diploid plasmodia within clones. Progeny of these plasmodia include both haploid and diploid amoebae. Haploid amoebae carrying a single heterothallic mating type allele very rarely give rise to plasmodia. These plasmodia are always haploid and usually yield amoebal progeny of the original mating type.

Although cell fusion is clearly necessary in the formation of crossed plasmodia, published data do not indicate whether amoebal fusions occur within clones or if such fusions are involved in clonal plasmodium formation.

Myxomycetes other than P. polycephalum also show both heterothallic and clonal plasmodium formation. Heterothallic behaviour follows the same pattern as in P. polycephalum, but there are some conflicting reports of the nature of clonal plasmodium formation. Conclusive evidence of homothallism is lacking, but apogamy is well documented in a mutant

Plasmodium formation and nuclear cycles in *Physarum polycephalum*.

Figure 3. Clonal plasmodium formation by amoebae carrying the mt_h allele.

Figure 4. Crossed plasmodium formation (mt_m and mt_n represent two different mating type alleles).

Figure 5. Clonal plasmodium formation by diploid amoebae heterozygous for mating type alleles.

Figure 6. Rare clonal plasmodium formation by amoebae carrying a single heterothallic mating type allele.

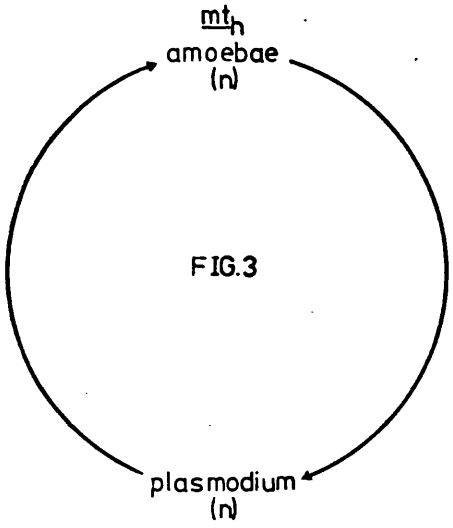


FIG.3

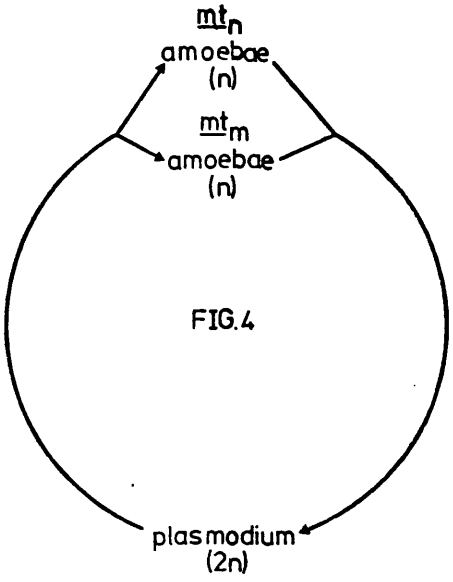


FIG.4

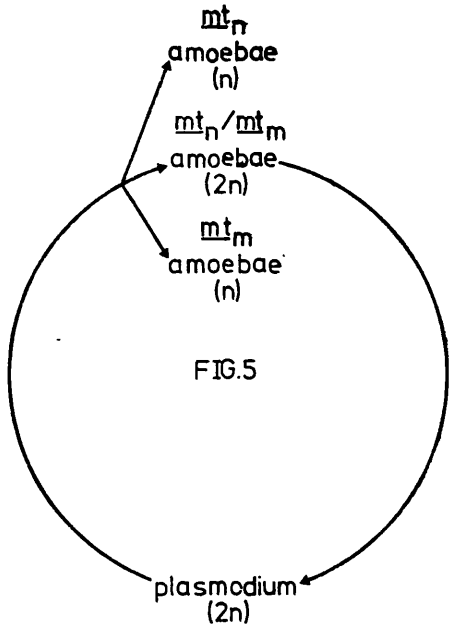


FIG.5

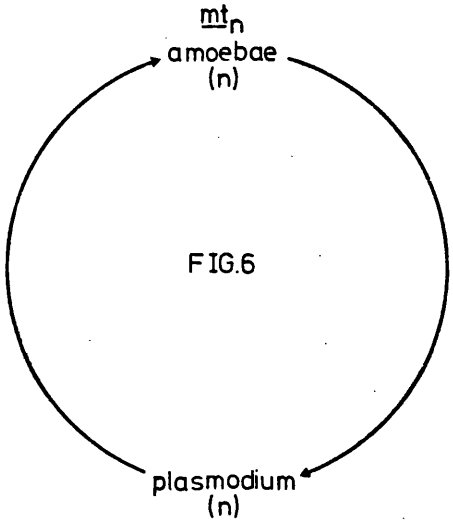


FIG.6

strain of D. nigripes.

It should be noted that the terms haploid and diploid are not used here in their strict sense; evidence from chromosome counts or nuclear DNA analyses in Myxomycetes are usually sufficiently exact to distinguish between haploid and diploid nuclei, but not accurate enough to distinguish between euploid and aneuploid nuclei.

1.3 INTRODUCTION TO WORK DESCRIBED IN THIS THESIS

P. polycephalum has long been recognised as a useful tool in eukaryote cell biology, principally because the plasmodium may be considered as a single giant cell. A number of biochemical analyses of the plasmodial phase have been carried out, mainly concerned with the mitotic cycle (Schiebel, 1973), sporulation (Sauer, 1973) and spherulation (Hutterman, 1973). Alexopoulos (1960) pointed to the fact that P.polycephalum is the first choice of Myxomycete for such investigations; no other plasmodium has been as extensively studied.

With recent advances in the axenic culture of amoebae (McCullough and Dee, 1976) it is now possible to make comparative biochemical studies of the amoebal and plasmodial phases (e.g. Hall et al., 1975).

In order to utilise fully the advantages of P. polycephalum in biochemical analysis, it is clearly desirable to carry out complementary genetical studies. As declared by Dee (1973), P. polycephalum "... is by no means an ideal organism for genetics .... it is a slow-moving, refractory, inelegant genetic tool". However, techniques for genetic analysis are available (Dee, 1973, 1975). They should be of value in the study of the

mitotic cycle (Grant, 1973) and have already been used in studies on plasmodial fusion and compatibility (Carlile, 1973), amoebal behaviour (Jacobson and Dove, 1975) and the subject of this thesis, the amoebal-plasmodial transition.

The unique Mycomycete life cycle includes a number of differentiation steps. Sporulation and spherulation have the advantage of being naturally synchronous processes occurring in the plasmodium, and have been much studied biochemically. Amoebal flagellation (Jacobson and Adelman, 1975) and encystment (Haars et al., 1975) have attracted relatively little interest. Genetic analysis of any of these processes would probably be exceedingly laborious, due to the difficulty of isolating and analysing mutants. Yet "... without [genetic analysis] there is no hope of ever dissecting out the mechanisms of differentiation", (Monod and Jacob, 1961).

That the amoebal-plasmodial transition is amenable to genetic analysis was shown by Wheals (1973). He screened 5×10^5 clones of amoebae, derived from mt_h cultures mutagenised by exposure to ultraviolet light and caffeine, and isolated four clones which failed to form plasmodia when cultured alone. These four strains appeared to complement one another, and one strain (APT1) was extensively analysed and shown to carry a mutation at a locus (apt-1) unlinked to the mating type locus.

Cooke and Dee (1975) used an enrichment technique without mutagenesis to isolate, from mt_h amoebae, two mutants which consistently formed plasmodia several days later than the wild type strains, and thus from somewhat larger amoebal colonies. Crosses of these mutants (CLd, CL348d) with heterothallic strains showed that they might carry mutations at or closely linked to the mating type locus, though it was not possible to test adequately the alternative hypothesis that a cytoplasmic

factor was responsible for the delay in plasmodium formation (Cooke, 1974).

This thesis describes work carried out to analyse further the mechanisms of the amoebal-plasmodial transition. To obtain information about the number and functions of genes involved, mutants defective in clonal plasmodium formation were isolated from $\underline{mt}_h$ strains and subjected to genetic analysis. This investigation is reported in Chapter 3. Satisfactory interpretation of the mutant analysis required information on whether amoebal fusions occurred within clones. Chapter 2 describes cytological and genetical studies undertaken to obtain this information. Chapters 4 and 5 are concerned with preliminary attempts to characterise physiologically and biochemically the mutant strains whose isolation is described in Chapter 3. Chapter 6 contains a discussion of the work described in this thesis. In particular, the role of the mating type locus is discussed and P. polycephalum is evaluated as a subject for further studies on development.

1.4 GENERAL MATERIALS AND METHODS

Routine methods employed in the course of this work are described below. Additional techniques, and modifications of those shown here, are given in the appropriate chapters.

1.4a Loci

$\underline{mt}$, amoebal mating type locus. Heterothallic alleles $\underline{mt}_1$, $\underline{mt}_2$, $\underline{mt}_3$, $\underline{mt}_4$ (Dee, 1966). $\underline{mt}_h$ (Wheals, 1970, 1973) confers the ability to form haploid plasmodia within amoebal clones (Cooke and Dee, 1974). Diploid crossed plasmodia normally arise when amoebae carrying different mating type alleles are cultured together (Mohberg and Rusch, 1971; Mohberg et al., 1973; Cooke and Dee, 1974). The amoebal mating type

locus has no known effect on the fusion of plasmodia.

fusA, fusB and fusC, plasmodial fusion loci (Carlile and Dee, 1967; Poulter and Dee, 1968; Poulter, 1969; Cooke, 1974; Adler and Holt, 1974b). Alleles fusA1 and fusA2 are co-dominant, fusB2 is dominant to fusB1, and fusC2 is dominant to fusC1. Plasmodia may fuse with one another only if they express the same fusion phenotype. Non-identity at any locus is sufficient to prevent fusion. Plasmodial fusion loci have no known effect on amoebal fusions. Plasmodial fusion genotypes are specified in this thesis by the minimum number of allele symbols necessary; thus all amoebal strains should be assumed to carry the fusB1 and fusC1 alleles, unless otherwise specified, and the designations fusA1 and fusA2 are used both for haploid strains and for diploid strains homozygous for fusA alleles.

apt1, a locus affecting the amoebal-plasmodial transition (Wheals, 1971, 1973). Amoebal strains of the genotype mt_h;apt-1^- fail to form plasmodia within amoebal clones. They cross with heterothallic apt-1^+ strains to give diploid plasmodia, but not with apt-1^- strains.

leu-1, a locus affecting leucine biosynthesis (Cooke and Dee, 1975). Homozygous leu-1^- plasmodia die on minimal defined medium agar (DM-1 agar; see 1.4q) unless supplemented with leucine (DM-1 + leucine; see 1.4q).

eme, a locus affecting amoebal growth in the presence of emetine chloride (Adler and Holt, 1974b). Amoebae carrying the mutant eme^r allele proliferate and form colonies on liver infusion agar (LIA: see 1.4q) containing 100 µg/ml emetine chloride; amoebae carrying the eme^s allele do not.

1.4b Strains

Table 1 shows laboratory stock strains used in this work. All

Table 1. Amoebal strains previously isolated and used in this work

Strains	References	Genotypes
a	Dee, 1966	$\underline{mt}_1$;*fusA1*
APT1	Wheals, 1973	$\underline{mt}_h$;*fusA2*;*apt*-1$^-$
CH188	See text	$\underline{mt}_3$;*fusA2*;*fusC2*;*eme*^{r}
CH207	See text	$\underline{mt}_4$;*fusA2*;*fusC2*
CL	Cooke & Dee, 1975	$\underline{mt}_h$;*fusA2*
CL5001	Cooke & Dee, 1975	$\underline{mt}_h$;*fusA2*;*leu*-1$^-$
i	Dee, 1966	$\underline{mt}_2$;*fusA2*;*fusB2*
LU14	Cooke, 1974	$\underline{mt}_2$;*fusA1*;*fusB2*
LU640	Cooke, 1974	$\underline{mt}_h$;*fusA1*
LU647	Cooke, 1974	$\underline{mt}_1$;*fusA2*
LU648	Cooke & Dee, 1975	$\underline{mt}_1$;*fusA1*
LU688	Cooke & Dee, 1975	$\underline{mt}_2$;*fusA1*
LU853	Cooke, 1974	$\underline{mt}_1$;*fusA2*;*leu*-1$^-$
LU858	Cooke, 1974	$\underline{mt}_2$;*fusA1*;*leu*-1$^-$
LU860	Cooke, 1974	$\underline{mt}_1$;*fusA1*;*leu*-1$^-$
LU861	Cooke, 1974	$\underline{mt}_2$;*fusA2*

All strains carried the *fusB1* and *fusC1* alleles unless shown otherwise. Only mutant alleles of *apt*-1, *eme* and *leu*-1 are shown.

strains except a,i and LU 14 are essentially of Colonia genetic background, either by direct descent, or by extensive backcrossing to Colonia strains. The original "Colonia isolate" was an amoebal culture, whose origin in nature is unknown, supplied by Dr. H.A. von Stosch (University of Marburg, Germany) in 1964. Strains a,i and LU14 are of the "Wisconsin 1 isolate", originally derived by Dee (1960) from a plasmodium supplied by Dr. H.P. Rusch (University of Wisconsin, U.S.A.) in 1957. Strains CH188 and CH207 were supplied to this laboratory by Dr. P.N. Adler and Dr. C.E. Holt. They are, respectively, 6th and 5th generation backcrosses to CL in the schemes described by Adler and Holt (1974b). Table 2 shows amoebal strains, derived in the course of this study, which are now maintained as laboratory stock cultures.

1.4c Nomenclature

The principles of nomenclature used in this thesis are a slightly modified version of those proposed by Cooke et al. (Cooke, 1974). Information essential to an understanding of this work is summarised below.

(i) Amoebal strains are normally represented by a two-lettered code, with a numerical suffix, e.g. CL5001, LU648. Exceptions to this are the Leicester University standard Colonia strain, CL, and strains described already in the literature, such as i.

(ii) Mutants are given a numerical suffix to the designation of the strain from which they were derived. Thus CL5001 is a mutant derived from CL, and CL5001/8 is a mutant derived from CL5001.

(iii) A plasmodium is normally designated by a bracketed description of the amoebal strain, or mixture of strains, from which it is derived, followed by an isolation number. Thus a clone of CL amoebae may give rise to the plasmodium (CL)1, while a cross between LU648 and LU688 may give

Table 2a. Amoebal strains isolated in the course of this work and now maintained as stock cultures

Strains	Genotypes
CL6049	mt_h;*fusA2*;*npfB1*$^-$
CL6082	mt_h;*fusA2*;*npfB2*$^-$
CL6089	mt_h;*fusA2*;*npfB3*$^-$
CL6099	mt_h;*fusA2*;*npfC1*$^-$
CL6100	mt_h;*fusA2*;*npfB7*$^-$
CL6111	mt_h;*fusA2*;*npfA1*$^-$
CL6115	mt_h;*fusA2*;*npfB8*$^-$
CL6129	mt_h;*fusA2*;*npfB4*$^-$
CL6130	mt_h;*fusA2*;*npfB5*$^-$
CL6134	mt_h;*fusA2*;*npfB6*$^-$
CL6136	mt_h;*fusA2*;*npfC2*$^-$
CL6143	mt_h;*fusA2*;*npfC3*$^-$
CL5001/8	mt_h;*fusA2*;*npfC4*$^-$;*leu-1*$^-$

All strains carry the *fusB1* and *fusC1* alleles. Only mutant alleles of *leu-1*, *npfA*, *npfB* and *npfC* are shown.

Table 2b. Amoebal strains isolated in the course of this work and now maintained as stock cultures

Strains	Alternative designations	Genotypes
LU862	(CH188 x LU640)2:20	mt_3;*fusA1*;*eme*^{r}
LU863	(CH207 x LU640)1:1	mt_4;*fusA1*
LU864	(APT1 x LU648)3:1	mt_h;*fusA1*;*apt-1*$^-$
LU865	(APT1 x LU648)3:15	mt_1;*fusA1*;*apt-1*$^-$
LU866	(APT1 x LU648)3:33	mt_1;*fusA2*;*apt-1*$^-$
LU867	(CL6111 x LU648)3:2	mt_h;*fusA1*;*npfA1*$^-$
LU868	(CL6111 x LU648)3:20	mt_h;*fusA2*;*npfA1*$^-$
LU869	(CL6111 x LU648)3:9	mt_1;*fusA1*;*npfA1*$^-$
LU870	(CL6111 x LU648)3:6	mt_1;*fusA2*;*npfA1*$^-$
LU871	(CL6049 x LU648)1:2	mt_h;*fusA1*;*npfB1*$^-$
LU872	(CL6082 x LU648)3:7	mt_h;*fusA1*;*npfB2*$^-$
LU873	(CL6089 x LU648)9:3	mt_h;*fusA1*;*npfB3*$^-$
LU874	(CL6129 x LU648)3:4	mt_h;*fusA1*;*npfB4*$^-$
LU875	(CL6130 x LU648)3:2	mt_h;*fusA1*;*npfB5*$^-$
LU876	(CL6134 x LU648)2:1	mt_h;*fusA1*;*npfB6*$^-$
LU877	(CL6100 x LU648)10:7	mt_h;*fusA1*;*npfB7*$^-$
LU878	(CL6115 x LU648)4:4	mt_h;*fusA1*;*npfB8*$^-$
LU879	(CL6099 x LU648)3:4	mt_h;*fusA1*;*npfC1*$^-$
LU880	(CL6136 x LU648)5:3	mt_h;*fusA1*;*npfC2*$^-$
LU881	(CL6143 x LU648)11:12	mt_h;*fusA1*;*npfC3*$^-$
LU882	(CL5001/8 x LU648)12:9	mt_h;*fusA1*;*npfC4*$^-$;*leu-1*$^-$
LU883	(CL5001/8 x LU648)12:6	mt_h;*fusA1*;*npfC4*$^-$
LU884	(LU688)1:21	mt_2;*fusA1**
LU887	(CL6082 x LU688)1:1	mt_2;*fusA1*;*whi-1*$^-$
LU896	(LU853 x LU887)1:11	mt_1;*fusA2*;*whi-1*$^-$;*leu-1*$^-$
LU897	(LU853 x LU887)1:35	mt_1;*fusA2*;*whi-1*$^-$

* LU884 forms plasmodia within plaques and carries a mutation in or linked to *mt*.

All strains carry the *fusB1* and *fusC1* alleles. Only mutant alleles of *apt-1*, *eme*, *leu-1*, *npfA*, *npfB*, *npfC* and *whi-1* are shown.

rise to the plasmodium (LU648 x LU688)4.

(iv) A heterokaryotic plasmodium is represented as the sum of the contributing plasmodia, enclosed within square brackets, with a numerical suffix. Thus fusion of the plasmodia (CL)19 and (CL5001)3 may give the heterokaryon [(CL)19 + (CL5001)3]2.

(v) Spores have the same designation as the plasmodium from which they are derived.

(vi) Amoebal progeny of a plasmodium are described by the plasmodium designation, followed by a colon and isolation number. Thus amoebal progeny clones of the plasmodium (LU648 x LU688)4 would be (LU648 x LU688)4:1, (LU648 x LU688)4:2, etc. Since the use of these designations becomes awkward for strains in frequent use, they may be replaced by the simpler codes described in (i). Leicester University strains normally have the code LU.

1.4d Amoebal culture

Amoebal strains were maintained in two-membered culture with Escherichia coli on liver infusion agar medium (LIA; 1 g Oxoid liver infusion powder per litre 2% agar). Plastic petri dishes of 9 cm diameter were used and incubation was normally in a constant-temperature room maintained at 25-26°C (referred to as "26°C").

The bacterial suspension was prepared by streaking nutrient agar plates (25 g Oxoid No. 2 nutrient broth powder per litre 1.2% agar) with E. coli, incubating overnight at about 37°C, and harvesting in 13 ml sterile distilled water by scraping with a glass spreader. The bacterial strain, Leicester University Department of Genetics Stock No. 145, was of the genotype thr⁻;leu⁻;thi⁻;his⁻;arg⁻;lac⁻;gal⁻;xyl⁻;malT⁻;str;T_1^R;T_2^s; T_6^R; λ^R; colI^R; F⁻(K12). The multiple nutritional requirements ensured

that amoebal colonies were not outgrown by the bacteria.

In early stages of this work amoebae were subcultured in essentially the same way as previously described (Wheals, 1970; Cooke, 1974). Amoebal suspensions of approximately 5×10^2 cells/ml were prepared by suspending a loopful of amoebae, taken from a single colony, in 2 ml of water. Amoebal and bacterial suspensions (0.1 ml of each) were inoculated on to a LIA plate and spread with a glass spreader. After 3-4 days incubation at 26°C, individual amoebal colonies were visible as small clear areas (plaques) in an opaque bacterial lawn.

The above technique proved rather slow for the handling of large numbers of amoebal strains and a quicker procedure, which gave the same result, was developed and used for the majority of the work described. A toothpick (Kaybee flat style, Keenan Industries, Canada) was held in a pair of forceps and the narrow end was used to pick amoebae from a plaque. The amoebae were stirred into a 0.2 ml "puddle" of bacterial suspension on a LIA plate and spread with a glass spreader. With practice, the density of amoebal plaques on a plate was easily controllable by varying the way in which the toothpick was stirred into the puddle.

Heterothallic strains in frequent use were incubated for 6-8 days at 26°C and stored, encysted, at 4°C until required, up to 3 months. Frequently-used strains able to form plasmodia efficiently within clones were incubated for 4-5 days only, since plasmodium formation occurred after this period.

Stock cultures of all strains were maintained on LIA slopes in screw-top McCartney bottles. These were inoculated in the way described by Haugli (1971) for the subculturing of amoebae on agar plates.

Amoebae were picked with a toothpick. The toothpick was dropped into a tube containing 0.3 ml of bacterial suspension and the tube was agitated with a Whirlimixer (Fisons Ltd., Loughborough, England). The resulting suspension was tipped on to a slope. The culture was incubated at 26°C, as described for plates, then stored at 4°C. Subculturing was normally carried out every 6 months, but some viable cells were recovered from slopes which had been stored for 18 months.

1.4e Plasmodium formation

Two tests of plasmodium formation were employed (see Fig. 7). In both cases dilute semi-defined medium agar (DSDM agar; see 1.4q) was used; this gave quicker and more reliable results than LIA.

The first test was to plate amoebae, with E. coli, as described in 1.4d. The plates were incubated for two weeks, during which time they were periodically inspected for the presence of plasmodia.

The second test of plasmodium formation was the "puddle test" (Cooke, 1974). Approximately 10^4 amoebae were inoculated into a 0.2 ml puddle of bacterial suspension on a DSDM plate. The puddle was not spread and was incubated for up to 6 weeks. Where two compatible strains were inoculated together, crossed plasmodia usually arose in 1-2 weeks. Clones of mt_n amoebae inoculated alone gave plasmodia in 3-4 days.

1.4f Plasmodial culture

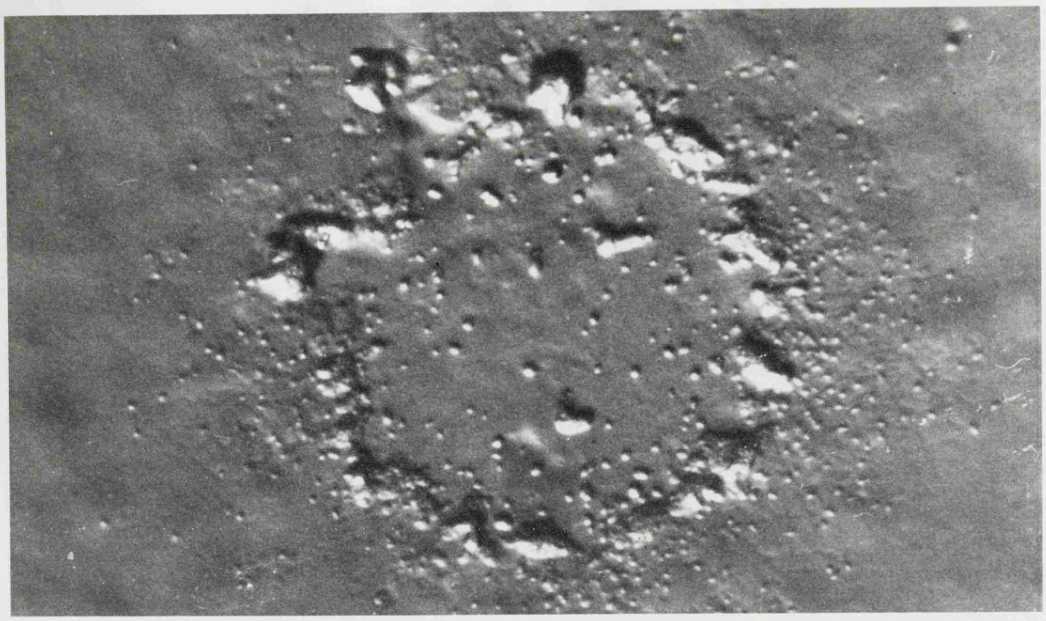
Plasmodia were routinely cultured on semi-defined medium agar (SDM agar; see 1.4q) in 9 cm petri dishes. They were incubated at 26°C in darkness and subcultured every 3-4 days, when the medium had been covered. A block of agar about 1 cm^2 was cut from the old culture and placed, plasmodial face up, in the centre of a fresh plate.

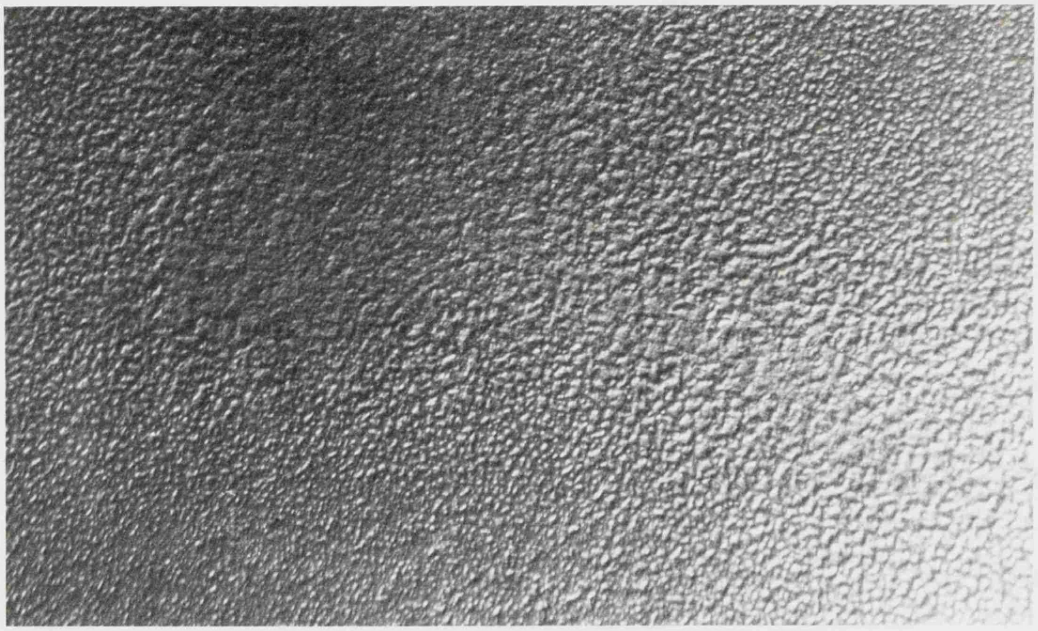
Figure 7. Plasmodium formation in Physarum polycephalum.

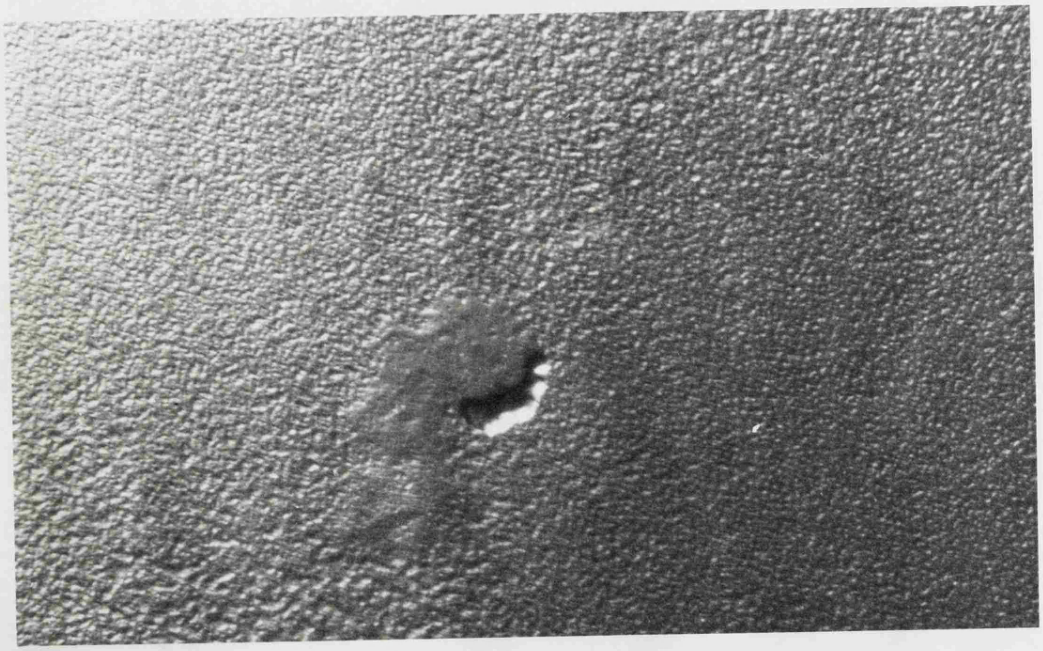
Top: Formation of plasmodia within a plaque. Amoebae appear as small dots and as a ridge around the edge of the plaque, where they are feeding on the bacterial lawn. Plasmodia appear as larger bodies which move about and ingest amoebae. Photographed 4 days after inoculation. x 50

Middle: Confluent amoebal growth. Amoebae have proliferated without forming plasmodia. Photographed 7 days after inoculation. x 50

Bottom: A single plasmodium formed within a population of amoebae. The plasmodium has migrated and ingested amoebae to leave a clear area. Photographed 9 days after inoculation. x 50







1.4g Sclerotisation

Plasmodia were subcultured to DM-1 agar plates (DM-1 + leucine was used for leu-1$^-$ strains). Sclerotia formed much more readily on this medium than on SDM agar, within 2 weeks. They were peeled off the agar and placed in petri dishes to dry for at least one week. They were then stored in McCartney bottles, at room temperature in darkness. Viability was normally retained for at least 2 years.

1.4h Sporulation

Plasmodia were allowed to cover SDM plates, which were then inverted and placed inside a "sporing chamber". This consisted of a box, in the base of which were three 20W ATLAS DAYLIGHT fluorescent tubes. Petri dishes were placed on a perspex sheet 30 cm above the fluorescent tubes. The sporing chamber was operated (12 hours on; 12 hours off) in a 26°C constant-temperature room. Air heated by the fluorescent tubes was removed by means of a fan.

Sporulation normally took 2-5 days, though some plates took up to 3 weeks. Sporangia were scraped from the surface of the agar with a spatula and placed in petri dishes. They were allowed to dry for at least 1 week, then transferred to screw-cap bottles. They were stored at room temperature and remained viable for at least 2 years.

1.4i Spore germination

A small lump of dried sporangia, 1-2 mm^3, was soaked in 0.05 ml of water for 30 minutes, crushed with the rounded end of a glass rod and suspended in a further 1 ml of water. This suspension was allowed to stand for 1-3 hours, then resuspended with a Whirlimixer. Bacterial suspension (0.2 ml) was inoculated on to 5 LIA plates and 0.1 ml of spore

suspension was added to one of these plates. The mixed suspension was spread with a glass spreader, which was then used, without resterilisation, to spread the remaining 4 plates. The 5 plates gave a range of dilutions of the spore suspension, sufficient to ensure that at least one plate would give rise to well-separated plaques after 4-5 days incubation at 26°C.

1.4j Isolation of progeny clones

Plaques arising from the plating of spores could not be considered as clones; a clump of spores might give rise to several amoebae which would form a single plaque, or a single spore might give rise to more than one amoeba (Dee, 1966). Thus amoebae from well-separated plaques were inoculated, with E. coli, on to an LIA plate. The suspension was spread and the plate was incubated for 4-5 days at 26°C. A single plaque was then chosen as a clone.

1.4k Microscopic observation of growing amoebal cultures

This has been described previously (Anderson et al., 1976). Two-membered cultures of amoebae and E. coli were set up on DSDM agar in filming slides (Fig. 8). Cool molten agar was inoculated into the slide by running a few drops under the edge of a cover slip. The agar solidified to form a circular block with flat, parallel surfaces against the cover slip and the slide base, and a surrounding air space. The cover slip was removed and a loopful of spores or amoebae, suspended in bacterial suspension, was gently spread over the agar surface. A fresh cover slip was placed over the cavity, in contact with the surface of the block, and its edges sealed to the perimeter of the cavity with paraffin wax.

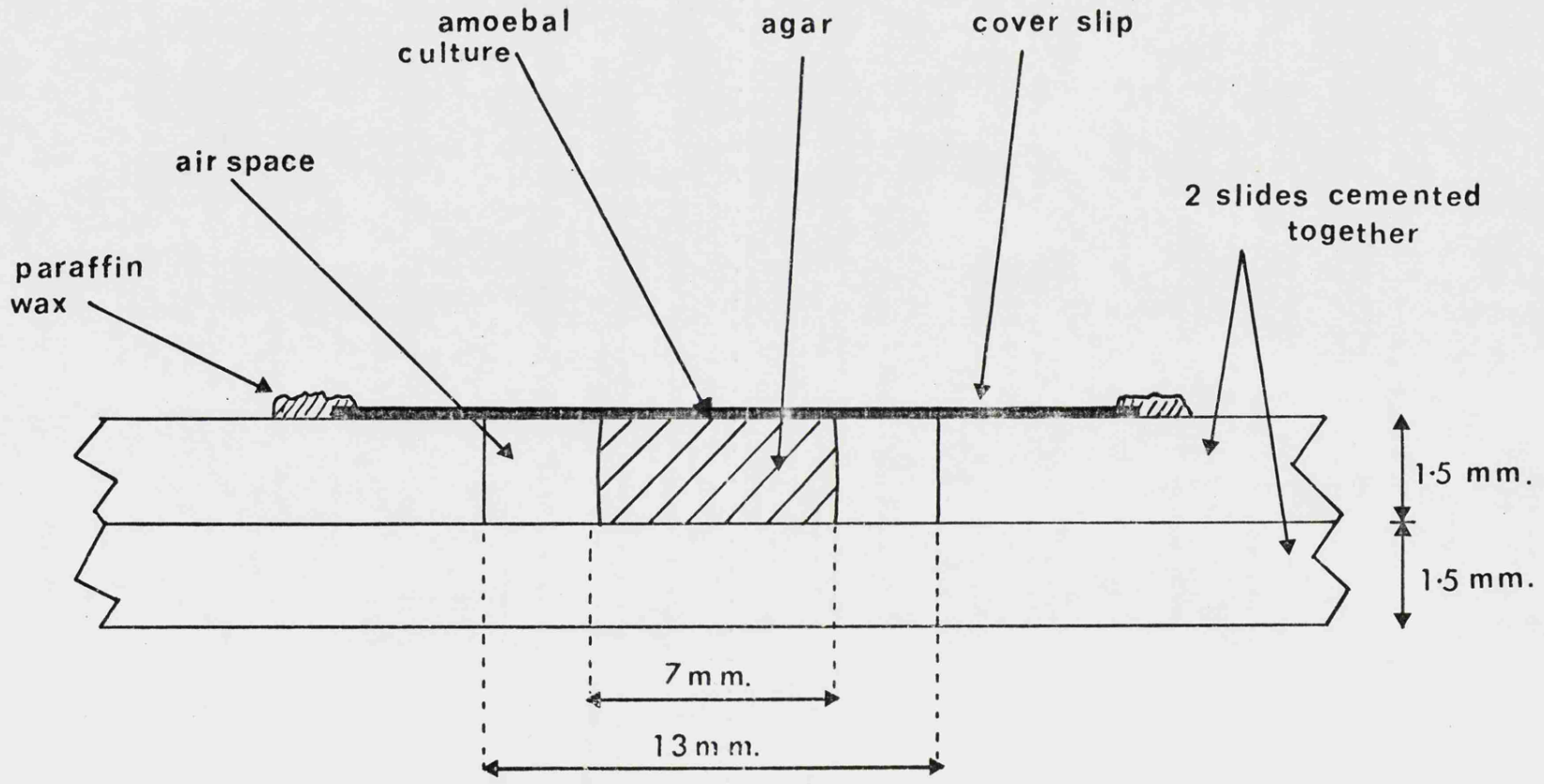

amoebal culture
agar
cover slip
air space
2 slides cemented together
paraffin wax
1·5 mm.
1·5 mm.
7 mm.
13 mm.

Figure 8. Longditudinal section through the central part of a filming slide to show the culture chamber.

Observation was with a Wild M-20 research microscope mounted on a Wild quartz-iodine illumination base. Phase-contrast optics were used, with magnifications of 100-750x. Still photographs were taken with a Nikon AFM unit mounted on the trinocular head of the microscope. Ilford Micro-Neg or Kodak Pan-X film were used.

1.41 Plasmodial fusion tests

These are illustrated in Figure 9. The fusion phenotype ("fusion type") of a plasmodium was determined by allowing it to grow into contact with tester plasmodia of known fusion types. Blocks approximately 4 x 4 x 30 mm were cut from SDM plates on which plasmodia were growing. Plastic petri dishes of 6 cm diameter, containing SDM agar, were each inoculated with an unknown plasmodium and a tester. The blocks were placed approximately 1 cm apart, with the sides carrying plasmodial growth facing one another. Plasmodia grew into contact in about 24 hours.

When the advancing edges of plasmodia of identical fusion type met, fusion occurred within a few minutes. Streaming of protoplasm between the plasmodia began almost immediately and prominent veins crossed the junction point within 1 hour.

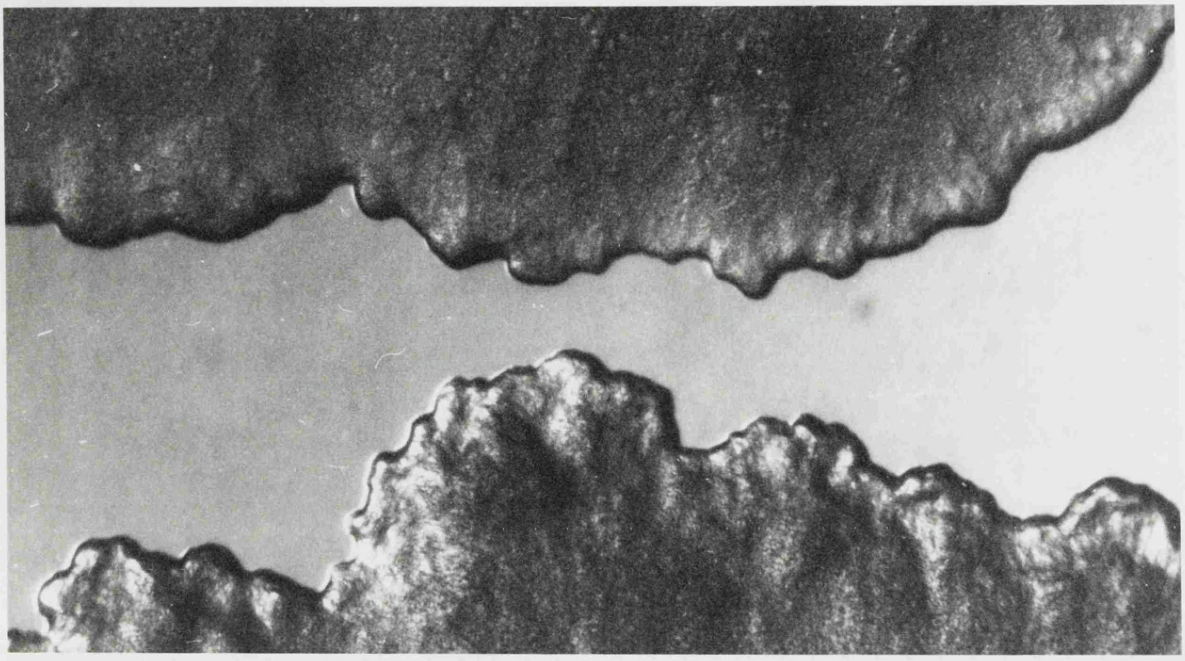
No fusion of the above type occurred when plasmodia of different fusion types came into contact. However, plasmodia differing only in their genotype at the fusA locus could occasionally participate in a limited and temporary fusion reaction; small veins appeared between heterozygous fusA1/fusA2 plasmodia and fusA2 plasmodia, but streaming was transient and a clear non-fusion reaction always resulted after a few hours. All tests were checked 8-16 hours after the initial observation of fusion or non-fusion, so that unambiguous results were always obtained.

Figure 9. Plasmodial fusion tests.

Top: The advancing edges of two plasmodia approach one another. x 15

Middle: Fusion. One hour after meeting the plasmodia are joined by prominent veins. x 15

Bottom: Non-fusion. One hour after meeting. x 15



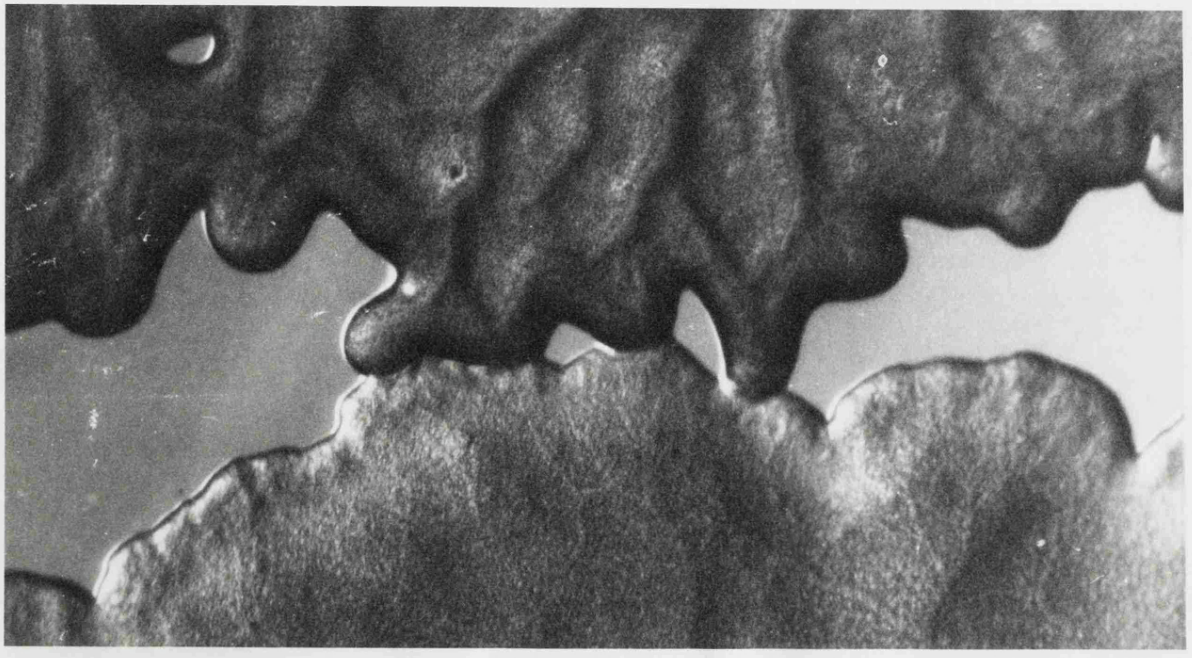


Table 3 shows the amoebal strains used to construct tester plasmodia of known genotype and phenotype. These plasmodia were remade from the amoebal strains every 6 months, since prolonged culture on agar led to senescence (Poulter, 1969).

1.4m Plasmodial leucine requirement tests

Two small blocks, each approximately 2 mm^3, were cut from an SDM agar plate carrying a growing plasmodium. The blocks were used to inoculate two 6 cm diameter petri dishes, one containing DM-1 agar and the other DM-1 + leucine agar. The plates were incubated at 26°C for 1 week. Plasmodia which grew on DM-1 + leucine but died on DM-1 were classified as leu-1^-, while those which grew on both media were classified as leu-1^+ (see Figure 10).

1.4n Isolation of plasmodial and amoebal nuclei

Plasmodial and amoebal nuclei were isolated essentially as described by Mohberg and Rusch (1971). Nuclei of plasmodia grown on SDM agar were observed in glycerol/ethanol fixed smears, using phase-contrast microscopy (Mittermayer et al., 1965). Plasmodia were harvested at least 1 hour after the completion of nucleolar reconstruction, to ensure that they were in G2 phase (Rusch, 1970).

One plasmodium, at least 5 cm in diameter, was scraped into 80 ml of ice-cold homogenising medium (0.25M sucrose; 0.01M $CaCl_2$; 0.01M Tris-HCl, pH 7; 0.1% $^w/_v$ Triton X-100). The suspension was homogenised for 15 sec. in an MSE Ato-Mix blender, operated on "high", with a 70 volt supply. The homogenate was kept on ice for 10-15 min to allow foam to subside, then centrifuged in 50 ml conical tubes for 5 min at 50g. The supernatant was filtered through a cotton-wool milk

Table 3. Plasmodial fusion testers

Fusion group	Genotype	Amoebae used to construct testers
I	fusA1;fusB1;fusC1	LU648 x LU688
II	fusA1;fusB2;fusC1	a x LU14
III	fusA1/fusA2;fusB1;fusC1	LU688 x LU853
IV	fusA1/fusA2;fusB2;fusC1	a x i
V	fusA2;fusB1;fusC1	CL
IX	fusA1/fusA2;fusB1;fusC2	CH207 x LU862

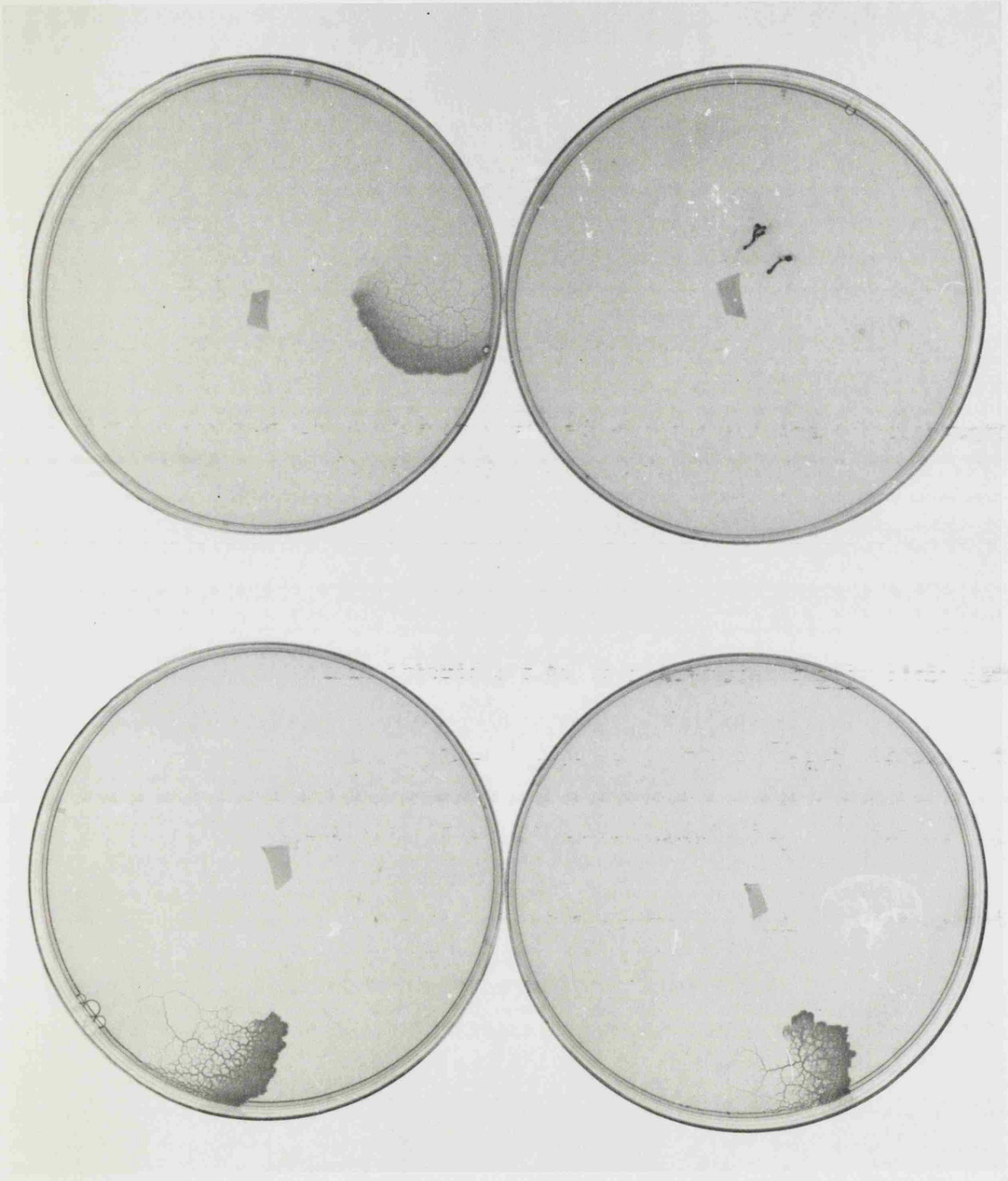
Figure 10. Plasmodial leucine requirement tests. Photographed 3 days after inoculation. x 0.8

Top left: *leu*-1$^-$ plasmodium on DM-1 + leucine agar.

Top right: *leu*-1$^-$ plasmodium on DM-1 agar.

Bottom left: *leu*-1$^+$ plasmodium on DM-1 + leucine agar.

Bottom right: *leu*-1$^+$ plasmodium on DM-1 agar.



filter pad sandwiched between two fabric milk filters (Grant and Poulter, 1973). The filter was saturated with homogenising medium before use. The filtered supernatant was centrifuged for 10 min at 500g. The resulting pellet was resuspended in 40 ml of homogenising medium and the centrifugation repeated. The final pellet was resuspended in 0.5 ml of homogenising medium, frozen in liquid N_2 and stored at -20°C.

Amoebal populations which contained mainly G2 cells were obtained by ensuring that cultures were harvested in logarithmic phase (Mohberg and Rusch, 1971). Approximately 10^4 amoebae were inoculated, with bacteria, on to an LIA plate and incubated at 26°C for 4 days. A further 0.2 ml of bacterial suspension was then added and the plate respread with a glass spreader. The culture was reincubated for 24 hours, then harvested in 10 ml of ice-cold water. This technique gave 10^6-10^7 cells from each plate. CL amoebae could not be cultured in this way as they formed plasmodia before harvesting. Thus for CL amoebae, 10 plates were incubated as above and respread after 3 days.

The amoebal suspension was made up to 40 ml with ice-cold water and centrifuged in a 50 ml conical tube for 5 min at 500g. The pellet was resuspended and centrifuged twice more, or until the wash water was free of bacteria. The final pellet was suspended in 40 ml of ice-cold homogenising medium and nuclei were isolated as for plasmodia, except that homogenisation was for 30 sec and the high speed centrifugations were each for 15 min at 1000g.

1.4o Isolation of chick erythrocyte nuclei

Blood from four 11-day chick embryos was haemolysed in 100 ml of 0.9% NaCl containing 0.1% saponin. The suspension was stirred slowly for 1 hour at 4°C, then centrifuged in 50 ml conical tubes for 10 min at

1000g. The pellet was resuspended in 40 ml of homogenising medium, filtered through a milk filter and centrifuged again for 10 min at 1000g. The final pellet (about 10^8 nuclei) was resuspended in 2 ml of homogenising medium, divided into 0.4 ml portions, frozen in liquid N_2 and stored at -20°C.

1.4p Estimation of nuclear DNA content

Nuclear DNA contents were estimated by measuring the light absorption of nuclei stained with Feulgen stain (Feulgen and Rossenbeck, 1924). This dye shows a specific stoichiometric Schiff-reaction with acid hydrolysed DNA (Swift, 1955), one molecule of dye reacting with two aldehyde radicals produced by acid hydrolysis of the deoxyribose.

Preparations of isolated nuclei were allowed to thaw at room temperature. Two drops of a suspension of Physarum nuclei and one drop of chick suspension were mixed on a slide and air dried for about 1 hour at room temperature. The material was fixed in acetic alcohol (1 vol glacial acetic acid + 3 vols ethanol) for 1 hour and stained by the Feulgen method (Darlington and LaCour, 1962). Hydrolysis was by 5N HCl at room temperature for 45 min (Decosse and Aiello, 1966; Deitch et al., 1968).

Stain intensities were measured with a Vickers M85 Scanning Microdensitometer (Vickers Instruments Ltd.). Individual nuclei were positioned in the centre of the field and enclosed within a masking frame (A/2 or A/3). The instrument was calibrated so that an area immediately adjacent to the nucleus gave 100% transmission, while interruption of the light path gave 0% transmission. All observations were made with the 100x objective, slit width 30, scanning spot size 2 and wavelength 55 (equivalent to 560 nm). The total absorption of light by each nucleus

was measured and given in arbitrary units. Fifty Physarum nuclei and 10 chick nuclei were measured on each slide (Figure 11 shows that they were easily distinguishable). The chick nuclei were used as a standard of 2.45 pg DNA/nucleus (Rasch et al., 1971; Mohberg, 1976), and the DNA contents of all the Physarum nuclei were calculated from the mean value of the standards.

1.4q Media

DM-1 agar (defined medium agar; Dee et al., 1973)

Stock solution:

	g/litre
L-glutamic acid	3.0
Glycine	0.5
L-methionine	0.1
Biotin	0.005
Thiamine	0.04
Citric acid.H_2O	3.54
Disodium EDTA	0.224
KH_2PO_4	2.0
$CaCl_2.2H_2O$	0.9
$MgSO_4.7H_2O$	0.6
$FeCl_2.4H_2O$	0.006
$ZnSO_4$	0.034

Adjust pH to 4.6 with 10% NaOH.

Sterilise by autoclaving (15 lb/in^2 for 20 min).

Store in dark.

Immediately before use, add 20.0 ml of 20% (^{w}/v) sterile glucose solution and 10.0 ml of haematin solution (see below) per litre of stock solution.

Figure 11. Feulgen stained nuclei. In the centre is a chick erythrocyte nucleus. Two P.polycephalum nuclei can be seen to the right, above and below the chick nucleus. P.polycephalum nuclei are clearly identifiable by the presence of lighter areas within them (nucleoli). x 2000

Mix equal quantities of this solution and molten 2.25% agar.

Haematin solution:

0.5g haematin per litre of 1% NaOH

Autoclave 15 lb/in^2 for 20 min.

Store at 4°C.

DM-1 + leucine agar

As DM-1 agar, but with 0.1g leucine per litre of stock solution.

DSDM agar (dilute semi-defined medium agar)

100 ml SDM (+ haematin) per litre of molten 2% agar.

LIA (liver infusion agar)

1g Oxoid liver infusion powder per litre of molten 2% agar.

Use 5.0% (^{w}/v) stock solution of liver infusion powder (autoclave 15 lb/in^2 for 20 min; store at 4°C).

Add 20 ml stock solution per litre of molten 2% agar.

Nutrient agar

25g Oxoid No. 2 nutrient broth powder per litre of molten 1.2% agar.

SDM (semi-defined medium; Dee and Poulter, 1970)

	g/litre
Glucose	10.0
Oxoid bacteriological peptone	10.0
Citric acid.H_2O	3.54
Disodium EDTA	0.224

(continued)

KH_2PO_4	2.0
$CaCl_2$	0.9
$MgSO_4.7H_2O$	0.6
$FeCl_2.4H_2O$	0.06
$ZnSO_4.7H_2O$	0.034
Vitamin mix	100 ml/litre

Adjust pH to 4.6 with 10% NaOH.

Sterilise by autoclaving (15 lb/in^2 for 20 min).

Add 10 ml of haematin solution per litre of SDM (use same haematin stock solution as for DM-1 agar) immediately before use.

Vitamin mix:

	mg/litre
Inositol	119.0
Choline chloride	85.7
Biotin	158.0
Thiamine HCl (Aneurin HCl)	424.0
Pyridoxal HCl	60.9
Pyridoxine HCl	87.2
Niacine (nicotinic acid)	42.2
D-Pantothenic acid (calcium salt)	45.0
p-Aminobenzoic acid	8.16
Folic acid	4.07
Vitamin B_{12} (cyanocobalamin)	0.049
Riboflavin	43.6

Store in deep freeze.

SDM agar

Mix equal quantities of SDM (+ haematin) and molten 2.25% agar.

1.4r Sources of chemicals

General laboratory chemicals were obtained from British Drug Houses or Fisons Scientific apparatus. Bacteriological peptone, nutrient agar and liver infusion powder were from Oxoid Ltd., vitamins were from Sigma Chemical Company and haematin was from Koch-Light Laboratories Ltd.

C H A P T E R 2

INVESTIGATION OF CELL FUSION IN PLASMODIUM FORMATION

CHAPTER 2 INVESTIGATION OF CELL FUSION IN PLASMODIUM FORMATION

2.1 INTRODUCTION

The nuclear events associated with plasmodium formation in P. polycephalum, and their dependence on the mating type locus, were demonstrated by work described in Chapter 1. However, these studies did not indicate the way in which the mating type locus controlled plasmodium formation. The studies described in Chapter 2 had two aims: to determine how plasmodia arose within clones of $\underline{mt}_h$ amoebae, and to determine whether the mating type locus was involved in the control of amoebal fusions.

Wheals (1970, 1971, 1973) assumed that the mating type locus controlled amoebal fusion, so that heterothallic amoebae fused only with amoebae carrying different mating type alleles. He also obtained preliminary evidence (1970, 1971) which indicated that $\underline{mt}_h$ amoebae underwent cell and nuclear fusion within clones (i.e. they were homothallic). He thus argued (1971) that the analysis of amoebal-plasmodial transition mutants would be of particular value in the investigation of the mechanisms of cell and nuclear fusion.

Cooke and Dee (1974) showed that nuclear fusion was not involved in the formation of plasmodia within clones of $\underline{mt}_h$ amoebae. Two possibilities remained for the formation of multinucleate plasmodia from uninucleate $\underline{mt}_h$ amoebae: cell fusion without nuclear fusion (coalescence), or repeated nuclear divisions within a single cell (apogamy).

Since nuclear fusion did not occur in the formation of plasmodia in clones of $\underline{mt}_h$ amoebae, it was clearly not possible to obtain mutants defective only in nuclear fusion by isolating amoebal-plasmodial transition mutants from $\underline{mt}_h$ strains. If plasmodium formation was by coalescence, then it would be expected that amoebal-plasmodial transition mutants defective only in cell fusion might be isolated from $\underline{mt}_h$ strains, but this class would be absent if plasmodium formation was by apogamy.

If $\underline{mt}_h$ plasmodium formation occurred through coalescence of amoebae, hybrid plasmodia should arise from mixtures of $\underline{mt}_h$ strains carrying different genetic markers. This was found to be the case by Wheals (1970, 1971) when he mixed strains differing in their plasmodial fusion alleles. However, Cooke (1974; Cooke and Dee, 1974) attempted to repeat and extend Wheals' observation, and obtained inconclusive results.

Section 2.2 reports further work, also using mixtures of $\underline{mt}_h$ amoebae carrying different genetic markers, to distinguish between apogamy and coalescence. For reasons which are discussed, this analysis proved inconclusive, and a direct demonstration of apogamy or coalescence was attempted, using time-lapse microcinematography. This is reported in Section 2.3.

Complementary to the search for evidence of cell fusion within clones of $\underline{mt}_h$ amoebae was the search for evidence of fusion within clones of amoebae carrying heterothallic mating type alleles. Dee (1966), referring to the formation of crossed plasmodia within mixtures of amoebae of different mating types, pointed out that "although it is probable that ..... the mating type factors determine whether or not a pair of amoebae which happen to meet will fuse, the mating type factors .... may also act at other stages, affecting for example .... the development of

the fused cells into a visible plasmodium". If the mating type locus controlled amoebal fusion, then fusions within heterothallic clones should not have been detectable; otherwise they should have occurred. An investigation to determine whether such fusions occurred is reported in Section 2.4.

2.2 GENETIC ANALYSIS OF CELL AND NUCLEAR FUSION IN PLASMODIUM FORMATION BY *mt*$_h$ AMOEBAE

Plasmodia expressing different fusion types do not fuse with one another. This, in principle, makes it possible to distinguish between apogamy and coalescence. Mixtures of *mt*$_h$ amoebae carrying different *fusA* alleles are mixed together; if plasmodia arise by apogamy, only plasmodia of groups I (*fusA1*) and V (*fusA2*) will be obtained, while if plasmodia arise by coalescence, some plasmodia of the fusion genotype *fusA1*/*fusA2* will be formed. Since the *fusA1* and *fusA2* alleles are co-dominant, hybrid plasmodia will be recognisable as they will express a third fusion type, group III. Wheals (1970, 1971) reported that mixtures of *mt*$_h$ amoebae carrying different alleles at the *fusA* locus gave rise to plasmodia of hybrid fusion type. This evidence was thus suggestive of coalescence.

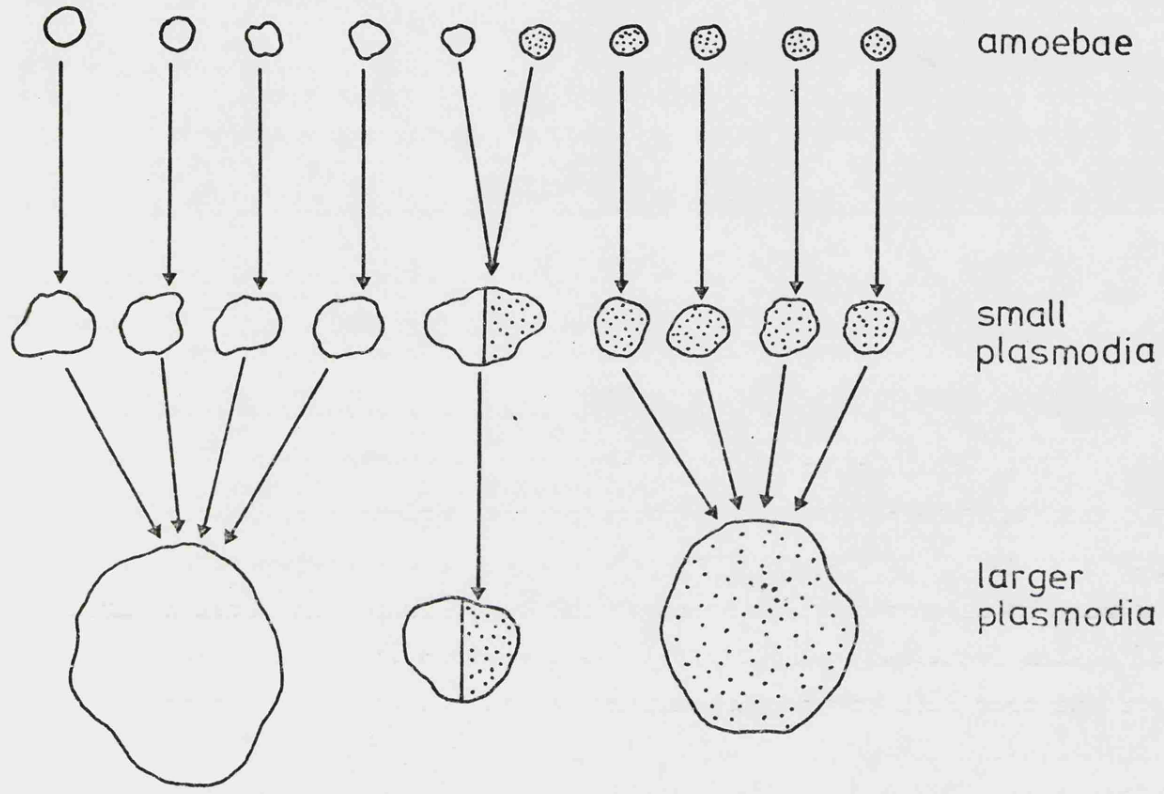
If all plasmodia in a culture arise by coalescence of pairs of amoebae, random fusions within a perfectly mixed population comprising equal numbers of two amoebal strains will theoretically yield "selfed" and hybrid plasmodia in the ratio 1 : 1. If more than two amoebae can coalesce together, the expected frequency of hybrid plasmodia will be greater than 50%. Alternatively, if some plasmodia arise apogamically,

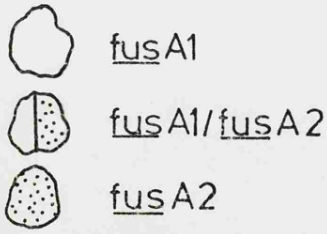
or if cells of the two strains are not perfectly mixed, or if there are unequal numbers of cells of the two strains, then the expected frequency of hybrid plasmodia will be less than 50%.

A significant proportion of hybrid plasmodia may be recovered even if almost all plasmodia arise by apogamy and only a few by coalescence. This is because, in practice, it is not possible to pick plasmodia as they arise; they must be allowed to grow until large enough to be identified and transferred to SDM agar plates. During this time plasmodia of like fusion type presumably meet and fuse with one another. Thus, as shown in Figure 12, the frequency of hybrid plasmodia recovered may be much higher than the frequency with which amoebae carrying different fusA alleles coalesce.

Cooke (1974) mixed amoebae which differed in their alleles at the fusA locus and also, in some cases, at the leu-1 locus. Out of a total of 136 plasmodia isolated from such mixtures and tested for their fusion behaviour, three were of hybrid fusion type. Analyses of nuclear DNA contents were consistent with these plasmodia being haploid heterokaryons and genetic analysis of progeny of one hybrid plasmodium failed to show any recombination of alleles at the unlinked fusA and leu-1 loci. Since less than 3% of plasmodia were of hybrid fusion type, Cooke interpreted his results as suggestive of apogamy. To explain the origin of the few hybrid plasmodia he obtained, he pointed out that "the transition from a uninucleate haploid amoeba to a uninucleate haploid plasmodium would not be instantaneous .... a period of time might exist when cell fusion would be prevented neither by identity at the mt_h locus nor by differences at the fusA locus. Contact of cells during this period might result in the production of a small proportion of hybrid plasmodia".

Figure 12. Possible mechanism by which a mixture of $\underline{mt}_h$;*fusA1* and $\underline{mt}_n$;*fusA2* amoebae could yield a significant proportion of group III (*fusA1*/*fusA2*) plasmodia, even if most plasmodia arose by apogamy. The rare occurrence of coalescence would yield small plasmodia of group III fusion behaviour. These group III plasmodia would be unlikely to meet and fuse with one another during the period of growth before larger plasmodia were picked from the culture. Group I (*fusA1*) plasmodia would be likely to meet and fuse with other plasmodia of the same fusion type in this period, thus reducing the total number of group I plasmodia. Similarly for group V (*fusA2*) plasmodia.


amoebae
small plasmodia
larger plasmodia


fusA1
fusA1/fusA2
fusA2

The experiments described in this section were carried out in an attempt to repeat and extend the above observations. The work was carried out jointly with Dr. Jennifer Dee.

2.2a Materials and Methods

Methods were as described in Chapter 1. All cultures were incubated at 26°C.

Strains	Amoebae:	LU640	mt_h;*fusA1*;*leu-1*$^+$
		CL5001	mt_h;*fusA2*;*leu-1*$^-$
	Plasmodia:	fusion testers of groups:	
		I	*fusA1*
		III	*fusA1*/*fusA2*
		V	*fusA2*

All strains carried the *fusB1* and *fusC1* alleles.

2.2b Results

(i) Preliminary attempt to mate mt_h strains carrying different *fusA* alleles In a direct attempt to repeat the observations of Cooke (1974) loopfuls of amoebae of strains LU640 (mt_h;*fusA1*) and CL5001 (mt_h;*fusA2*) were mixed in 0.05 ml puddles of bacterial suspension on DSDM agar plates. Control puddles of the two strains alone were also set up. After incubation for one day the puddles were stirred with a wet loop to promote inter-strain contact. After three days each puddle contained many small plasmodia, which were too close together to allow them to be picked individually. Five blocks, approximately 1 mm^3, were cut from each puddle and placed on separate SDM agar plates; each block carried one or more plasmodia. Plasmodia which arose on the

SDM agar plates were tested for fusion with plasmodia of groups I (fusA1), III (fusA1/A2) and V (fusA2). Where two non-fusing plasmodia arose on a single SDM plate (indicating that the inoculum block had carried plasmodia of more than one fusion type) both plasmodia were tested. The results (Table 4) agreed with those obtained by Cooke (1974) in that all but one of the 111 plasmodia were apparently derived directly from one or other of the two amoebal strains.

Since the formation of plasmodia within plaques on LIA was routinely about one day slower than on DSDM agar, it was reasoned that amoebae might have more opportunity to undergo inter-strain coalescence if the experiment described above was repeated on LIA plates. This was done, with the modification that the cultures were stirred after two days; it was expected that plasmodia would appear after four days, so that in both experiments the stirring would be carried out two days before the picking of plasmodia. However, many plasmodia were visible in nearly all puddles on LIA plates after only three days, including control puddles of the strains alone. Plasmodia were picked and tested for fusion behaviour as before (see Table 5). The results from puddles on LIA did not agree well with those obtained on DSDM agar; about 15% of plasmodia tested could not be attributed to only one or other of LU640 and CL5001.

The observation that some plasmodia fused with both group III and group V testers was unexpected. It was concluded that plasmodia of this class were probably derived in some way from both amoebal strains. Further work to investigate the basis of this behaviour is reported in (iv).

The ratio of numbers of group I (fusA1) to group V (fusA2)

Table 4. Fusion behaviour of plasmodia isolated after mixing LU640 and CL5001 amoebae on DSDM agar

Fusion behaviour with tester plasmodia of groups: I	III	V	Number in class	Deduced fusion group		Deduced amoebal origin of plasmodia
F	NF	NF	31	I	(*fusA1*)	LU640
NF	F	NF	0	III	(*fusA1*/*fusA2*)	LU640 + CL5001
NF	F	F	1	III/V	(*fusA1*/*fusA2*)	LU640 + CL5001
NF	NF	F	79	V	(*fusA2*)	CL5001
		TOTAL	111			

F = fusion. NF = non-fusion.

Genotype of LU640 amoebae mt_h;*fusA1* (control puddles of LU640 amoebae yielded group I plasmodia).

Genotype of CL5001 amoebae mt_h;*fusA2* (control puddles of CL5001 amoebae yielded group V plasmodia).

Table 5. Fusion behaviour of plasmodia isolated after mixing LU640 and CL5001 amoebae on LIA

Fusion behaviour with tester plasmodia of groups: I	III	V	Number in class	Deduced fusion group		Deduced amoebal origin of plasmodia
F	NF	NF	6	I	(*fusA1*)	LU640
NF	F	NF	9	III	(*fusA1*/*fusA2*)	LU640 + CL5001
NF	F	F	12	III/V	(*fusA1*/*fusA2*)	LU640 + CL5001
NF	NF	F	118	V	(*fusA2*)	CL5001
		TOTAL	145			

F = fusion. NF = non-fusion.

Genotype of LU640 amoebae mt_h;*fusA1* (control puddles of LU640 amoebae yielded group I plasmodia).

Genotype of CL5001 amoebae mt_h;*fusA2* (control puddles of CL5001 amoebae yielded group V plasmodia).

plasmodia was significantly less than 1 : 1 ($p < 0.01$) for puddles on LIA and DSDM agar. This was presumably because there were fewer fusA1 amoebae in the puddles than fusA2. The frequency of hybrid plasmodia predicted if plasmodia were formed only by coalescence of pairs of amoebae would thus be less than 50%. However, assuming perfect mixing, the frequency of hybrid plasmodia should always have been greater than that of fusA1 plasmodia, as was found for puddles on DSDM agar.

No firm conclusions about the nature of plasmodium formation by mt_h amoebae could be drawn from this experiment, though the result suggested that the frequency of hybrid plasmodia might be influenced by the agar medium or the time of stirring the puddles.

(ii) Effect of different agar media and stirring times on the frequency of hybrid plasmodia The preliminary experiment described in (i) indicated two factors which might affect the proportion of hybrid plasmodia recovered from mixtures of fusA1 and fusA2 amoebae: the agar medium and the time at which the puddles were stirred. If hybrids appeared mostly in puddles stirred at the time of plasmodium formation, it would be evidence for fusion of incipient plasmodia. In order to investigate these possibilities a further series of puddles was set up on both DSDM agar and LIA, again using amoebae of strains LU640 and CL5001. For each medium different sets of puddles were stirred after one, two, three, and four days, and a further set was not stirred. Plasmodia were picked after four days and tested as before. The results are shown in Table 6.

The ratio of numbers of group I plasmodia to group V plasmodia was significantly different from 1 : 1 for only one of the ten sets of puddles ($p > 0.05$ except for LIA/day 3, where $p < 0.01$). It was thus assumed that most puddles contained approximately equal numbers of amoebae

Table 6. Fusion behaviour of plasmodia isolated after mixing LU640 and CL5001 amoebae on DSDM agar and LIA, the cultures being stirred after different times

Medium	Day stirred	Deduced fusion group of plasmodia:* I	III	V
DSDM	1	14	1	18
"	2	13	1	16
"	3	12	0	17
"	4	17	0	12
"	NS**	13	0	13
	TOTALS	69	2	76

Medium	Day stirred	Deduced fusion group of plasmodia: I	III	V
LIA	1	14	1	13
"	2	17	2	10
"	3	7	0	22
"	4	18	1	18
"	NS	10	0	17
	TOTALS	66	4	80

* Fusion groups deduced as shown in Tables 4 and 5.

** NS = not stirred.

Genotype of LU640 amoebae $\underline{mt}_h$;$\underline{fusA1}$ (control puddles of LU640 amoebae yielded group I plasmodia).

Genotype of CL5001 amoebae $\underline{mt}_h$;$\underline{fusA2}$ (control puddles of CL5001 amoebae yielded group V plasmodia).

of the two strains at the time of plasmodium formation. No plasmodia were of fusion type III/V. Only four out of 150 plasmodia picked from puddles on LIA were of group III, and only two group III plasmodia were picked from DSDM agar, out of a total of 147.

This experiment failed to establish a connection between the type of medium or time of stirring and the frequency of hybrid plasmodia. In all treatments hybrid plasmodia arose at low frequency or not at all.

(iii) Effect of pre-incubating amoebal strains at 4°C In the experiment described in (ii), all the puddles were set up at the same time, from the same stock plates of growing amoebae. In the preliminary experiment described in (i), the puddles on DSDM agar were set up from plates of growing amoebae but the puddles on LIA were set up from the same plates after they had been stored at 4°C overnight. Although microscopic observation of filming slide cultures had indicated that cold-encysted amoebae excysted within a few hours at 26°C, it was thought possible that the cold treatment might have increased the frequency of hybrid plasmodia in (i). Thus a further set of puddles was inoculated with LU640 and CL5001. All puddles were set up on LIA, half from plates of growing amoebae and half from the same plates after storage overnight at 4°C. Within each set of puddles some were stirred after one day, some after two days and the remainder left unstirred. Plasmodia were picked after four days and tested as before. The results are shown in Table 7.

Out of a total of 77 plasmodia picked from puddles inoculated with growing amoebae, 10 were of fusion group III and 11 of group III/V. The overall ratio of group I to group V plasmodia from these puddles was not significantly different from 1 : 1 ($0.05 > p > 0.01$). In contrast, only a small proportion of plasmodia picked from puddles inoculated with

Table 7. Fusion behaviour of plasmodia isolated after mixing LU640 and CL5001 amoebae on LIA, the cultures being inoculated with growing or cold-treated amoebae and stirred after different times

Cultures inoculated with growing amoebae				
Day stirred	Deduced fusion group of plasmodia:* I	III	III/V	V
1	8	4	9	6
2	4	4	2	14
NS**	8	2	0	16
TOTALS	20	10	11	36

Cultures inoculated with cold-treated amoebae				
Day stirred	Deduced fusion group of plasmodia: I	III	III/V	V
1	0	0	0	20
2	9	0	3	10
NS	6	0	0	16
TOTALS	15	0	3	46

* Fusion groups deduced as shown in Tables 4 and 5.

** NS = not stirred.

Genotype of LU640 amoebae $\underline{mt}_h$;$\underline{fus}$A1 (control puddles of LU640 amoebae yielded group I plasmodia).

Genotype of CL5001 amoebae $\underline{mt}_h$;$\underline{fus}$A2 (control puddles of CL5001 amoebae yielded group V plasmodia).

cold-treated amoebae were of hybrid fusion type; none out of 64 was of group III and only three were of group III/V. Four puddles inoculated with cold-treated amoebae and stirred after one day gave rise only to group V plasmodia. This might have been due to failure of LU640 amoebae to form plasmodia under these conditions, or to inoculation of the puddles with only CL5001 amoebae. The ratio of group I to group V plasmodia from the remaining puddles (15 : 26) was not significantly different from 1 : 1 ($p > 0.05$).

Puddles set up with cold-treated amoebae gave fewer plasmodia of groups III and III/V than puddles set up with growing amoebae. Since this was the reverse of the predicted result, it was concluded that the evidence did not support the view that cold-treatment affected the frequency of hybrid plasmodia.

(iv) Analysis of plasmodia expressing hybrid fusion types

Cooke (1974) showed, from evidence of nuclear DNA contents and progeny analysis, that the hybrid plasmodia he isolated from mixtures of mt_h;*fusA1* and mt_h;*fusA2* amoebae were haploid heterokaryons. Poulter (1969) showed that the fusion behaviour of heterokaryons containing nuclei carrying different fusion alleles depended upon the relative proportions of the nuclear types. He constructed heterokaryons from plasmodia expressing different *fusA* alleles (but not *fusA1* and *fusA2*) and found that where either of the two nuclear types comprised more than about 10% of the total, the heterokaryon expressed hybrid fusion behaviour; where the minority class was less than about 10% of the total, the plasmodium expressed the fusion type of the majority class.

It was reasoned that the group III/V plasmodia might be haploid heterokaryons in which the proportions of *fusA1* and *fusA2* nuclei were at some critical level, leading to expression of both III and V fusion

behaviour (though it was not clear why a corresponding I/III class was not found). To test this hypothesis, five group III/V plasmodia and five group III plasmodia were caused to sporulate (the plasmodia were chosen from those isolated in (i) and (iii)). Approximately 20 amoebal progeny clones of each plasmodium were isolated and allowed to form clonal plasmodia. These plasmodia were tested for fusion behaviour and leucine requirement. Table 8 shows details of the analysis of one group III/V plasmodium. Table 9 summarises the results of similar analyses of all ten plasmodia.

The results confirm the assumption that the group III/V plasmodia were derived from both LU640 and CL5001, since progeny of two of the five III/V plasmodia included both fusA1 and fusA2 types. The proportion of fusA1 progeny from these two plasmodia was very low, probably explaining the failure of recover fusA1 progeny from the other three III/V plasmodia.

The ratio of fusA1 to fusA2 progeny of the group III/V plasmodia differed greatly from 1: 1, indicating that the III/V plasmodia were probably heterokaryons of some sort. Progeny showed only the combinations of fusA and leu-1 alleles carried by LU640 and CL5001, and the absence of recombinant types was consistent with the III/V plasmodia being haploid heterokaryons. However, there was only a small number of fusA1 progeny; more progeny would have to be examined to exclude the possibility that the plasmodia contained some diploid fusA1/fusA2 nuclei. As expected from Cooke's (1974) results, no recombination of fusA and leu-1 alleles was detected in the progeny of group III plasmodia.

If the fusion behaviour of group III/V plasmodia was due to their carrying fusA1 and fusA2 nuclei in critical proportions, then group III/V plasmodia would be expected to contain a smaller proportion of fusA1

Table 8. Analysis of plasmodia formed from amoebal progeny clones of the plasmodium (LU640 x CL5001)1

Fusion behaviour with plasmodia of groups: I	III	V	Deduced *fusA* genotype	Growth of plasmodia on: DM-1	DM-1 + leucine	Deduced *leu*-1 genotype	Number in class
F	NF	NF	*fusA*1	+	+	*leu*-1^{+}	1
				-	+	*leu*-1^{-}	0
NF	NF	F	*fusA*2	+	+	*leu*-1^{+}	0
				-	+	*leu*-1^{-}	17
						TOTAL	18

F = fusion. NF = non-fusion.

Genotype of LU640 amoebae *mt*$_h$;*fusA*1;*leu*-1^{+} (control puddles of LU640 amoebae yielded group I plasmodia which grew on DM-1 agar).

Genotype of CL5001 amoebae *mt*$_h$;*fusA*2;*leu*-1^{-} (control puddles of CL5001 amoebae yielded group V plasmodia which died on DM-1 agar).

Table 9. Analysis of plasmodia formed from amoebal progeny clones of the plasmodia (LU640 x CL5001)1-10

Progeny derived from group III/V plasmodia				
Plasmodia	Deduced genotypes of progeny clones:**			
	fusA1;*leu-1*$^+$	*fusA1*;*leu-1*$^-$	*fusA2*;*leu-1*$^+$	*fusA2*;*leu-1*$^-$
(*)1	1	0	0	17
(*)2	0	0	0	20
(*)3	0	0	0	20
(*)4	0	0	0	20
(*)5	1	0	0	19
TOTALS	2	0	0	96

Progeny derived from group III plasmodia				
Plasmodia	Deduced genotypes of progeny clones:			
	fusA1;*leu-1*$^+$	*fusA1*;*leu-1*$^-$	*fusA2*;*leu-1*$^+$	*fusA2*;*leu-1*$^-$
(*)6	0	0	0	20
(*)7	0	0	0	20
(*)8	16	0	0	2
(*)9	1	0	0	16
(*)10	0	0	0	19
TOTALS	17	0	0	77

(*) = (LU640 x CL5001)

** Genotypes deduced as shown in Table 8.

Genotype of LU640 amoebae mt_h;*fusA1*;*leu-1*$^+$.

Genotype of CL5001 amoebae mt_h;*fusA2*;*leu-1*$^-$.

nuclei than group III plasmodia. In addition, the proportion of fusA1 nuclei in all group III/V plasmodia would probably be very similar, while that in group III plasmodia might vary over a wide range. The results shown in Table 9 were consistent with this view, since progeny of III/V plasmodia were almost all fusA2, while the ratio fusA1 : fusA2 in progeny of group III plasmodia varied widely. However, it was clear that larger numbers of progeny of more plasmodia would have to be tested for any firm conclusions to be drawn. The results indicated that the critical proportion of fusA1 nuclei for group III/V behaviour might be much less than the 10% which the results of Poulter (1969) suggested. However, nuclear ratios in the heterokaryons may have changed subsequent to fusion testing, or there may have been differential viability of fusA1 and fusA2 nuclei during sporulation; maintenance of nuclear ratios through subculturing and sporulation of heterokaryotic plasmodia seems to occur in some cases, but not in others (Haugli, 1971; Anderson, 1973; Cooke, 1974).

2.2c Discussion

The work described in this section provided no clear support for either apogamy or coalescence. Cooke (1974) interpreted his results as supporting apogamy, since he obtained only 3% hybrid plasmodia instead of the 50% he expected from coalescence. In (i)-(iii) plasmodia of fusion groups III and III/V represented less than 10% of the total tested. This, also, was less than half, but coalescence predicted 50% hybrids only under certain defined conditions (i.e. when all plasmodia were formed by coalescence which always involved fusion of pairs of amoebae in puddles where equal numbers of the two strains were perfectly mixed). Deviation from these conditions could have reduced the proportion of

hybrids. Although the three factors tested as possible influences on the frequency of hybrid plasmodia were not shown to have any effect, there were large differences in hybrid frequencies between treatments and experiments, and in one case the frequency approached 50%.

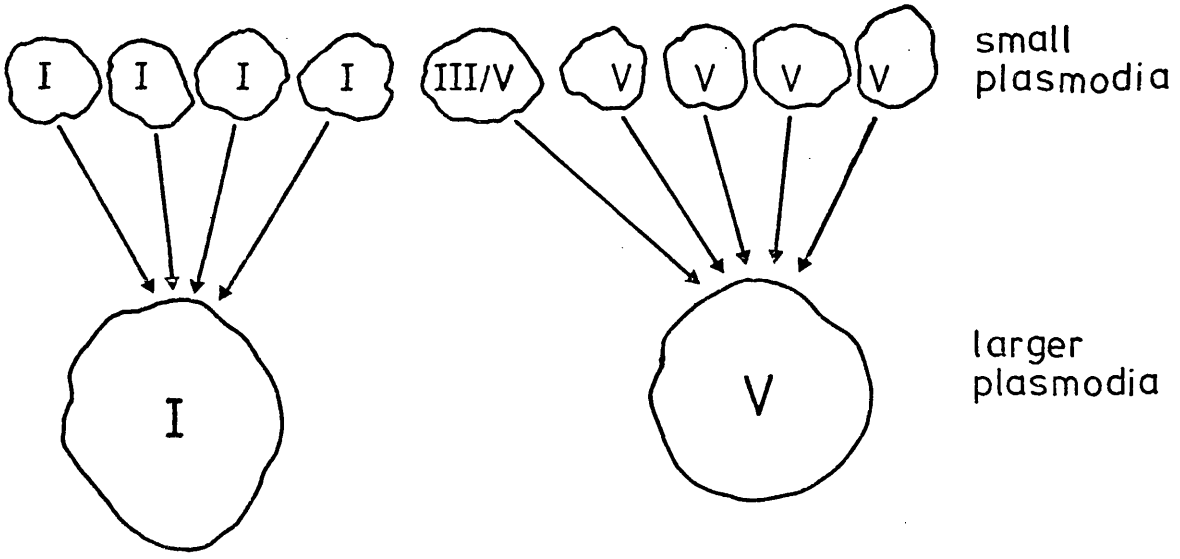
As already noted, it could be argued that these experiments gave no real measure of the frequency of hybrid plasmodium formation. A high proportion of group III plasmodia might be generated if most plasmodia formed apogamically, but a few amoebal fusions occurred also (see Figure 12). The frequency of group III/V plasmodia could not have been enhanced in the same way (see Figure 13), so that the frequencies of both group III and group III/V plasmodia may in fact have been a fair reflection of the frequencies of inter-strain fusions. However, it is not clear whether these fusions involved amoebae; as Cooke (1974) pointed out, the recovery of hybrid plasmodia could be due not only to coalescence of amoebae but also to fusion of cells in the process of forming plasmodia by apogamy.

Although the refinement of this type of experiment, leading to discovery of the factors which influenced the hybrid frequency, might have given evidence more strongly suggestive of apogamy or coalescence, it was decided to abandon indirect genetic analysis and attempt a direct demonstration of the way in which $\underline{mt}_n$ plasmodia were formed.

2.3 CINEMATOGRAPHIC ANALYSIS OF PLASMODIUM FORMATION WITHIN CLONES OF $\underline{mt}_n$ AMOEBAE

N. Kerr (1967) reported conclusive evidence showing plasmodium formation in the minute S3 mutant of D. nigripes by apogamy. The main evidence was a low power time-lapse film which recorded continuously the

Figure 13. Possible fate of group III/V plasmodia arising in mixtures of $\underline{mt}_h$;$\underline{fusA1}$ and $\underline{mt}_h$;$\underline{fusA2}$ amoebae.


I
I
I
I
III/V
V
V
V
V
small plasmodia
I
V
larger plasmodia

behaviour of cells within a colony. Nuclei could not be observed, but plasmodia were seen to arise only from individual products of amoebal divisions. Discontinuous high power phase contrast observations confirmed the results of the low power study; binucleate cells were seen to form by nuclear division within uninucleate cells, and at least one of these uninucleate cells was the product of an amoebal division. Binucleate cells gave rise to plasmodia by further nuclear divisions and fusions with other binucleate cells. Uninucleate cells were normally engulfed and digested by binucleate cells, but on one occasion a uninucleate cell fused with a binucleate cell. Kerr suggested that this uninucleate cell had already differentiated into a plasmodium. Kerr also presented pictures of what he interpreted as delayed cytoplasmic fission in some binucleate cells, yielding pairs of uninucleate cells. These divisions were characterised by long cytoplasmic strands between the two halves of the dividing cell; this was quite distinct from the normal mode of division in uninucleate cells.

The aim of the work described in this section was to analyse the formation of mt_h plasmodia by the method successfully used by Kerr for D. nigripes. Filming was carried out in conjunction with Mr. (now Dr.) D.J. Cooke, who has presented a preliminary report (Cooke, 1974). Detailed analysis of results was carried out jointly with Dr. Jennifer Dee. The work has been fully presented elsewhere (Anderson et al., 1976).

2.3a Materials and methods

Methods were as described in Chapter 1, with additional methods as shown below:

(i) Strains Amoebae: CL mt_h

CL5001 mt_h

(ii) Microcinematography A Wild-Bolex time-lapse system was used, with Bolex-Wild Variotimer modules (MBF-B and MBF-C) controlling the camera (Bolex cine camera H16R X-5), motor (MBF-A) and an electromagnetic shutter which prevented illumination of cultures except during exposures. The system allowed exposures of 0.2 - 10 sec, with intervals between exposures of 0.3 sec-6 hours. Exposures were usually set at 0.2 sec to give minimum blurring of image through movement of cells. A Wild exposure meter and calibration chart were used to set the appropriate light intensity. Ilford Pan F 16 mm negative film (100 ft spools) was used and photographs reproduced in this section were printed from individual frames. Bulk processing and printing were by Brent Laboratories Ltd., London.

Low power films were made with the camera mounted on a Wild M-5 stereo microscope. Magnifications of x12-50 on the microscope corresponded to x3-12.5 in the film plane. Low power observations were of colonies growing on E. coli in DSDM agar plates. High power films of filming slide cultures were made with the camera mounted on a Wild M-20 research microscope with phase-contrast optics. Illumination was by a Wild quartz-iodine base. Observation was with x10 or x20 objectives and x10 photo eyepiece. This gave magnifications of x25-50 in the film plane.

All filming was carried out in a 26°C constant-temperature room and a small fan was used to ensure that cultures did not become overheated.

(iii) Analysis of films Films were analysed using a Specto Motion Analysis Projector Mk. III, with a capacity for forward and reverse

projection at speeds of 2, 4, 8, and 16 frames per second, and single frame projection. These facilities proved essential in the detailed analysis of films, since single frames could often be misinterpreted.

Cell and nuclear areas were estimated by projecting the film onto a screen divided into approximately 4000 squares, each equivalent to a cell area of 7.4 μm^2.

2.3b Results

(i) Low power films N. Kerr (1967) used a strain of D.nigripes which formed plasmodia within colonies of only a few cells when he demonstrated apogamy. No comparable mutant strains existed to facilitate the analysis in P. polycephalum. Plaques of mt_h strains, such as CL and CL5001, each contain up to about 10^4 amoebae at the time of plasmodium formation, and Figure 7 (see Chapter 1) shows that most of these amoebae are concentrated in a ring around the periphery of the plaque. Individual cells cannot be distinguished in the ring, but the cells in the central area are mostly well spaced out. Plasmodia are seen in the dense part of the plaque and also in the central area. It was thought that the origin of plasmodia in the central parts of mt_h colonies might be traced by low power time-lapse microcinematography.

Two films were made, showing the development of a CL plaque and a CL5001 plaque. Filming was begun two and three days after inoculation, respectively. Magnifications were in the range x3-12.5 in the film plane, progressively lower magnifications being used to keep plaques within the field as they grew larger. Intervals between exposures were 45 sec.

Some individual amoebae (but not nuclei) and amoebal divisions were clearly visible in the central area of each plaque. However,

plasmodia did not appear to form in the sparsely populated central areas; they arose in the dense outer ring and then sometimes migrated inwards to the centre. Low power filming was thus abandoned.

(ii) High power films In order to follow individual cells in $\underline{mt}_h$ cultures undergoing the amoebal-plasmodial transition, it was necessary to use high power phase-contrast observation of filming slide cultures. Problems were encountered in choosing optimum conditions. At a magnification in the film plane of x50 it was possible to observe clearly cells and their nuclei, but the field of observation was limited to approximately 0.2 x 0.15 mm (25-50 cells). Since cells were constantly entering and leaving the field, due to active amoeboid movement, individual cells could only be observed for a few hours unless followed by periodic field changes. At a magnification in the film plane of x25, individual cells and nuclei became indistinguishable in dense parts of the culture and when the microscope went slightly out of focus (it was found necessary to refocus the apparatus every 20-30 minutes). However, the field of observation was four times greater than at x50, so that it was theoretically feasible to follow individual cells for longer periods.

Fourteen films were made of the development of CL plaques at magnifications of x25 and x50. Many of these did not show plasmodia because of unsatisfactory cultures, equipment malfunction or, usually, failure to form plasmodia in the part of the culture under observation. Most films showed amoebal divisions and several also showed binucleate cells forming by nuclear division within uninucleate cells. No fusions of uninucleate cells were observed. It was usually impossible to trace the origin or fate of individual binucleate cells, for the reasons already described. However, one film provided an almost complete record of the

events of plasmodium formation. Detailed analysis of this film is described in (iii).

(iii) Detailed analysis of a high power film showing plasmodium formation in strain CL The development of a CL culture over the period 48-80 hours after inoculation was analysed in detail. Times of events described below are expressed as the number of hours after inoculation. In the early stages of colony growth, the field of observation was not changed since the cells which gave rise to plasmodia were not then identifiable. Intervals between exposures of the film were of 10 seconds duration.

Early stages of the film showed a population of active, uninucleate amoebae. Eighteen amoebae divided during the film (Figure 14), all but one in the period 48-64 hours. The first indication that a cell was about to divide came when locomotion ceased. The cell became rounded. The nucleolus moved to the side of the nucleus and disappeared. The nucleus itself became indistinct. Cell division occurred approximately 15 minutes after nucleolar disappearance. Locomotion of the daughter cells began and their nucleoli became visible. Nucleolar reconstruction was completed at a minimum of 40 minutes after division.

From 51 hours onwards, uninucleate cells larger than those initially present were discernible in the field. Thirteen of these cells became binucleate as a result of nuclear division (Figure 15). All binucleate cells whose formation was observed arose in this way; no fusions of uninucleate cells were seen.

The first stage observed in the nuclear division from which each binucleate cell arose was movement of the nucleolus to the side of the nucleus. However, as in amoebal divisions, the nucleolus disappeared after locomotion had ceased and the cell had rounded up. After nucleolar disappearance, the nucleus itself became indistinct, though in many cases

Figures 14 and 15.

Figure 14 (left column). Division of uninucleate cell.

Top: Nucleolus (arrow) still visible. x 1000

Middle: Nucleolus no longer visible in nucleus (arrow). x 1000

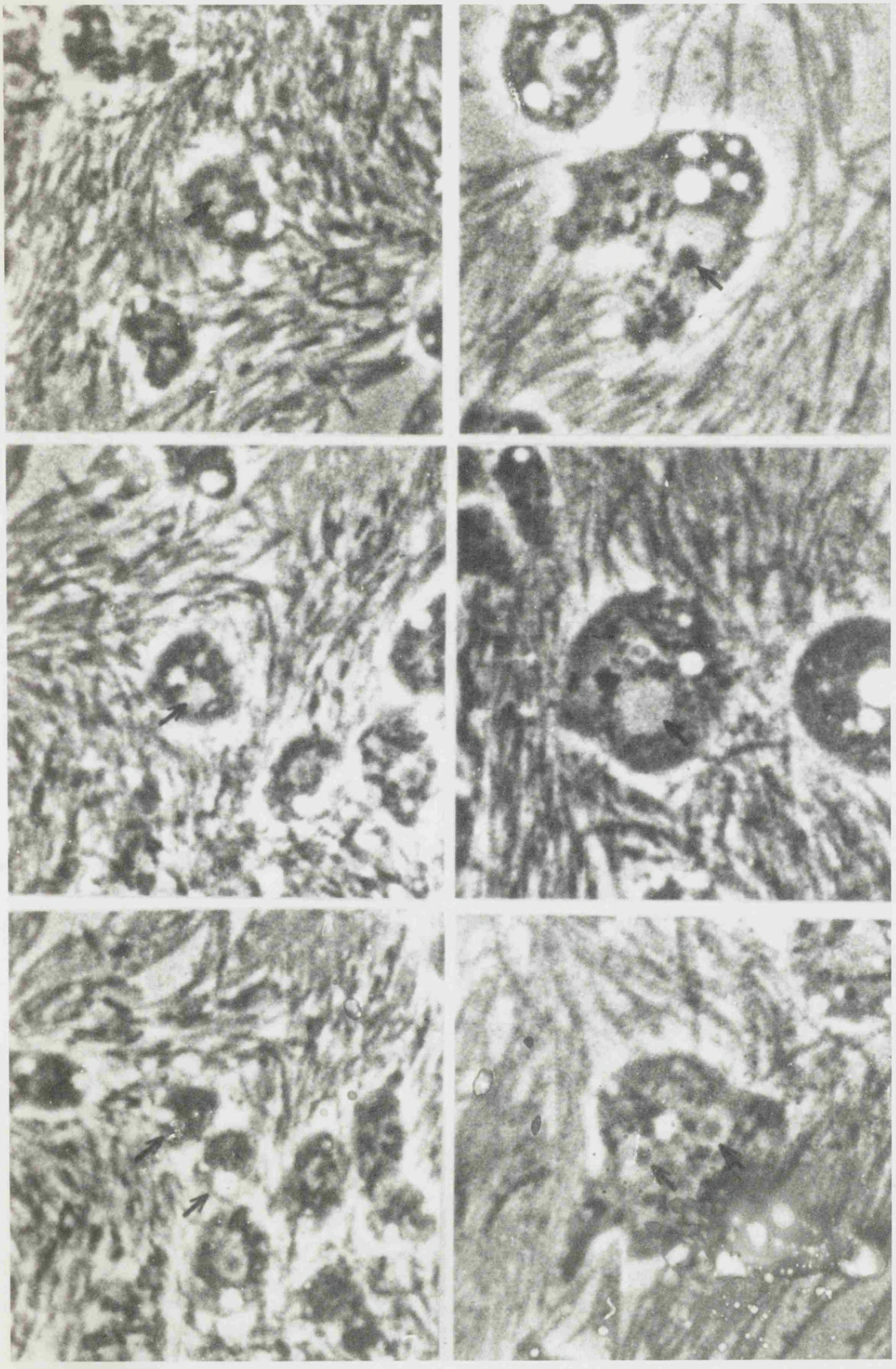
Bottom: Daughter cells (arrows). White "holes" are contractile vacuoles. x 1000

Figure 15 (right column). Binucleate cell formation.

Top: Nucleolus (arrow) has moved to side of nucleus. x 1000

Middle: Nucleolus no longer visible in nucleus (arrow). x 1000

Bottom: Binucleate cell. Nucleoli visible in nuclei (arrows). x 1000



the metaphase plate could be seen. Anaphase was also visible in one cell. Locomotion was resumed and two nuclei appeared in the cell. Nucleolar reconstruction was completed approximately 55 minutes after the original nucleolus had disappeared. While the nucleus was indistinct, cells frequently appeared to begin cleavage, but this was not completed.

All cells which were observed to become binucleate or to divide were measured in order to compare their cell and nuclear areas (Figure 16). Effects of growth during the cell cycle were eliminated by measuring all cells just before nucleolar disappearance. Comparison of means (using t-tests) indicated that cell and nuclear areas of cells which became binucleate were significantly larger than those of cells which divided.

Attempts were made to trace each large uninucleate cell back to its origin. In each case the cell was found to have entered the field a short time before becoming binucleate; thus it was not possible either to follow cell growth or to observe a previous cell division.

The first binucleate cell formed subsequently rounded up and failed to develop further. All other binucleate cells, arising from 57 hours onwards, continued active movement. Three of these cells participated in the formation of a plasmodium (see below). The development of most of the other cells could not be followed because of their active migration, but 2 of them were seen to undergo abnormal divisions. These were clearly distinguishable from amoebal divisions, since they occurred after nucleolar reconstruction in the binucleate cells was complete, when they had resumed locomotion. Two portions of the cytoplasm, each containing a nucleus, pulled out in opposite directions, leaving a thin cytoplasmic bridge between them. This was eventually broken to give 2 uninucleate cells whose subsequent fate was not followed.

At about 70 hours, 2 binucleate cells each underwent a nuclear division to become quadrinucleate (Figure 17). In each cell the nuclei

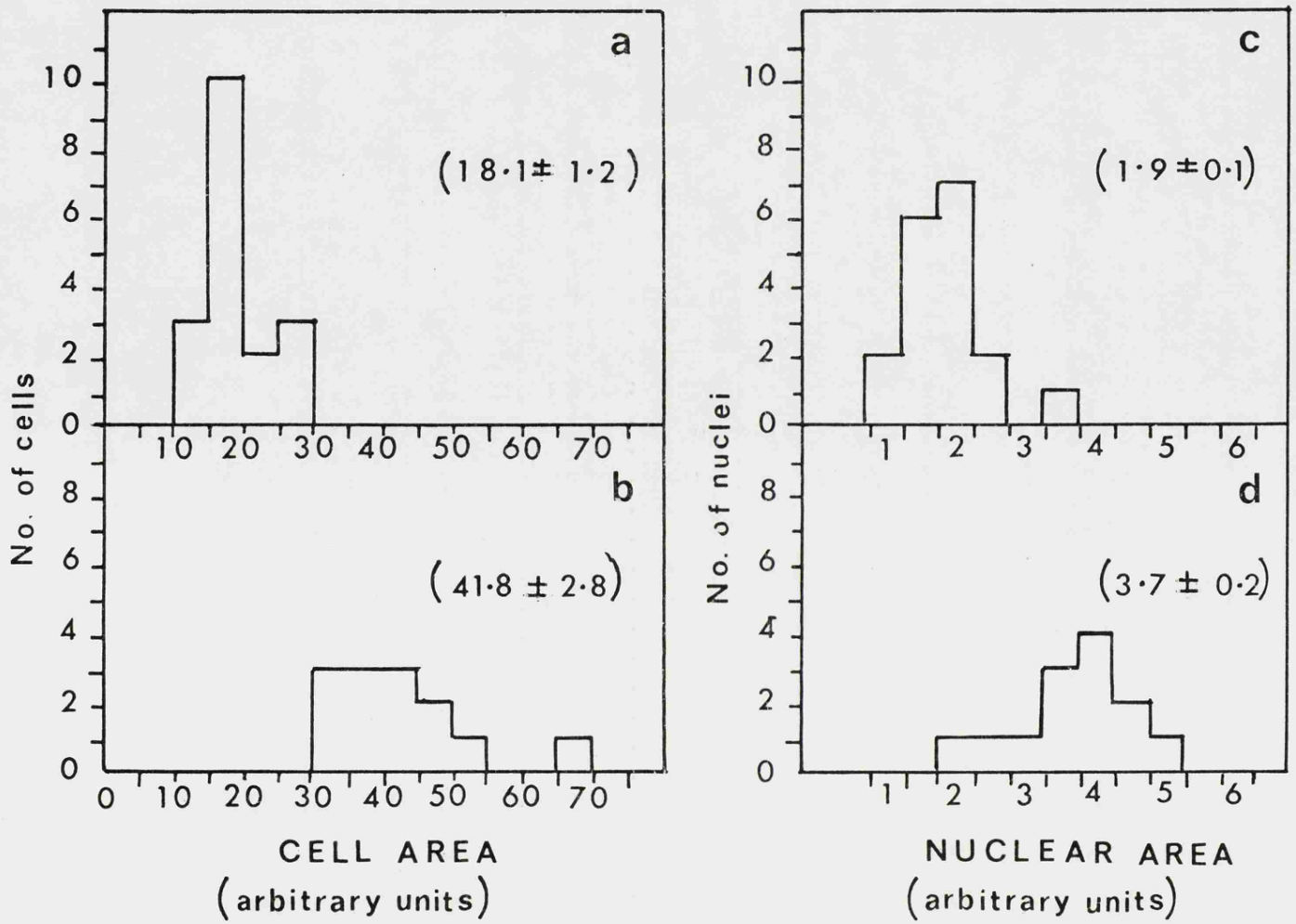

a
(18·1± 1·2)
b
(41·8 ± 2·8)
No. of cells
CELL AREA
(arbitrary units)
c
(1·9 ±0·1)
d
(3·7 ± 0·2)
No. of nuclei
NUCLEAR AREA
(arbitrary units)

Figure 16. Cell and nuclear areas of strain CL, measured before nuclear division. Figures in parentheses are means and standard errors. a Cell area before division. b Cell area before binucleate cell formation. c Nuclear area before cell division. d Nuclear area before binucleate cell formation. The means for a and b differed significantly ($p<0.001$) when compared using a t-test modified to allow for their differing standard deviations (Bailey, 1959 p.51). The means for c and d were found to differ significantly ($p<0.001$) when compared using a t-test.

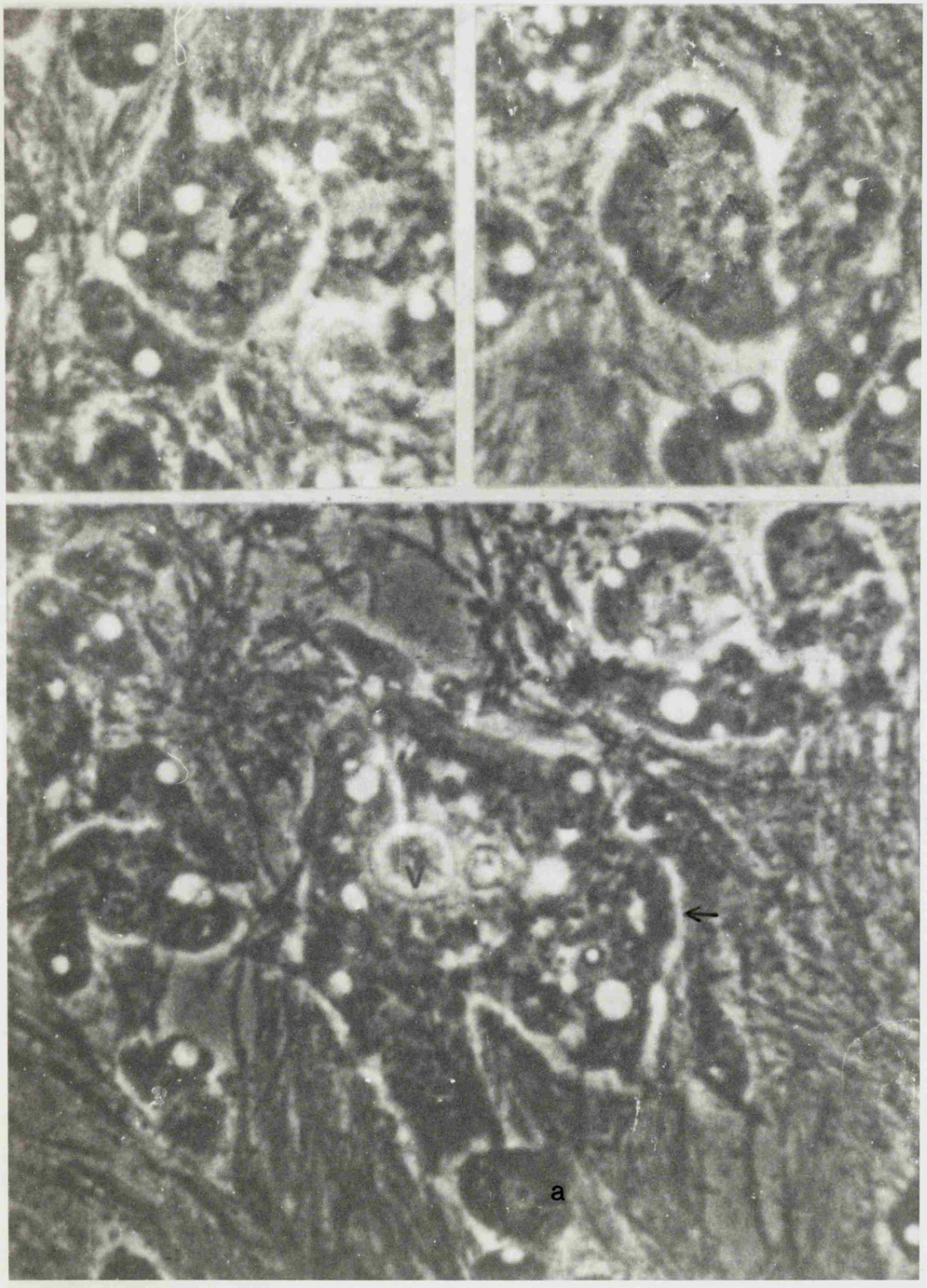
Figures 17 and 18.

Figure 17 (top). Quadrinucleate cell formation.

Left: Binucleate cell. Nucleoli no longer visible in nuclei (arrows). x 1000

Right: Quadrinucleate cell. Nuclei (arrows) have not yet completed nucleolar reconstruction. x 1000

Figure 18. (bottom). Multinucleate cell exhibiting plasmodial characteristics. Amoeba (a) is being pursued across the field and a pseudopodium has been extended to ingest it. A large food vacuole (v) contains an ingested amoeba. The transparent outer layer (arrow) may represent slime production. Nuclei are not visible due to the presence of other cell inclusions. x 1000

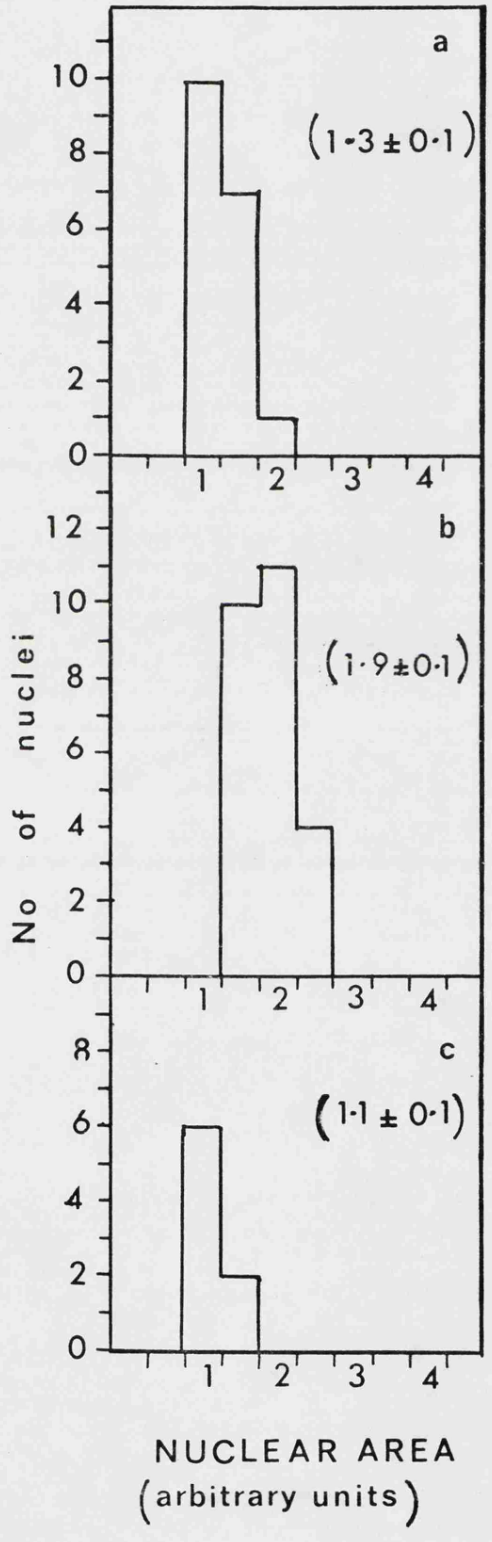

a

divided synchronously. Measurements of these 8 nuclei showed that they had the same area as amoebal nuclei measured after division, but were significantly smaller than the nuclei of binucleate cells just after their formation (Figure 19). Thus the increase in nuclear area at the time of binucleate cell formation was not permanent. The nuclei of cells containing more than 4 nuclei could not be seen clearly, due to the presence of other cell inclusion, and it was not possible even to estimate the number of nuclei in such cells.

One cell, which had become binucleate at 63 hours and quadrinucleate at 70 hours, was followed by periodic field changes until 80 hours (Figure 20). During this time successive fusions with 2 binucleate cells and a multinucleate cell occurred. Both binucleate cells had formed from uninucleate cells. After fusion between one of these binucleate cells and the quadrinucleate cell, ingestion of active amoebae began. The multinucleate cell appeared to pursue the amoebae before engulfing them (Figure 18). Similar pursuit of amoebae was frequently observed in binucleate and quadrinucleate cells, but this did not end in ingestion. After ingestion, the amoebae became visible in food vacuoles (Figure 18). In one case only part of an amoeba was engulfed. Rhythmic pulsations of the multinucleate cell began at the same time as ingestion of amoebae. Unlike uninucleate and binucleate cells, the multinucleate cell appeared to have a thick, transparent outer layer (Figure 18).

Plasmodia are multinucleate, readily fuse with one another, ingest amoebae and undergo rhythmic cytoplasmic streaming. Figure 20 traces the acquisition of these characteristics during the development of a cell which was clearly a plasmodium by the end of the film. Plasmodia also secrete slime, and the transparent outer layer of the multinucleate cell may have represented slime production.

Figure 19. Nuclear areas of strain CL, measured after nuclear division. Figures in parentheses are means and standard errors. a Uninucleate cells. b Binucleate cells. c Quadrinucleate cells. When compared, using t-tests, the means for a and c did not differ significantly ($p>0.1$) while b differed significantly ($p<0.001$) from both a and c.


a
(1·3 ± 0·1)
b
(1·9±0·1)
c
(1·1 ± 0·1)
No of nuclei
NUCLEAR AREA
(arbitrary units)

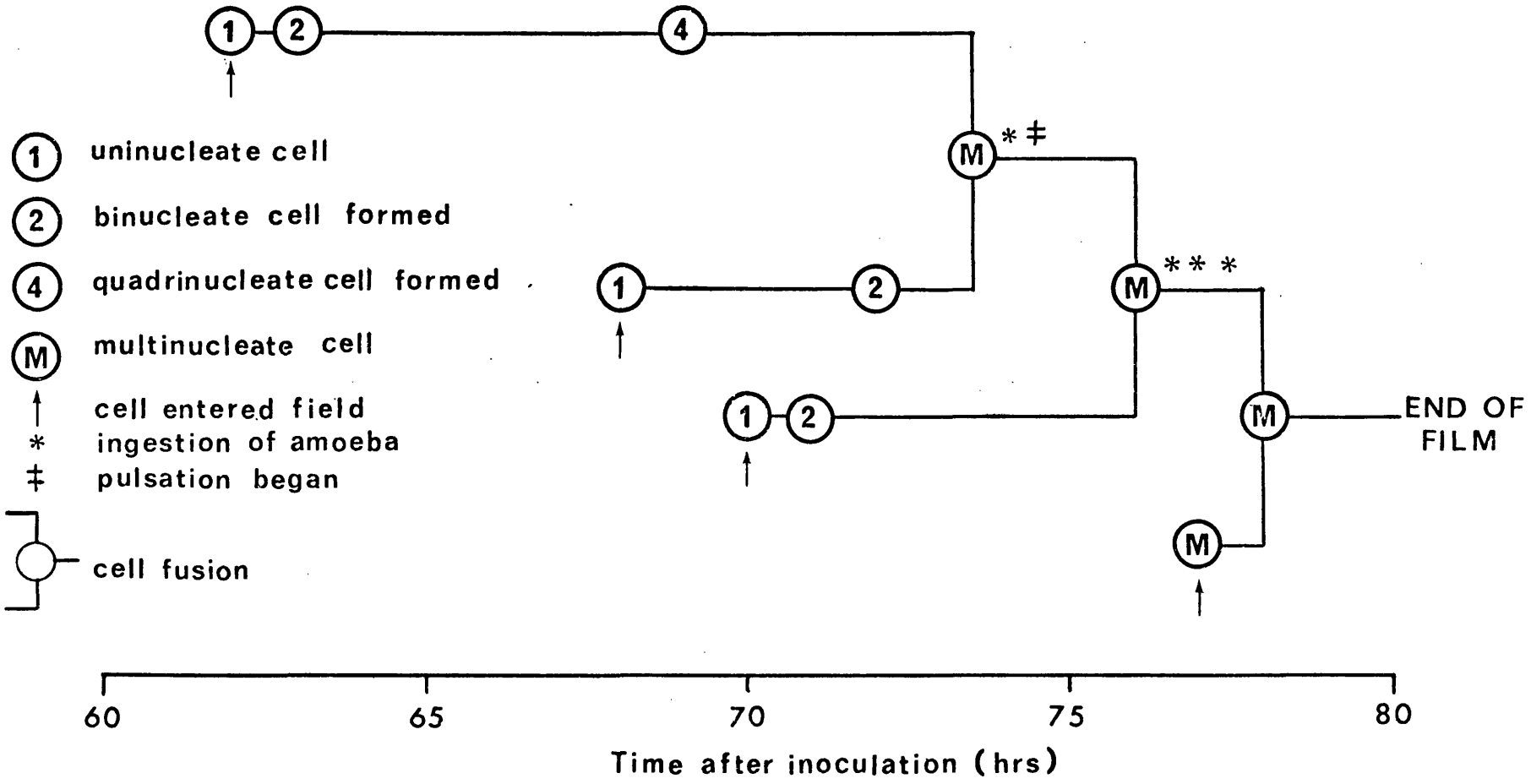

1 uninucleate cell
2 binucleate cell formed
4 quadrinucleate cell formed
M multinucleate cell
cell entered field
* ingestion of amoeba
‡ pulsation began
cell fusion
END OF FILM
60
65
70
75
80
Time after inoculation (hrs)

Figure 20. Developmental history of a multinucleate cell identifiable as a plasmodium by the end of a high power film of plasmodium formation in strain CL.

2.3c Discussion

The results reported in (iii) were convincing evidence that plasmodia developed by apogamy rather than coalescence. The observation that binucleate cells arose by nuclear division in uninucleate cells, and the subsequent participation of a number of such binucleate cells in the formation of a cell which was undoubtedly a plasmodium were consistent with the predictions of apogamy; no evidence was obtained in favour of coalescence, since no fusions of uninucleate cells were observed. Events shown in other films were also consistent with apogamy, since binucleate cells were seen to form by nuclear division in uninucleate cells and no fusions of uninucleate cells were observed.

Although it was not possible to trace the origin of the uninucleate cells which gave rise to the plasmodium, it was assumed that they arose from amoebal cell division of the type observed. It would have been interesting to study the period between binucleate cell formation and the preceding cell division, in order to follow cell growth and to compare the intermitotic time with that of proliferating amoebae.

It was unexpected that binucleate cells arose from cells whose nuclei appeared distinctly larger than those of cells which divided. It was not possible to measure directly the volumes of cells and nuclei on the film, but it seemed very likely that a difference in area reflected to some extent a difference in volume. Thus it seemed that the nuclear volume increased prior to binucleate cell formation and subsequently returned in the quadrinucleate cells to a value similar to that for amoebae. It was possible that the large nuclei contained more than the haploid amount of DNA, in which case the extra DNA must have been eliminated during plasmodium formation in order to restore the haploid DNA content of CL plasmodial nuclei. It was unlikely, however, that the large nuclei were

diploid as a result of zygote formation, and that meiosis occurred during quadrinucleate cell formation. This would have meant that meiosis occurred at a different stage in the life cycle from that at which it occurred in heterothallic strains, and would not have been consistent with the results reported by Cooke (1974) and in Section 2.2 of this thesis, indicating that recombination of genetic markers did not occur in hybrid plasmodia formed in mixtures of $\underline{mt}_h$ amoebae. It was probable that the transient increase in nuclear size was due to metabolic changes associated with the developmental transition which the cell was undergoing. For example, increased nuclear size can be associated with increased RNA synthesis (Harris, 1967).

The fact that plasmodial characteristics were acquired progressively during development confirmed that the amoebal-plasmodial transition was far from instantaneous. The demonstration of apogamy thus suggested that the hybrid plasmodia isolated from mixtures of $\underline{mt}_h$ amoebae (see Section 2.2b) probably arose by fusion of incipient plasmodia rather than of amoebae. However, the cinematographic analysis did not exclude the possibility that amoebal fusions occasionally occurred within cultures of $\underline{mt}_h$ cells, and that the products of such fusions could give rise to plasmodia.

2.4 ATTEMPTS TO DETECT CELL FUSIONS WITHIN CLONES OF HETEROTHALLIC AMOEBAE

The ability of CL amoebae to form plasmodia within clones was apparently due to possession of the $\underline{mt}_h$ allele. Thus the observation that amoebae of strain CL gave rise to plasmodia by apogamy was incompatible with the view that the only function of the mating type locus was to determine whether particular cells would fuse when they met. The mating

type locus apparently played a role in determining whether individual cells would undergo the amoebal-plasmodial transition. However, the demonstration of apogamic development in CL did not exclude the possibility that one function of the mating type locus was to control amoebal fusions.

If one role of the mating type locus was to restrict amoebal fusions to mixed mating type cultures, amoebal fusions should not have been detectable within clones of heterothallic amoebae. The absence of such fusions, in conditions which led to amoebal fusions within mixed mating type cultures would indicate that the mating type locus was involved in the control of amoebal fusions. This section reports preliminary attempts to determine whether amoebal fusions occurred within clones of heterothallic amoebal strains.

2.4a Materials and Methods

Methods were as described in Chapter 1 and in Section 2.3a. All cultures were incubated at 26°C.

Strains	Amoebae:		
		CL	mt_h
		LU647	mt_1
		LU648	mt_1
		LU688	mt_2
		LU862	mt_3
		LU863	mt_4
		i	mt_2

2.4b Results

(i) Attempt to identify binucleate cells in cultures of heterothallic strains Before attempting to observe directly the fusion of pairs of amoebae, a preliminary test was carried out to determine whether

there was indirect evidence suggestive of amoebal fusions within clones. The immediate result of fusion of two cells within a culture of uninucleate amoebae would be the formation of a binucleate cell twice the size of a uninucleate cell. Thus clonal cultures of heterothallic strains were examined for the formation of binucleate cells. Clearly the presence of binucleate cells would not, in itself, constitute proof of cell fusion; as shown in Section 2.3, binucleate cells could also arise by nuclear division within uninucleate cells.

To test for the formation of binucleate cells, a number of filming slide cultures of CL and the heterothallic strains shown in 2.4a were set up. Daily observations were made for approximately one week; each culture (up to several thousand cells) was first scanned rapidly at a magnification of x200, then about fifty individual cells were examined at x400.

All cultures started with cells which were uninucleate. No binucleate or multinucleate cells could be seen initially in any culture. A small proportion of cells did not contain any visible nuclei. In some cases these were undergoing mitosis; cell division subsequently occurred and a single nucleus appeared in each of the daughter cells. Nuclei could not be seen in some other cells because of optical imperfections in parts of the cultures. After proliferation for about three days, CL cultures invariably contained a few binucleate cells which were easily detected during rapid scanning of the slides. Quadrinucleate cells and plasmodia were present on subsequent days. Proliferating cultures of single strains of heterothallic amoebae continued to show only uninucleate cells and a few cells in which nuclei could not be clearly distinguished. When the bacterial food source was exhausted, after about one week, amoebae encysted.

The failure to observe binucleate cells in these cultures, even though similar cultures of CL amoebae invariably gave rise to binucleate cells and plasmodia, was consistent with the hypothesis that amoebal fusions (or binucleate cell formation of the type observed in CL cultures) did not occur within clones of heterothallic amoebae. However, it was possible that a number of fusions did occur, but that the fusion products were visibly binucleate for only a short time or not at all. Since clones of heterothallic amoebae gave rise to plasmodia extremely infrequently, the products of fusions would not normally be expected to develop into plasmodia. However, it was possible that binucleate cells produced by fusion of amoebae of like mating type would immediately lyse or undergo nuclear fusion or cytoplasmic cleavage to yield uninucleate cells. If this were so, then daily observations might be unlikely to detect binucleate cells. Further possibilities were that the cultural conditions were in some way inappropriate for the occurrence of amoebal fusions, or that fusions occurred at a frequency too low to be detected.

(ii) Frequency of amoebal fusions within mixed mating type cultures of heterothallic strains The preliminary study described in (i) obtained no evidence to suggest that fusions occurred within amoebal clones. However, it was possible that amoebal fusions did not occur because of unsuitable cultural conditions, or that they occurred at very low frequency. If either of these possibilities was true, mixed mating type cultures of heterothallic strains would also be expected to contain only uninucleate cells.

Pairs of heterothallic amoebal strains were inoculated together into filming slide cultures. Strains LU648($\underline{mt}_1$), LU688($\underline{mt}_2$), LU862($\underline{mt}_3$) LU863($\underline{mt}_4$) were each coinoculated with the other three strains. Dee (1966) had noted that crosses between distantly related strains usually

gave rise to plasmodia more rapidly than crosses of closely related strains. Thus LU647(mt_1) amoebae were coinoculated on to filming slide cultures with amoebae of strain i, which was not of Colonia genetic background. All LU647 x i cultures gave rise to binucleate cells and plasmodia within 2-4 days. The other cultures mostly gave rise to binucleate cells and plasmodia in 3-6 days, though amoebae in about one third of these cultures encysted before any plasmodia were formed. In cultures where plasmodia arose, 5-20 plasmodia were observed. These plasmodia rapidly grew, fused with one another and ingested all amoebae and bacteria. This behaviour may have prevented larger numbers of plasmodia being observed.

These results suggested that the failure to observe binucleate cells in clonal cultures of heterothallic strains was not due to cultural conditions which led to few or no amoebal fusions; the majority of similar cultures containing amoebae of mixed mating types gave rise to several plasmodia, each of which presumably originated from the fusion of two amoebae.

(iii) Attempts to observe amoebal fusions in crosses of heterothallic strains The possibility still remained that amoebal fusions occurred within clonal cultures of heterothallic strains but that the binucleate products of fusions were short-lived and thus not detected. In order to test this possibility, it was desirable to obtain time-lapse cinematographic evidence that amoebal fusions consistently occurred within mixed mating type cultures. Only if these were consistently observed would the absence or presence of detectable fusions within clonal cultures be strong evidence for or against the view that amoebal fusions were controlled by the mating type locus.

Attempts were made to obtain cinematographic evidence of amoebal fusions within mixed mating type cultures. Since LU647 x i

cultures had been shown in (ii) to produce plasmodia consistently and rapidly, a number of these cultures was set up. After 1-2 days incubation a slide was placed on the microscope stage and adjusted so that a densely populated part of the culture was within the field of view which would have been recorded by time-lapse cinematography at a magnification in the film plane of x50. The culture was checked two or more times daily and the field adjusted to follow the migration of cells as they ingested the bacterial lawn. In three of the four slides observed in this way, 1-3 plasmodia appeared in the field within two days. Although these plasmodia might have been immigrants formed outside the field, it was concluded that there was a good chance of observing amoebal fusions and plasmodium formation if the cultures were filmed.

Three attempts were made to film the development of mixed mating type cultures. In each case technical problems, with the time-lapse apparatus or the constant-temperature room, prevented success. Further attempts were prevented by lack of time.

2.4c Discussion

This study failed to obtain conclusive evidence for or against the hypothesis that the mating type locus controlled the ability of amoebae to fuse with one another. The results were consistent with the view that amoebae of like mating type did not fuse with one another, since no binucleate cells were observed in clonal cultures of heterothallic amoebae. Since binucleate cells and plasmodia usually arose in similar cultures containing amoebae of mixed mating types, it seemed that the presence or absence of binucleate cells in filming slide cultures was primarily a function of the mating type alleles present. These results did not exclude the possibility that amoebal fusions occurred in clonal

cultures but did not result in detectable binucleate cells.

Studies of the type attempted in (iii) should ultimately provide conclusive evidence for or against the view that one function of the mating type locus is to control amoebal fusions. However, it must be noted that a demonstration that amoebal fusions did not occur within clonal cultures of heterothallic strains would not prove that the mating type factors determined whether or not a particular pair of amoebae which happened to meet would fuse. A second possible interpretation of such a result would be that amoebal fusions only occurred within mixed mating type cultures but that fusions within such cultures were at random; only products of fusions of amoebae carrying different mating type alleles would develop into plasmodia. The first possibility implies that the mating type of a cell is expressed at its surface, while the second possibility implies that the mating type is expressed as diffusible factors of some kind and also within the cell. One obvious way to distinguish between these possibilities would be to label amoebae of two different mating types before mixing them, then to observe whether fusions are restricted to cells carrying different labels. This kind of approach may prove difficult, as amoebae proliferate for several generations before plasmodium formation occurs in filming slide cultures. Ross (1967c) studied cultures of D. iridis in which amoebae of one of the two heterothallic strains present had been labelled. He used neutral red, which did not stain the amoebae but which stained bacteria in food vacuoles. Amoebal labelling was lost before cell fusions were observed.

C H A P T E R 3

ISOLATION AND ANALYSIS OF AMOEBAL-PLASMODIAL TRANSITION MUTANTS

CHAPTER 3 ISOLATION AND ANALYSIS OF AMOEBAL-PLASMODIAL TRANSITION MUTANTS

3.1 INTRODUCTION

The aim of work described in this chapter was to isolate and genetically analyse mutants defective in plasmodial formation. It was hoped that the analysis of such mutants would yield information on the number and functions of genes involved in the amoebal-plasmodial transition.

The work of Wheals (1973) showed that mutants defective in the amoebal-plasmodial transition could be isolated from mutagenised cultures of mt_h amoebae. In addition to four strains unable to form clonal plasmodia, Wheals also recovered about 250 strains with some inherited defect in plasmodium formation. These strains characteristically formed plasmodia within clones 7-15 days after inoculation on to DSDM agar plates, while plaques of the wild-type strain all produced plasmodia within five days. Cooke and Dee (1975) isolated two strains which also showed characteristically delayed plasmodium formation. These strains were isolated without mutagenesis, but with the use of an enrichment procedure. The isolation procedure used in the current work included both mutagenesis and enrichment, since it was reasoned that the combined methods would be more efficient than each alone.

At the time the work described in this chapter was begun, it was clear (see Chapter 1) that the mating type (*mt*) locus and another, unlinked, locus (*apt*-1) were involved in plasmodium formation. There was also suggestive evidence that at least three loci other than *apt*-1

might be involved, since Wheals obtained plasmodium formation in all mixtures of different pairs of the four mutant strains he investigated, but not in the individual mutants. However, he did not show that the plasmodia were in any way hybrid. Of all Wheals' mutant strains, only APT1 (mt_h;*apt*-1$^-$) was still available for further investigation, so that his evidence for four loci remained arguable. Strain APT1 was included in the analysis of strains isolated in the current work. The mutant strains isolated by Cooke and Dee (CLd, CL348d) probably carried mutations in or closely linked to the mating type locus. These strains were not included in the current analysis as their "delayed" phenotype made them unsuitable for some of the procedures employed. Some of the work described in this chapter has been reported elsewhere (Anderson and Dee, 1976).

3.2 MATERIALS AND METHODS

Methods were mostly as described in Chapter 1, with additional methods as described below. Incubation of all cultures was at 26°C unless otherwise stated.

3.2a Strains

Amoebae:	CL	mt_h;*fusA2* .
	CL5001	mt_h;*fusA2*,*leu*-1$^-$
	APT1	mt_h;*fusA2*;*apt*-1$^-$
	LU647	mt_1;*fusA2*
	LU648	mt_1;*fusA1*
	LU688	mt_2;*fusA1*
	LU858	mt_2;*fusA1*;*leu*-1$^-$
	LU860	mt_1;*fusA1*;*leu*-1$^-$

	LU861	$\underline{mt_2}$;$\underline{fusA2}$
	LU862	$\underline{mt_3}$;$\underline{fusA1}$;$\underline{eme}^r$
	LU863	$\underline{mt_4}$;$\underline{fusA1}$
	CH188	$\underline{mt_3}$;$\underline{fusA2}$;$\underline{fusC2}$;$\underline{eme}^r$
	i	$\underline{mt_2}$;$\underline{fusA2}$;$\underline{fusB2}$
Plasmodia:	testers of groups	
	I	$\underline{fusA1}$
	III	$\underline{fusA1}$/$\underline{fusA2}$
	IV	$\underline{fusA1}$/$\underline{fusA2}$;$\underline{fusB2}$
	V	$\underline{fusA2}$
	IX	$\underline{fusA1}$/$\underline{fusA2}$;$\underline{fusC2}$

All strains carried the $\underline{fusB1}$ and $\underline{fusC1}$ alleles unless otherwise stated.

3.2b Mutagenesis

The mutagenesis procedure employed was essentially the same as employed in a number of previous studies (Haugli, 1971; Haugli and Dove, 1972; Haugli et al., 1972; Wheals, 1973; Cooke, 1974; Cooke and Dee, 1975). Cultures of CL or CL5001 amoebae growing on LIA plates were flooded with 10 ml of water and scraped with a glass spreader. The density of the resulting amoebal suspension was determined on a haemocytometer slide and adjusted to approximately 3×10^5 cells/ml. Oxoid membrane filters (6 cm diameter; 0.45 µm pore size) were each inoculated with 0.1 ml of amoebal suspension and 0.1 ml of bacterial suspension. The suspensions were spread with a glass spreader. Each membrane filter was supported on a filter paper disc which was itself supported on a monolayer of glass beads in a 9 cm plastic petri dish. The filter paper was underlaid with 13 ml of liver infusion solution (1 g Oxoid liver infusion powder per litre of water) so that the culture

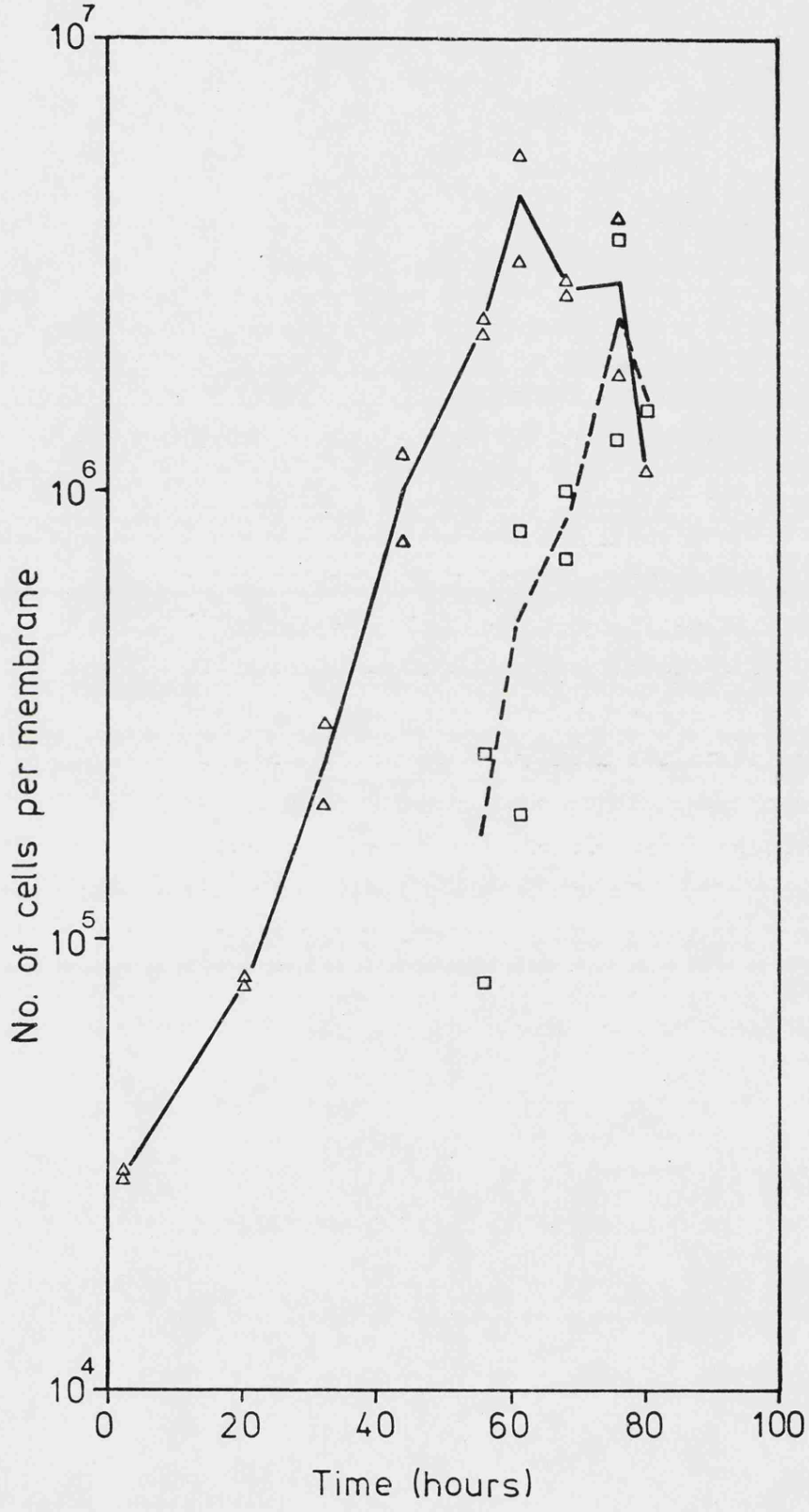
was kept moist. After incubation for 48 hours the cell density had reached about 10^6 amoebae per membrane, but plasmodium formation had not yet begun (see Figure 21). These cultures of exponentially growing amoebae were exposed to the chemical mutagen N-methyl-N-nitro-N-nitroso-guanidine (NMG; Sigma Chemical Company). The membrane filters were transferred to dishes containing glass beads and filter papers, but with NMG dissolved in phosphate buffer (pH 7.0) instead of liver infusion solution (the NMG was dissolved with stirring for 30 minutes at 37°C to give a concentration of 250-300 µg/ml). After 30 minutes over NMG solution, the membranes were transferred for 10 minutes to sodium thiosulphate solution (2% $^{w}/v$), then back to liver infusion solution (three transfers of 10 minutes each). The membranes were each harvested in 2 ml of water by scraping with a glass spreader. Cells plated on LIA plates at this stage normally showed viabilities in the range of 1-8% of controls treated similarly but without NMG.

3.2c Enrichment procedure

Cooke (1974; Cooke and Dee, 1975) used an enrichment method to isolate variants requiring a longer incubation period for plasmodium formation than the $\underline{mt}_h$ strains from which they were derived. He allowed dense cultures of amoebae to grow on agar plates until plasmodia began to form. He then flooded the plates with water to obtain an amoebal suspension which was plated in the same way as before. The process was repeated a number of times and the final amoebal suspension was plated out to give separate plaques. It was found that the majority of plaques showed delayed plasmodium formation.

Cooke's method was clearly effective in enriching a population of $\underline{mt}_h$ amoebae for variants which showed low efficiency in plasmodium

Figure 21. Growth and plasmodium formation by CL amoebae. Amoebae were inoculated on to membrane filters (as described in text) and incubated at 26°C. Cultures were harvested at intervals in 10 ml of water by scraping with a glass spreader. Dilutions were inoculated on to LIA plates with E.coli, spread and incubated for 5 days. Where 30-100 amoebal plaques were visible on a plate, the number was used to calculate the viable count of amoebae on the membrane at the time of harvesting (a reconstruction experiment indicated that about 100% of amoebae on each membrane were harvested and remained viable on replating). Each triangle (△) represents the mean value of counts on duplicate plates from a single membrane. Where plasmodia arose on dilution plates without amoebal plaques being first visible ("P clones"; N.Kerr, 1961) it was assumed that these arose from plasmodia already present on the membranes when harvested. Squares (□) represent viable counts of plasmodia (calculated as for amoebae except that only 8-50 plasmodia were counted per plate). The efficiency of harvesting and replating of plasmodia could not be determined.


10^7
10^6
10^5
10^4
No. of cells per membrane
0
20
40
60
80
100
Time (hours)

formation. Two mechanisms could have been responsible. First, plasmodia were formed mainly by "non-delayed" cells, so that this part of the cell population was selectively depleted at each round of plasmodium formation. Second, although amoebae did not grow in these cultures as individual colonies, it is likely that the growth of clones led to areas with a high proportion of "delayed" amoebae. Relatively few plasmodia would form within such areas, so that the probability of "delayed" amoebae being ingested by newly-formed plasmodia was less than the corresponding probability for "non-delayed" amoebae. Since the enrichment operated over many cell generations, only variants which were both stable and retained the ability to proliferate at nearly the same rate as the wild-type strain could be recovered.

The enrichment procedure employed in the current work was essentially the same as that used by Cooke, but with several modifications. Immediately after mutagenesis had been carried out, amoebae were harvested from each membrane filter by flooding with 2 ml of water and scraping with a glass spreader. The whole 2 ml of amoebal suspension from a single membrane (about 10^4 viable cells) was either inoculated on to a single DSDM agar plate or divided between two plates. These plates were each additionally inoculated with 0.1 ml of bacterial suspension. Each plate ("enrichment plate") was taken as the start of an enrichment line; amoebae were incubated for 5-7 days, until many small plasmodia had formed, held at $4^{\circ}C$ for 24 hours, then harvested in 2 ml of water by gentle agitation. The cold treatment was found to reduce the small number of viable plasmodia which were harvested with the amoebae (this reduction was due either to plasmodial death or to reduced efficiency of harvesting. The harvested amoebae (about 10^4) were inoculated, with E. coli, on to a fresh DSDM agar enrichment plate. Four further DSDM

agar plates were each inoculated with 0.2 ml of bacterial suspension. A glass spreader was used to spread the inoculum over the enrichment plate and was then immediately used, without resterilisation, to inoculate the other four plates. Thus these plates represented serial dilutions of the amoebal suspension inoculated on to the enrichment plate. The enrichment plate was incubated for 5-7 days until small plasmodia were visible, then amoebae were harvested and replated as before, up to five more times. Dilutions inoculated at each replating were incubated until plasmodia formed (4-5 days on these plates, which were drier than the enrichment plates). In about 75% of enrichment lines from mutagenised cultures, some plaques which did not form plasmodia at the same time as the majority of plaques were found; these generally appeared in dilution plates set up from the third to fifth enrichment plates in a line. Of ten enrichment lines set up with non-mutagenised amoebae only one gave rise to plaques with a "delayed" plasmodium formation phenotype.

In a reconstruction experiment, carried out to test the enrichment procedure before its use in mutant isolation, a mixture of 0.1% APT1 + 99.9% CL amoebae was enriched for APT1 cells by a factor of ten on each of two successive enrichment cycles (Table 10). However, reconstruction experiments could only give a rough indication of efficiency, since even small differences in growth rates of the two strains would have markedly affected the result (log phase doubling times of CL and APT1 amoebae were both estimated at 6 hours). In practice, the average number of enrichment cycles required to recover variant plaques from mutagenised cultures was in agreement with enrichment of about 10 times per cycle.

Table 10. Reconstruction experiment to test enrichment of APT1 amoebae in APT1 + CL mixtures

	Plaque types on dilution plates*		
	Amoebal (deduced origin APT1)	Plasmodial (deduced origin CL)	% APT1
Before Enrichment	1	1036	0.1
First Enrichment	9	1023	0.9
Second Enrichment	42	386	9.8

* see text for details of enrichment technique.

3.3 RESULTS

3.3a Isolation of mutants

In order to carry out genetic analysis of mutants defective in clonal plasmodium formation, it was necessary that these mutants should retain the ability to cross with other strains. Since mutations abolishing clonal plasmodium formation might also render mutants unable to cross, it was desirable to isolate strains in which clonal plasmodium formation was conditionally defective. Thus enrichments for most lines were carried out at high temperature in the hope that the mutants isolated would include some temperature-sensitive strains. Adler and Holt (1974b) showed that plasmodium formation by $\underline{mt}_h$ strains was delayed at 29.5°C, as compared with 26°C. This observation was confirmed and it was also noted that the synchrony of plasmodium formation in different plaques on the same plate was less good at 29-30°C than at 26°C. Since the enrichment procedure depended upon "rapid" and synchronous formation of plasmodia by non-mutant cells, high temperature enrichments were carried out at 28.5 ± 0.5°C (referred to subsequently as "28.5°C"); at this temperature $\underline{mt}_h$ amoebae consistently formed plasmodia in all plaques after 4-5 days.

A total of 130 enrichment lines was set up from mutagenised cultures of $\underline{mt}_h$ amoebae; 70 lines of mutagenised CL amoebae and 20 lines of CL5001 amoebae were enriched at 28.5°C. Forty lines of mutagenised CL amoebae were enriched at 21-23°C (referred to subsequently as "22°C"). The 130 lines represented screening of about 2×10^6 cells.

A total of 96 lines yielded dilution plates on which some individual plaques contained only amoebae when plasmodium formation had occurred in the other plaques. Cells from such amoebal plaques were subcultured to LIA plates and recloned. The clones were designated CL6003 to CL6143 and CL5001/1 to CL5001/13.

Cells from each clone were inoculated, with E. coli, on to two DSDM agar plates and spread with a glass spreader. One plate was incubated at 22°C (the "permissive" temperature) for two weeks and the other at 28.5°C. All strains grew well as amoebae at both temperatures. Most mutant clones gave rise to plasmodia on plates incubated at both temperatures, one or more days later than CL and CL5001 controls (the control plates showed plasmodia at both temperatures after 4-5 days). This group of mutant strains was classified as "delayed". Clones with the most extreme "delayed" phenotype did not give rise to plasmodia until 10-12 days after inoculation, when a confluent lawn of about 10^7 amoebae had formed; as few as 5-6 plasmodia formed per plate. Other strains formed plasmodia sooner and in larger numbers. Clones with the least extreme "delayed" phenotype formed plasmodia after 6-7 days, in plaques which were somewhat larger than those in which plasmodia formed on control plates of CL and CL5001 amoebae.

Some mutant strains did not give rise to any plasmodia within two weeks, at one or both temperatures: 10 strains isolated from separate enrichment lines formed no plasmodia at either temperature, and three formed no plasmodia at 28.5°C but, after 6-10 days, formed 10-100 plasmodia per plate at 22°C (and 26°C). The 13 strains which did not give rise to plasmodia at one or both temperatures were designated NPF (no plasmodium formation). Most of the work described in this chapter was aimed at the analysis of these strains (see Table 11).

The NPF strains appeared to have arisen independently, since all were isolated from separate enrichment lines. However, in two cases a pair of mutants could be traced back to a single mutagenised culture. The pairs of strains involved were CL6129/CL6143 and CL6134/6136. Since it seemed that NMG acted during DNA replication in eukaryotes (Dawes and

Table 11. NPF strains

Amoebal strains	Derived from	Plated on DSDM agar	
		Plasmodia at 22°C	Plasmodia at 28.5°C
CL6049	CL	-	-
CL6082	CL	-	-
CL6089	CL	-	-
CL6099	CL	-	-
CL6100	CL	"delayed"	-
CL6111	CL	"delayed"	-
CL6115	CL	"delayed"	-
CL6129	CL	-	-
CL6130	CL	-	-
CL6134	CL	-	-
CL6136	CL	-	-
CL6143	CL	-	-
CL5001/8	CL5001	-	-
CL		+	+
CL5001		+	+

+ = plasmodia in plaques. - = no plasmodia.

Carter, 1974) and that growing amoebae spent most of each cell generation in G2 phase (Mohberg and Rusch, 1971), it was probable that the cultures had been divided before proliferation of mutant cells. Further analysis of the strains (see 3.3h) indicated that in both cases the potentially non-independent mutants carried mutations of different genes.

The three temperature-sensitive NPF strains which were isolated from enrichment lines incubated at 22°C were sufficiently "delayed" at this temperature to allow selection to operate.

3.3b Microscopic observation of NPF strains

Work described in Chapter 2 showed that plasmodium formation in filming slide cultures of CL amoebae was characterised by several observable stages: amoebae, large uninucleate cells, binucleate cells, quadrinucleate cells and multinucleate plasmodia. Mutants blocked at a particular stage of the amoebal-plasmodial transition (e.g. the binucleate cell stage) might be easily recognisable. Wheals (1973) examined cells of the mutant strains APT1 to APT4 and reported that they were indistinguishable from amoebae of the wild-type strain from which they were derived.

Amoebae of the 13 NPF strains and APT1 were inoculated on to filming slide cultures. Cultures of CL amoebae were also set up. Daily observations were made with phase-contrast optics; first, a whole slide was scanned at x200, then about 50 individual cells were observed closely at x400. Cultures of CL amoebae at first contained uninucleate cells and then binucleate cells, quadrinucleate cells and multinucleate plasmodia. Two cultures of each NPF strain were observed; all strains proliferated as uninucleate cells indistinguishable from those initially present. These cells encysted when the bacterial food source was exhausted, after 6-7 days. No binucleate cells were seen. Two out of

four cultures of APT1 amoebae behaved in the same way as those of the NPF strains; the other two each showed a single binucleate cell on the third day of incubation, though these cells were not visible on day 4. No quadrinucleate cells or plasmodia were seen and it was concluded that the binucleate cells either underwent cell division to give pairs of uninucleate cells, or they lysed. Uninucleate cells in these cultures eventually encysted, as seen in other cultures.

These observations gave no clear indication that any of the mutants was blocked at an intermediate stage of plasmodium formation. The result for APT1 suggested that plasmodium formation in this strain did not progress past the binucleate cell stage, but it also seemed that very few cells ever reached this stage. The failure to observe cells arrested at any intermediate stage of development might have indicated that the mutants were defective in the initiation of plasmodium formation. However, they may have been blocked at stages which were not cytologically identifiable; it is possible that most of the events in the amoebal-plasmodial transition have already occurred by the time of binucleate cell formation.

3.3c Phenotypes of temperature-sensitive NPF strains

Amoebae of the three temperature-sensitive NPF strains grew as well as CL amoebae at 28.5°C (judged by plaque size). Thus the failure to form plasmodia at high temperature must have been due to temperature-sensitivity of the amoebal-plasmodial transition and/or of plasmodial growth. The fact that filming slide cultures showed only uninucleate cells indicated that plasmodium formation may have been temperature-sensitive, but since differentiation to become a plasmodium could have been essentially completed in the uninucleate state it was not possible

to conclude definitely that the amoebal-plasmodial transition was temperature-sensitive. In order to determine whether plasmodial growth was temperature-sensitive, plasmodia carrying the temperature-sensitivity factors were made from the NPF strains and tested for growth at high temperature (see Table 12 and below).

Amoebal strains CL6100, CL6111, CL6115 and CL were allowed to form clonal plasmodia on DSDM agar plates at 22°C. Since these plasmodia arose at the permissive temperature it was thought that they might have formed from the mutants without reversion to non-temperature-sensitivity. A single plasmodium from each strain was inoculated on to SDM agar plates and incubated at 32°C. This was the maximum temperature for growth of CL plasmodia. Plasmodia formed from the three temperature-sensitive strains grew as well as the CL plasmodium.

The four plasmodia were caused to sporulate and 20 amoebal progeny clones derived from the spores of each. The progeny clones were inoculated, with bacteria, on to DSDM agar plates which were incubated at 28.5°C. Amoebal plaques formed on all plates. Progeny of the CL plasmodium formed plasmodia within plaques, but no plasmodia formed from progeny of the mutants. Thus plasmodia formed at 22°C from the temperature-sensitive strains were not revertants.

These results strongly suggested that the three temperature-sensitive NPF strains showed sensitivity of the amoebal-plasmodial transition, rather than of plasmodial growth.

3.3d Reversion of "delayed" strains

Cooke (1974) showed that clonal plasmodium formation in one of the two "delayed" strains he isolated (CLd) was associated with reversion; amoebal progeny of clonal plasmodia formed by CLd amoebae always gave rise to plasmodia in plaques at the same time as CL amoebae, while the "delayed"

Table 12. Analysis of plasmodia formed at 22°C from temperature-sensitive NPF strains

Plasmodium	Plasmodial growth at 32°C	Plasmodium formation by progeny at 28.5°C
(CL6100)1	YES	NO
(CL6111)1	YES	NO
(CL6115)1	YES	NO
(CL)R1	YES	YES*

* plasmodia in plaques.

character was always retained in progeny of crosses. Cooke suggested that clonal plasmodia formed in CLd cultures from revertant amoebae which had regained the ability to form plasmodia in plaques as rapidly as CL amoebae (i.e. they were "CL-like"); CLd amoebae were able to form crossed plasmodia with other strains in the normal way. Plasmodia formation in the second strain (CL348d) occurred without reversion; progeny of both clonal and crossed plasmodia retained the "delayed" phenotype.

A number of "delayed" strains isolated in the current work were tested to determine whether the same two types of behaviour were present. Plasmodia were allowed to form on DSDM agar plates, grown on SDM agar plates and caused to sporulate. Five amoebal progeny clones were derived from spores of each plasmodium, plated on DSDM agar to give well-separated plaques and incubated for two weeks. Each plate was scored for whether plasmodia formed at the same time as on CL control plates, or whether amoebae retained the "delayed" phenotype. The results are shown in Table 13. Both revertant and non-revertant behaviour was found and, in all but one of the 23 sets of progeny tested, all clones showed the same behaviour. In one case four clones retained the "delayed" phenotype, while all plaques on the fifth plate formed plasmodia at the same time as CL plaques.

These results showed that the two classes of "delayed" plasmodium formation identified by Cooke were also present in mutants isolated in the current work. In one case it seemed that plasmodia had formed in both ways; this could also have been true in other cases, since only five progeny of any one plasmodium were tested. The reversion rate was assumed to be equivalent to the frequency of plasmodium formation. The number of plasmodia formed in each case was 10^{-4}-10^{-6} of the maximum amoebal population.

Table 13. Reversion of "delayed" strains

Plasmodium	Plasmodium formation* by progeny clones		Reversion deduced
	CL-like	"delayed"	
(CL6004)1	5	0	YES
(CL6008)1	5	0	YES
(CL6009)1	5	0	YES
(CL6014)1	5	0	YES
(CL6015)1	5	0	YES
(CL6016)1	5	0	YES
(CL6017)1	5	0	YES
(CL6018)1	5	0	YES
(CL6019)1	5	0	YES
(CL6021)1	5	0	YES
(CL6022)1	5	0	YES
(CL6023)1	5	0	YES
(CL6024)1	5	0	YES
(CL6025)1	5	0	YES
(CL6026)1	5	0	YES
(CL6027)1	5	0	YES
(CL6013)1	1	4	YES
(CL6003)1	0	5	NO
(CL6005)1	0	5	NO
(CL6006)1	0	5	NO
(CL6007)1	0	4	NO
(CL6011)1	0	5	NO
(CL6012)1	0	5	NO

* CL-like: plasmodia formed in plaques after 4 days, as in CL controls.

"Delayed": plasmodia formed as in controls of appropriate mutant strains.

3.3e Reversion of NPF mutants

(i) Frequency of plasmodium formation The NPF mutants were defined as a group by their failure, in an initial screening (described in 3.3a), to form plasmodia when they were plated to give well separated plaques on DSDM agar. In order to determine whether these strains gave rise to plasmodia at some low frequency, each mutant (and also APT1) was inoculated into 0.2 ml bacterial puddles on 50 DSDM agar plates. The cultures were incubated at 26°C, or 28.5°C for temperature-sensitive strains, for six weeks. Since heterothallic strains were also phenotypically NPF, amoebae of $\underline{mt}_1$ and $\underline{mt}_2$ strains were tested in the same way.

Plates were checked at weekly intervals and scored for plasmodium formation. Plasmodia appeared in some puddles after 2-6 weeks. Where plasmodia were present it was usually clear that only one or, occasionally, two plasmodia had formed; CL amoebae under the same conditions formed many plasmodia in every puddle within one week. Table 14 shows the numbers of puddles of each strain in which plasmodium formation was observed.

Viable counts for some strains (CL6049, APT1, LU648) were obtained at weekly intervals by washing off control puddles in 10 ml of water and plating appropriate dilutions on LIA plates. Net growth was completed in about one week and viable populations then remained steady at about 10^7 cells per puddle. Thus the numbers of plasmodia formed were roughly 10^{-7}-10^{-9} of the maximum amoebal population, with essentially no difference between the mutants and the heterothallic strains. After 1-2 weeks the majority of cells in the puddles were encysted and presumably not available for plasmodium formation. Thus the effective frequency of plasmodium formation may have been considerably higher than the results suggested.

Table 14. Puddle tests of NPF strains, APT1 and heterothallic strains incubated for six weeks

Amoebal strains	No. of puddles out of 50 in which plasmodia formed
CL6049	1
CL6082	3
CL6089	4
CL6099	38
CL6100*	3
CL6111*	4
CL6115*	3
CL6129	9
CL6130	4
CL6134	9
CL6136	11
CL6143	10
CL5001/8	9
APT1	16**
LU648 ($\underline{mt}_1$)	6
LU688 ($\underline{mt}_2$)	6
LU861 ($\underline{mt}_2$)	41

* Tested at 28.5°C.

** Plasmodia mostly died when less than 1 mm^2.

(ii) Nuclear DNA contents of clonally formed plasmodia Plasmodia obtained in (i) could have arisen in several possible ways. It seemed very unlikely that the cultures had been contaminated with amoebae of other strains since such contamination had never been observed in this laboratory. The possibility was dismissed when it was found that the fusion behaviour of the plasmodia corresponded in every case with the known plasmodial fusion genotypes of the amoebal strains.

If plasmodium formation had occurred by cell and nuclear fusion of pairs of amoebae, the plasmodia would have been diploid. Cell and nuclear fusion could have occurred as a result of mutation within a clonal culture to give a new NPF cell type able to cross with the strain inoculated. Alternatively, homothallic plasmodium formation might have occurred at low frequency.

In order to determine whether plasmodia obtained in (i) were diploid, amoebal and plasmodial nuclei of appropriate strains were isolated and stained by the Feulgen method. DNA contents of 50 nuclei from each preparation were determined by scanning microdensitometry and the results are shown in Table 15 and Figures 22 and 23. Nuclear DNA values for each amoebal strain fell in a single distribution of mean value 0.5-0.8 pg DNA/nucleus. This corresponded approximately to the haploid DNA content (Mohberg, 1976). Most nuclear DNA values for each clonally formed plasmodium also fell in a single distribution of mean value 0.5-0.8 pg DNA/nucleus; in some cases a proportion of nuclei contained higher values (this is typical of haploid $\underline{mt}_h$ plasmodia; Cooke, Mohberg, personal communications). A control plasmodium formed by crossing amoebae of strains LU648 ($\underline{mt}_1$) and LU688 ($\underline{mt}_2$) gave a mean nuclear DNA value approximately twice that of amoebae and clonally formed plasmodia (1.2 pg DNA/nucleus).

Table 15. Mean nuclear DNA contents of amoebae and clonally formed plasmodia

No.*	Amoebae	DNA (pg/nucleus)
1	CL6049	0.61
2	CL6082	0.68
3	CL6089	0.75
4	CL6099	0.57
5	CL6100	0.65
6	CL6111	0.61
7	CL6115	0.72
8	CL6129	0.58
9	CL6130	0.57
10	CL6134	0.47
11	CL6136	0.54
12	CL6143	0.73
13	CL5001/8	0.62
14	APT1	0.73
15	CL	0.61
16	LU648	0.69
17	LU688	0.72

No.*	Plasmodia	DNA (pg/nucleus)
18	(CL6049)1	0.56
19	(CL6082)2	0.71
20	(CL6089)1	0.71
21	(CL6099)3	0.65
22	(CL6099)9	0.67
23	(CL6111)3	0.58
24	(CL6111)4	0.67
25	(CL6129)1	0.63
26	(CL6130)1	0.62
27	(CL6136)4	0.62
28	(CL6143)6	0.73
29	(CL5001/8)7	0.68
30	(CL)19	0.75
31	(LU648)1	0.64
32	(LU648)5	0.72
33	(LU688)1	0.74
34	(LU648 x LU688)R1	1.20

* See Figures 22 and 23.

Figure 22. Nuclear DNA contents of amoebal strains. See Table 15 for key to strains.

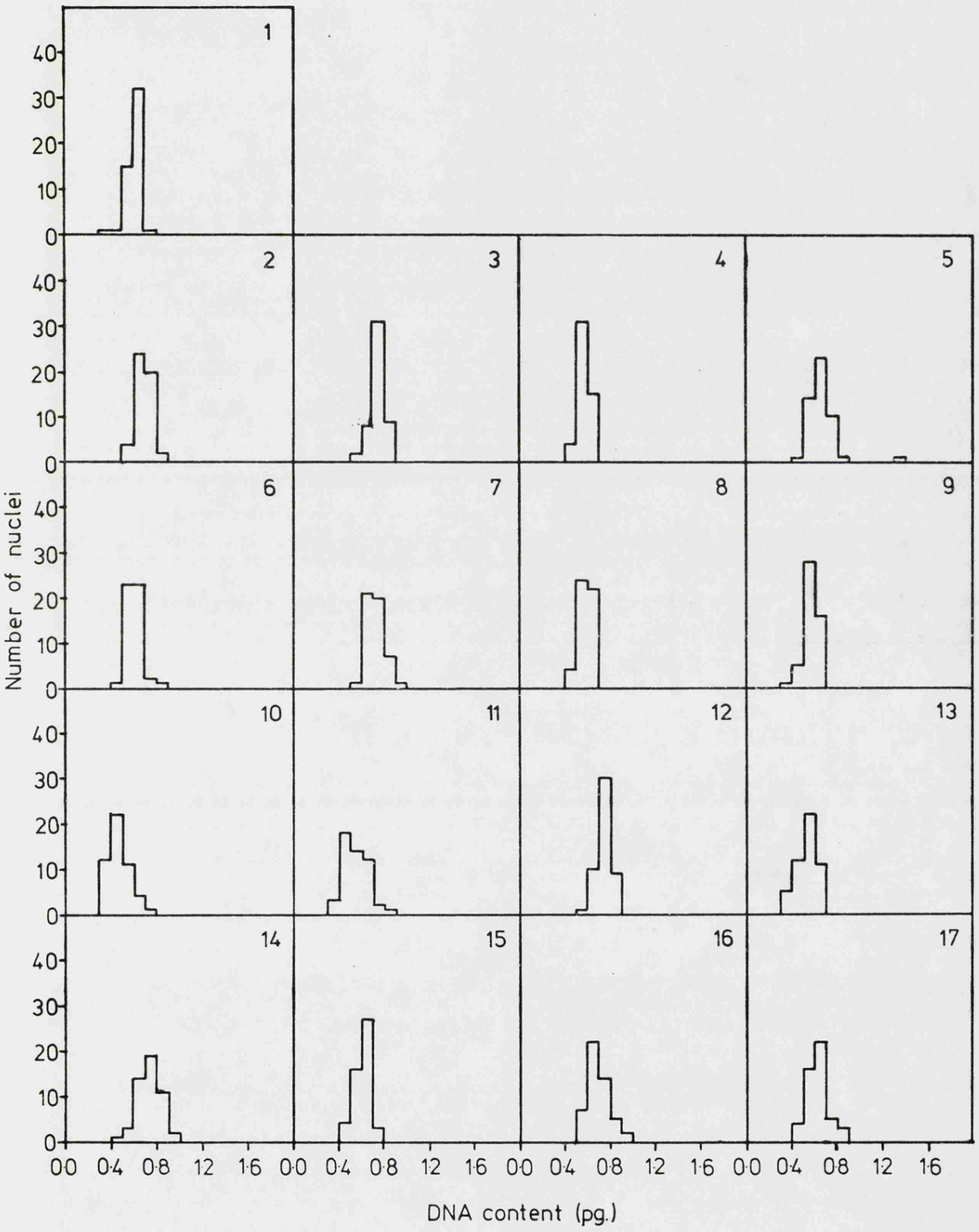
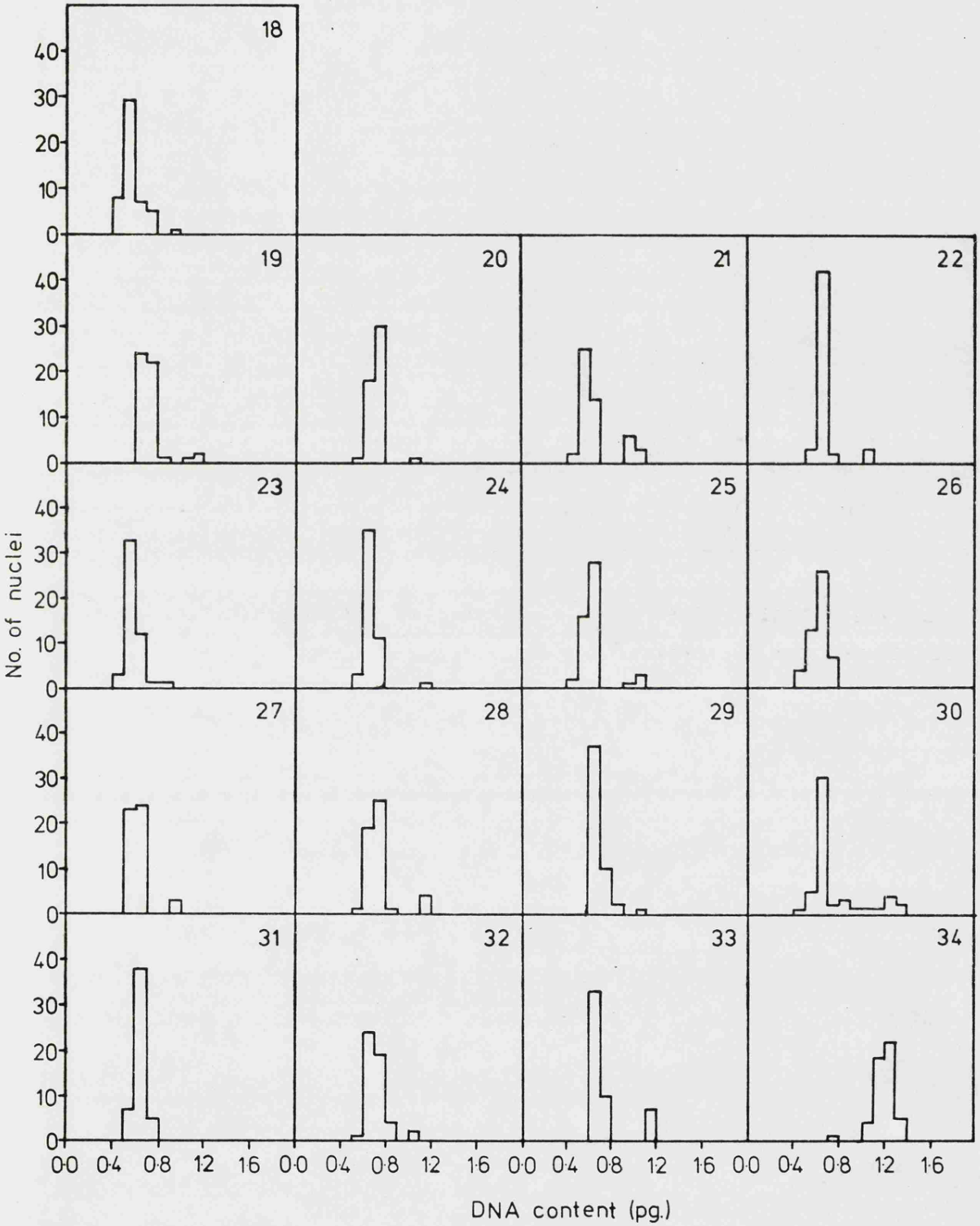

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Number of nuclei
0
10
20
30
40
0·0
0·4
0·8
1·2
1·6
DNA content (pg.)

Figure 23. Nuclear DNA contents of clonally formed plasmodia. See Table 15 for key to strains.


18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
No. of nuclei
0
10
20
30
40
0·0 0·4 0·8 1·2 1·6
DNA content (pg.)

It was concluded that the clonally formed plasmodia were haploid rather than diploid and arose from haploid amoebal strains. Thus the possibility of nuclear fusion was excluded.

(iii) Analysis of progeny of clonally formed plasmodia The haploid plasmodia obtained in (i) could have arisen from mutant amoebae which had acquired the ability to form plasmodia without nuclear fusion. Alternatively, the formation of plasmodia by apogamy or coalescence could have occurred at low frequency without any heritable change in plasmodium forming behaviour.

To distinguish between these two possibilities, progeny of a number of clonally formed plasmodia were analysed. Where possible (see below for exceptions) about 20 amoebal progeny clones were derived from the spores of each plasmodium and plated on DSDM agar to give well separated plaques. The plates were incubated at 26°C, or 28.5°C for progeny of plasmodia derived from temperature-sensitive strains. The results are shown in Table 16.

All progeny from six plasmodia formed plasmodia in plaques at the same time as CL controls. It was concluded that the six plasmodia had probably arisen from mutant amoebae which had acquired the ability to form plasmodia in plaques (in the case of NPF strains these mutant amoebae were thus revertants). All progeny of ten plasmodia grew only as amoebae. Six of the ten plasmodia were known to be haploid and it thus seemed that they had formed without a heritable change in plasmodium forming ability. Progeny of two plasmodia included some clones which formed plasmodia in plaques and some clones which did not form plasmodia. The two plasmodia were known to be haploid and they might have arisen from fusion of revertant and spontaneously formed plasmodia. Spores of four clonally formed plasmodia did not give rise to amoebal progeny in

Table 16. Analysis of progeny of clonally formed plasmodia

Plasmodia derived from NPF strains	Plasmodium formation by progeny clones**	
	CL-like	None
(CL6099)9	20	0
(CL6100)3	20	0
(CL6115)3	19	0
(CL6129)1	19	0
(CL6134)1	20	0
(CL6136)4	9	11
(CL6143)6	17	1
(CL6049)1	0	20
(CL6089)1	0	19
(CL6089)3	0	20
(CL6099)3	0	20
(CL6111)4	0	19
(CL6130)1	0	19
(CL6136)7	0	20
(CL6143)7	0	20
(CL6082)2	*	*
(CL6111)3	*	*
(CL5001/8)7	*	*

Plasmodia derived from heterothallic strains	Plasmodium formation by progeny clones**	
	CL-like	None
(LU688)1	20	0
(LU648)1	0	20
(LU688)2	0	19
(LU648)5	*	*

* plasmodia arose from spores without prior formation of visible amoebal colonies.

** CL-like: plasmodia formed in plaques after 4 days as in CL controls.

None: no plasmodia formed within 14 days.

the normal way; spores plated on LIA plates with E. coli gave rise to plasmodia without prior formation of visible amoebal colonies (a few amoebal colonies also formed on some plates but the total number of these colonies was never more than 5% of the number of plasmodia). It was concluded that this behaviour might represent mutation to give a new plasmodium formation phenotype.

Adler and others (Adler and Holt, 1975; Adler et al., 1975), working only with heterothallic strains, carried out essentially the same tests as described in (i)-(iii) and found the same kinds of result. They designated strains which gave rise to plasmodia without prior formation of amoebal colonies "ALC" strains (amoeba-less life cycle). Comparison of their results with those reported in (i)-(iii) indicated that plasmodium formation by mutant NPF amoebae and by heterothallic amoebae was indistinguishable on the bases of frequency, nuclear DNA content of plasmodia and behaviour of progeny clones.

(iv) Reversion of APT1 As noted in Table 14, growth of clonal plasmodia derived from amoebae of strain APT1 was very poor. One such plasmodium (APT1)1, was maintained for several months in culture on SDM agar plates supplemented with a few rolled oat flakes. In the absence of oats growth ceased.

It was found that the plasmodium (APT1)1 would not sporulate. This was not surprising, since APT1 was derived from the mt_h strain C5.1 (Wheals, 1973), which was also a non-sporulator. In order to determine whether amoebal progeny of (APT1)1 formed plasmodia in plaques, the plasmodium was allowed to grow into contact and fuse with a plasmodium derived from CL5001 (mt_h;leu-1$^-$;fusA2). The resulting heterokaryon, [(APT1)1 + (CL5001)1]1, sporulated. Fifty amoebal progeny clones were isolated and plated on DSDM agar to test for the formation of plasmodia

in plaques. Twenty-eight clones formed plasmodia in plaques at the same time as CL5001 controls. The plasmodia grew on SDM agar without oat flakes, or on DM-1 + leucine agar, but all died when subcultured to DM-1 agar. The behaviour of these clones thus suggested that they were derived from CL5001 nuclei. Twenty clones formed plasmodia in plaques 2-3 days later than CL5001 controls. These plasmodia died on SDM agar unless supplemented with rolled oat flakes, and thus appeared to be derived from (APT1)1 nuclei. Two clones did not form plasmodia, so that there was no indication of their origin.

These results (see Table 17) showed that most, at least, of the amoebal progeny derived indirectly from (APT1)1 formed plasmodia in plaques, albeit with a "delayed" phenotype. It was probable, therefore, that (APT1)1 was formed from revertant amoebae which had acquired the ability to form plasmodia in plaques. However, it was also possible that (APT1)1 arose without any heritable change, and that plasmodium formation by its progeny was due to some cytoplasmic factor transmitted from (CL5001)1 to (APT1)1 in the heterokaryon. The poor growth of plasmodia derived from APT1 amoebae might have been due to pleiotropic effects of a suppressor mutation causing revertant plasmodium formation. Alternatively, the clone of APT1 amoebae used in this experiment might have carried a mutant allele at a locus affecting plasmodial growth. Analyses by Wheals (1973) and Dee et al., (1973), and evidence reported in 3.3f, indicated that APT1 amoebae did not normally transmit such a factor to progeny of crosses with other strains.

If the heterokaryon analysis is taken as evidence of reversion, then evidence of reversion was found for 8 of the 14 mutant strains (see also (iii)). Since three strains showed evidence of both revertant and non-revertant plasmodium formation, the failure to detect reversion in

Table 17. Analysis of progeny of the heterokaryotic plasmodium [(APT1)1 + (CL5001)1]1

Plasmodium formation by progeny clones*	Phenotypes of plasmodia derived from progeny clones	Number in class	Deduced origin
CL5001-like	Growth on SDM Growth on DM-1 + leucine Death on DM-1	28	(CL5001)1
"delayed"	Growth on SDM + oat flakes Death on SDM	20	(APT1)1
none	-	2	-

* CL5001-like: plasmodia formed in plaques after 4 days, as in CL5001 controls.
"Delayed": plasmodia formed in plaques after 6-7 days.
None: no plasmodia formed within 14 days.

(CL5001)1 control plasmodium grew on SDM and DM-1 + leucine, died on DM-1.

(APT1)1 control plasmodium grew on SDM + oat flakes, died on SDM.

any particular strain was no indication that the strain was unable to revert.

(v) The genetic basis of clonal plasmodium formation in NPF and heterothallic strains Other work (presented later in this chapter) indicated that most of the NPF strains carried mutations in one or other of two nuclear genes (*npfB* and *npfC*) in or closely linked to the mating type locus; no recombination of alleles of *npfB* or *npfC* and *mt* was detected. Thus, if reversion of the NPF strains occurred by back-mutation, crosses of revertant amoebae with heterothallic strains (e.g. mt_3) would yield amoebal progeny which were all either $mt_h;npf^+$ or mt_3. No $mt_h;npf^-$ recombinant progeny would arise, so that all clones which failed to form plasmodia in plaques would carry the mt_3 allele and would fail to cross with other mt_3 clones. Alternatively, if reversion of the NPF strains was due to acquisition of suppressor mutations (su^-) unlinked to *mt* and the *npf* genes, crosses of revertant amoebae with a mt_3 strain would yield some amoebal progeny which failed to form plasmodia in plaques but were able to cross with mt_3 strains (i.e. $mt_h;npf^-;su^+$ recombinants).

In addition to the investigation of reversion by the NPF strains, the mutation of LU688 to CL-like behaviour was analysed in the same way; the similarity already noted between behaviour of heterothallic and NPF strains suggested that mutation of LU688 might have occurred in the same way as reversion of the mutants.

The two NPF strains studied were CL6099 ($mt_h;npfC1^-$) and CL6129 ($mt_h;npfB4^-$). The plasmodia (CL6099)9 and (CL6129)1 both gave rise to amoebal progeny which gave rise to plasmodia in plaques on the same day as CL controls. A single amoebal progeny clone of each of (CL6099)9 and (CL6129)1 was selected for analysis (these clones were designated LU888 and LU889, respectively). In addition an amoebal clone derived from the plasmodium (LU688)1 was selected for analysis and designated LU884.

Amoebal clones LU888 and LU889, which both carried the *fusA2* allele, were crossed with LU862 (*mt*$_3$;*fusA1* - the derivation of LU862 is described in Appendix 2), while LU884, which carried the *fusA1* allele, was crossed with LU647 (*mt*$_1$;*fusA2*). All these crosses were carried out at 29-30°C, since Adler and Holt (1974b) had shown in *mt*$_h$ x *mt*$_3$ crosses that the proportion of clonally formed *mt*$_h$ plasmodia was reduced at this temperature. Plasmodia arising in the cross puddles were tested for their fusion behaviour with known plasmodia of fusion groups I, III and V. Crossed plasmodia were identified by fusion only with a group III (*fusA1*/*fusA2*) tester plasmodium.

A single group III plasmodium from each cross was caused to sporulate and about 50 amoebal progeny clones were derived from the spores of each plasmodium. The analysis of these progeny is shown in Tables 18-20. They were first plated on DSDM agar to give well separated plaques and incubated for two weeks. In each case about half the progeny formed plasmodia in plaques and the remainder did not form plasmodia. The fusion behaviour of the plasmodia was tested; about half were group I (*fusA1*) and half group V (*fusA2*).

Those progeny which did not form clonal plasmodia were inoculated into 0.2 ml bacterial puddles on DSDM agar with appropriate heterothallic strains (i.e. *mt*$_3$ for progeny of crosses involving LU888 and LU889, *mt*$_1$ for progeny of the cross involving LU884). These plates were incubated for six weeks. No plasmodia of appropriate hybrid fusion type were obtained. Since it was known that CL6099 and CL6129 would cross with *mt*$_3$ strains (see 3.3i) and that LU688 would cross with *mt*$_1$ strains (Cooke and Dee, 1974), the failure of these attempted crosses indicated that all the progeny which did not form clonal plasmodia carried the *mt*$_1$ or *mt*$_3$ allele (depending upon the cross). The 1 : 1 segregation of *fusA1* and

Table 18. Analysis of progeny of the plasmodium (LU888* x LU862)2

Plasmodium formation by progeny clones plated on DSDM agar and incubated for two weeks	CL-like**	Fusion type of plasmodia formed by progeny clones	No. in class	
		I	7	
		V	11	
	none	Formation of hybrid$^{\alpha}$ plasmodia with:- LU862 CH188	No. in class	Deduced mating type of progeny clones
		none none	32	$\underline{mt}_3$

* LU888 = (CL6099)9:21

** plasmodia formed in plaques after 4 days, as in CL controls.

$^{\alpha}$ hybrid fusion type : expected group III with LU862 ($\underline{mt}_3$;$\underline{fus}$A1;$\underline{fus}$B1;$\underline{fus}$C1)
OR group IX with CH188 ($\underline{mt}_3$;$\underline{fus}$A2;$\underline{fus}$B1;$\underline{fus}$C2)

Segregation "CL-like" : "none" not significantly different from 1:1 ($p \approx 0.05$).

For explanation see text.

Table 19. Analysis of progeny of the plasmodium (LU889* x LU862)1

Plasmodium formation by progeny clones plated on DSDM agar and incubated for two weeks	CL-like**	Fusion type of plasmodia formed by progeny clones	No. in class	Deduced mating type of progeny clones
		I	13	
		V	16	
	none	Formation of hybrid$^{\alpha}$ plasmodia with:- LU862 CH188	No. in class	
		none none	21	$\underline{mt}_3$

* LU889 = (CL6129)1:21

** plasmodia formed in plaques after 4 days, as in CL controls.

$^{\alpha}$ hybrid fusion type : expected group III with LU862 ($\underline{mt}_3$;$\underline{fus}$A1;$\underline{fus}$B1;$\underline{fus}$C1)
OR group IX with CH188 ($\underline{mt}_3$;$\underline{fus}$A2;$\underline{fus}$B1;$\underline{fus}$C2)

Segregation "CL-like" : "none" not significantly different from 1:1 ($p > 0.05$).

For explanation see text.

Table 20. Analysis of progeny of the plasmodium (LU884* x LU647)1

Plasmodium formation by progeny clones plated on DSDM agar and incubated for two weeks	CL-like**	Fusion type of plasmodia formed by progeny clones	No. in class	
		I	13	
		V	17	
	none	Formation of hybrid$^{\alpha}$ plasmodia with:- LU647 LU648	No. in class	Deduced mating type of progeny clones
		none none	19	$\underline{mt}_1$

* LU884 = (LU688)1:21

** plasmodia formed in plaques after 4 days, as in CL controls.

$^{\alpha}$ hybrid fusion type : expected group III with LU647 ($\underline{mt}_1$;$\underline{fus}$A2;$\underline{fus}$B1;$\underline{fus}$C1)
OR group III with LU648 ($\underline{mt}_1$;$\underline{fus}$A1;$\underline{fus}$B1;$\underline{fus}$C1)

Segregation "CL-like" :"none" not significantly different from 1:1 ($p > 0.05$).

For explanation see text.

fusA2 types among the progeny which formed plasmodia in plaques (in each case $p > 0.05$) confirmed that the progeny were derived from heterozygous diploid plasmodia.

The failure to detect recombinant progeny which did not form plasmodia in plaques and were not mt_1 or mt_3 indicated that, in all three cases, the change to CL-like plasmodium formation was probably due to mutation of a nuclear gene in or linked to the mating type locus. This was consistent with the results expected if (CL6099)9 and (CL6129)1 were formed as a result of back-mutations of the mutant strains CL6099 and CL6129.

(vi) *Microscopic analysis of plasmodium formation by ALC strains*

Adler *et al.* (1975) carried out a microscopic analysis of plasmodium formation in ALC strains and concluded that the life cycle of these strains lacked the amoebal phase; spores germinated to yield plasmodia rather than amoebae.

To determine whether the four ALC strains identified in (iii) showed plasmodium formation direct from spores, filming slide cultures were set up with about 10^4 spores per culture. Spores of (CL6082)2, (CL6111)3, (CL5001/8)7 and (LU648)5 were observed daily, and also (CL)20 spores as a control. (CL)20 spores began to germinate within one day, yielding uninucleate amoebae. These amoebae proliferated and after 4 days some binucleate cells were visible within dense colonies of amoebae (probably 10^3 cells or more). Plasmodia were seen after 5-6 days. Spores of ALC strains germinated after 1-6 days and uninucleate amoebae were observed in all cultures (Adler *et al.* also reported that amoebal colonies developed in some ALC strains). Plasmodia were observed after 5-7 days. Two plasmodia developed well away from any amoebae, so that it was clear that they had either germinated from a spore or developed from

an amoeba directly after germination. Due to the presence of amoebae in the cultures, the possibility that plasmodia arose from groups of 5-10 amoebae could not usually be excluded.

It was concluded that these observations were consistent with the conclusions of Adler *et al.* that plasmodia arose directly from spores. However, in no case was there proof that this was so; plasmodia might have been formed within very small amoebal colonies. No attempt was made to distinguish these alternatives, though it would probably have been possible with time-lapse cinematographic analysis.

3.3f Crosses of NPF mutants with a heterothallic strain

Wheals (1973) showed that an amoebal-plasmodial transition mutant carrying a single nuclear gene mutation unlinked to the mating type locus could be identified by analysing progeny of a plasmodium formed by crossing the mutant strain with a heterothallic strain. He crossed amoebae of strain APT1 with a mt_1 strain. One quarter of the amoebal progeny of the cross formed plasmodia in plaques (mt_h;*apt*-1^+ recombinants) and the remainder failed to form plasmodia. A similar analysis was carried out on all the NPF strains to detect mutant alleles at putative *npf* loci unlinked to the mating type locus. APT1 was also included in this analysis.

(i) *Preliminary screening for npf⁻ alleles at loci unlinked to mt* Crosses were set up at 26°C or 28.5°C of all the mutants (which carried the *fusA2* allele) with LU648 (mt_1;*fusA1*). In each case plasmodia which fused with group III (*fusA1*/*fusA2*) tester plasmodia were recovered, indicating the formation of hybrids in the crosses. Plasmodia which fused with group V (*fusA2*) testers were also recovered from some mixtures, and from at least some mutants these apparently uniparental plasmodia arose at frequencies higher than those at which plasmodia were formed in

puddles of the mutants alone. This stimulation of "selfing" is described in detail in Chapter 4.

Spores were obtained from one hybrid plasmodium derived from each cross. About 100 amoebal progeny clones were isolated from each spore batch, plated on DSDM agar and incubated for two weeks at 26°C or 28.5°C, as appropriate. The results indicated that only CL6111 and APT1 carried mutant alleles at loci unlinked to *mt*, since only progeny of the crosses CL6111 x LU648 and APT1 x LU648 included one quarter which formed plasmodia in plaques (see Table 21). Four other crosses each gave rise to one amoebal progeny clone which formed plasmodia in plaques. These four clones were shown not to be normal haploid products of meiosis and were excluded from consideration in further analyses of the crosses. The nature of these anomalous clones is discussed in (viii).

The failure to detect mt_n;*npf*$^+$ recombinants, except from the crosses involving CL6111 and APT1, could be explained in several ways. The most likely explanation was that NPF strains other than CL6111 carried mutations at or closely linked to the mating type locus. However, two other possibilities existed; either the plasmodia which were assumed to be diploid heterozygotes were in fact haploid heterokaryons, so that no recombination of nuclear genes was possible, or the mutants carried extranuclear mutations inherited by all progeny of the crosses with LU648.

(ii) Attempt to determine whether the crosses with LU648 yielded diploid heterozygotes If hybrid plasmodia formed in crosses of the mutants with LU648 were haploid heterokaryons, no recombination of nuclear genes would be detected in the progeny. Amoebal progeny of all the crosses, except those involving CL6111 and APT1, were classified for their *mt* and *fusA* alleles (Tables 22-25). To do this, progeny were each

Table 21. Formation of plasmodia in plaques by progeny of hybrid plasmodia derived from NPF x LU648 crosses

Hybrid plasmodia	Progeny plated on DSDM agar	
	Plasmodia in plaques	Amoebae only
(CL6049 x LU648)1	1	104
(CL6082 x LU648)3	0	99
(CL6089 x LU648)9	0	96
(CL6099 x LU648)3	0	89
(CL6100 x LU648)10*	0	100
(CL6111 x LU648)3*	21	79
(CL6115 x LU648)4*	0	100
(CL6129 x LU648)3	0	105
(CL6130 x LU648)3	1	99
(CL6134 x LU648)2	0	98
(CL6136 x LU648)5	1	98
(CL6143 x LU648)11	0	100
(CL5001/8 x LU648)12	1	99
(APT1 x LU648)3	23	77

* Tested at 28.5°C.

Table 22. Segregation of mt_h and mt_1 alleles in progeny of the plasmodium (CL6049 x LU648)1

Plasmodium formation when progeny clones inoculated in puddles:- Alone	with LU648	with LU688	Deduced genotype	Number in class
- -	+ +	- -	$\underline{mt_h};\underline{npf}^-$	35
- -	+ +	+ -	$\underline{mt_h};\underline{npf}^-$	1
+ -	+ +	- -	$\underline{mt_h};\underline{npf}^-$	4
				40
- -	- -	+ +	$\underline{mt_1}$	43*
- -	+ -	+ +	$\underline{mt_1}$	3
+ -	- -	+ +	$\underline{mt_1}$	13
+ -	+ -	+ +	$\underline{mt_1}$	2
- -	- -	+ -	$\underline{mt_1}$	1**
- -	- -	- -	$\underline{mt_1}$	1**
+ +	+ +	+ +	$\underline{mt_1}$	1**
				64

Each test carried out in duplicate: + + = plasmodia in both puddles, + - = plasmodia in one puddle, - - = no plasmodia.

** Retested: result as *

Segregation $\underline{mt_h}$: $\underline{mt_1}$ significantly different from 1:1 ($0.05 > p > 0.01$).

Genotype of CL6049 $\underline{mt_h};\underline{npf}^-$.
Genotype of LU648 $\underline{mt_1}$.
Genotype of LU688 $\underline{mt_2}$.

Table 23. Segregation of fusA1 and fusA2 alleles in progeny of the plasmodium (CL6049 x LU648)1

Fusion behaviour of derived* plasmodia with testers of groups:- I	III	V	Deduced progeny genotype	Number in class
F	NF	NF	fusA1	54
				54
NF	F	NF	fusA2	44
NF	NF	F	fusA2	6
				50

* plasmodia derived by crossing progeny clones with LU648 (mt_1;fusA1) or LU688 (mt_2;fusA1).

F = fusion. NF = non-fusion.

Segregation fusA1 : fusA2 not significantly different from 1:1 ($p > 0.05$).

Genotype of CL6049 fusA2.
Genotype of LU648 fusA1.

Table 24. Recombination of mt and fusA alleles in progeny of the plasmodium (CL6049 x LU648)1

Classes	No. in class
Parental:-	
$\underline{mt}_h$;$\underline{fusA}2$	16
$\underline{mt}_1$;$\underline{fusA}1$	30
	46
Recombinant:-	
$\underline{mt}_h$;$\underline{fusA}1$	24
$\underline{mt}_1$;$\underline{fusA}2$	34
	58

Segregation recombinants:parentals not significantly different from 1:1 ($p > 0.05$).

Table 25. Recombination of mt and fusA alleles in progeny of hybrid plasmodia derived from NPF x LU648 crosses

Hybrid plasmodia	Recombinants		Parentals	
	mt_h;fusA1	mt_1;fusA2	mt_h;fusA2	mt_1;fusA1
(CL6049 x LU648)1	24	34	16	30
(CL6082 x LU648)3	20	26	29	24
(CL6089 x LU648)9	30	19	20	27
(CL6099 x LU648)3	22	25	17	25
(CL6100 x LU648)10	4	3	6	7
(CL6115 x LU648)4	2	4	2	2
(CL6129 x LU648)3	27	25	22	31
(CL6130 x LU648)3	4	4	1	1
(CL6134 x LU648)2	3	1	3	3
(CL6136 x LU648)5	2	2	2	4
(CL6143 x LU648)11	4	3	5	8
(CL5001/8 x LU648)12	7	7	2	4

Progeny classified as shown in Tables 22-24.

inoculated into two 0.2 ml bacterial puddles alone, two puddles with LU648 amoebae and two puddles with LU688 amoebae (*mt*$_2$). The puddles were incubated for six weeks.

Amoebal clones which formed plasmodia in both puddles with LU648 were classified as *mt*$_h$;*npf*$^-$. Clones which formed plasmodia in both puddles with LU688 were classified as *mt*$_1$. None of the mutants crossed readily with LU688 (see 3.3i), so that each progeny clone normally formed plasmodia with one or other of LU648 and LU688. The classification for *mt* alleles of progeny of the cross CL6049 x LU648 is shown in Table 22. Each test cross was carried out in duplicate to exclude mis-scoring as a result of low frequency clonal plasmodium formation; examination of progeny inoculated alone showed that the frequency of clonal plasmodium formation was not markedly different from that found for the parent strains (see 3.3e).

Plasmodia formed in puddles with LU648 or LU688 were tested for their fusion behaviour and the *fusA* alleles carried by the progeny clones were deduced from the results. Table 23 shows the classification for *fusA* alleles of progeny of the cross CL6049 x LU648. A single plasmodium derived from each progeny clone was tested. LU648 and LU688 both carried the *fusA*1 allele, so that recovery of group III (*fusA*1/*fusA*2) or group V (*fusA*2) plasmodia from test cross puddles indicated crossing or "selfing", respectively, of *fusA*2 progeny. Group I (*fusA*1) plasmodia were assumed to arise from crossing or "selfing" of *fusA*1 progeny, though clonal plasmodium formation by LU648 or LU688 when in mixtures with *fusA*2 progeny could have led to *fusA*2 clones being incorrectly designated *fusA*1. This source of error was probably of little significance, since 12 clones classified in this way as *fusA*1 all gave rise to group III plasmodia when mixed with known *fusA*2 strains.

Recombinant types were identified within each set of progeny tested. Table 24 summarises the classification for mt and fusA alleles of progeny of the cross CL6049 x LU648. Table 25 summarises the results of analyses of all the NPF strains except CL6111. Where sufficient numbers were classified allele ratios were not usually significantly different from 1 : 1 ($p > 0.05$ except in one case where $p > 0.01$) and there was no significant deviation from free recombination of mt and fusA alleles ($p > 0.05$). It was concluded that the progeny were derived from heterozygous diploid plasmodia. Thus the failure to detect mt_h;npf^+ recombinants, except from the cross CL6111 x LU648, was not due to failure to form diploid crossed plasmodia.

(iii) Crosses to determine whether NPF mutants carried extranuclear mutations If some of the NPF strains failed to form plasmodia because they carried extranuclear mutations, the cytoplasmic factors involved were presumably equally distributed to both mt_h and mt_1 progeny of the crosses with LU648. Since all the mt_h progeny of most crosses failed to form plasmodia, it followed that in these crosses all progeny must have carried the cytoplasmic factors.

It seemed likely that two strains carrying the same factor preventing the formation of clonal plasmodia would be unable to cross with one another. Thus a mt_1;fusA1 clone was selected from among the progeny of each cross with LU648 which did not yield mt_h;npf^+ recombinants. These clones were inoculated into 0.2 ml bacterial puddles with the corresponding mutant strains (fusA2).

Plasmodia of group III (fusA1/fusA2) fusion behaviour were recovered from each of these crosses, indicating the production of hybrid plasmodia. This seemed to suggest that extranuclear mutations were not involved, and that the NPF strains must carry nuclear gene mutations at or

closely linked to the mating type locus. However, the basis of the test was shown to be doubtful when it was found that strains carrying the (nuclear) mutant allele present in CL6111 were able to cross with one another (see (vi)).

(iv) Heterokaryon tests to determine whether NPF mutants carried extranuclear mutations Two non-revertant plasmodia formed clonally from CL6089 and CL6099 (see 3.3e) were each allowed to grow into contact and fuse with CL5001 plasmodia (mt_h;leu-1^-) to form heterokaryons. Fifty amoebal progeny of each heterokaryon were isolated, plated on DSDM agar and incubated for two weeks. Some progeny formed plasmodia in plaques and were shown to be leu-1^-, while the remainder failed to form plasmodia in plaques and were shown to be leu-1^+ (see Tables 26 and 27). Thus none of the leu-1^- progeny acquired the mutant phenotype. This was strong evidence that CL6089 and CL6099 did not carry extranuclear mutations.

Since CL6089 and CL6099 were representatives of the two complementation groups subsequently identified in the strains carrying apparently mt-linked mutations (see 3.3h), it was concluded that all the mutant strains, except CL6111 and APT1, probably carried nuclear mutations at or closely linked to the mating type locus.

(v) Analysis of progeny of the cross APT1 x LU648 Progeny of the plasmodium (APT1 x LU648)3 were analysed. Clones which formed plasmodia in plaques were classified as mt_h;apt-1^+. These plasmodia were classified for fusA alleles. The remaining clones (mt_h;apt-1^- and mt_1) were classified for mt and fusA alleles as described in (ii). Amoebal clones carrying mt_1 were classified for apt-1 alleles by incubating them in puddles with mt_h;apt-1^- strains; mt_1;fusA1 progeny were mixed with APT1 (mt_h;fusA2;apt-1^-) and mt_1;fusA2 clones were mixed with LU864 (a mt_h;fusA1;apt-1^- progeny clone of the cross). Clones giving rise to

Table 26. Analysis of progeny of the heterokaryotic plasmodium [(CL6089)1 + (CL5001)1]1

Progeny clones plated on DSDM agar and incubated for two weeks	Plasmodia in plaques	Growth on DM-1 and DM-1 + leucine of plasmodia formed from progeny clones:- DM-1	DM-1 + leucine	Deduced genotype	Number in class
		-	+	leu-1⁻	12
		+	+	leu-1⁺	0
	No plasmodia	Growth on DM-1 and DM-1 + leucine of plasmodia formed from progeny x LU860*:- DM-1	DM-1 + leucine	Deduced genotype	Number in class
		-	+	leu-1⁻	0
		+	+	leu-1⁺	38

Genotype of LU860 mt_1;fusA1;leu-1⁻

Genotype of CL6089 mt_h;npf⁻;fusA2;leu-1⁺

Genotype of CL5001 mt_h;fusA2;leu-1⁻

*group III (fusA1/fusA2) plasmodia selected from crosses of progeny with LU860, tested for leu-1 alleles.

+ = growth. - = death.

Table 27. Analysis of progeny of the heterokaryotic plasmodium [(CL6099)3 + (CL5001)1]1

Progeny clones plated on DSDM agar and incubated for two weeks	Plasmodia in plaques	Growth on DM-1 and DM-1 + leucine of plasmodia formed from progeny clones:- DM-1	DM-1 + leucine	Deduced genotype	Number in class
		-	+	leu-1$^-$	38
		+	+	leu-1$^+$	0
	No plasmodia	Growth on DM-1 and DM-1 + leucine of plasmodia formed from progeny x LU860*:- DM-1	DM-1 + leucine	Deduced genotype	Number in class
		-	+	leu-1$^-$	0
		+	+	leu-1$^+$	12

Genotype of LU860 mt_1;fusA1;leu-1$^-$

Genotype of CL6099 mt_h;npf$^-$;fusA2;leu-1$^+$

Genotype of CL5001 mt_h;fusA2;leu-1$^-$

*group III (fusA1/fusA2) plasmodia selected from crosses of progeny with LU860, tested for leu-1 alleles.

+ = growth. - = death.

group III plasmodia in these crosses were designated $\underline{mt}_1;\underline{apt}\text{-}1^+$, and those not giving rise to group III plasmodia were designated $\underline{mt}_1;\underline{apt}\text{-}1^-$. The results of the analysis are summarised in Table 28.

(vi) Analysis of progeny of the cross CL6111 x LU648 Amoebal progeny clones of the plasmodium (CL6111 x LU648)3 were classified for mt, fusA and a proposed gene, npfA (see Table 29). Clones which formed plasmodia in plaques when plated at 28.5°C were designated $\underline{mt}_h;\underline{npfA}^+$. The remaining clones were tested for the formation of plasmodia in bacterial puddles at 26°C, and those which gave plasmodia (within two weeks) were classified as $\underline{mt}_h;\underline{npfA1}^-$. All other clones were classified as $\underline{mt}_1$. The segregation of $\underline{mt}_h$ and $\underline{mt}_1$ did not differ significantly from 1 : 1 ($p > 0.05$), and the segregation of $\underline{npfA}^+$ and $\underline{npfA}^-$ in $\underline{mt}_h$ clones was also not significantly different from 1 : 1 ($p > 0.05$).

All progeny were classified for their fusA alleles ($\underline{mt}_1$ progeny were crossed with LU688 to give plasmodia to be tested). Two clones initially classified as $\underline{mt}_h;\underline{npfA1}^-$ were excluded from the analysis when it was found that they were heterozygous at the fusA locus. Further information about these clones is given in (viii). Segregation of fusA1 and fusA2 did not differ significantly from 1 : 1 ($p > 0.05$).

To detect the segregation of $\underline{npfA}^+$ and $\underline{npfA1}^-$ in the $\underline{mt}_1$ progeny, attempts were made to cross them (at 28.5°C) with $\underline{npfA1}^-$ clones. It was expected that, if $\underline{npfA1}^-$ strains were unable to cross with one another, half the $\underline{mt}_1$ progeny ($\underline{npfA1}^-$) would not form plasmodia of hybrid fusion type with $\underline{mt}_h;\underline{npfA1}^-$ strains carrying different fusA alleles (the $\underline{mt}_h;\underline{npfA1}^-$ strains used were LU867,fusA1, and LU868,fusA2). However, all the $\underline{mt}_1$ clones gave rise to hybrid fusA1/fusA2 plasmodia in these crosses. To determine whether any of the $\underline{mt}_1$ clones carried the $\underline{npfA1}^-$ allele, it was necessary to analyse $\underline{mt}_h$ progeny of these hybrid plasmodia. Twenty

Table 28. Analysis of progeny of the plasmodium (APT1 x LU648)3

		apt-1$^+$	apt-1$^-$	Totals		
mt_h	fusA1	16	11	27	46 mt_h	56 fusA1
	fusA2	7	12	19		
mt_1	fusA1	15	14	29	54 mt_1	44 fusA2
	fusA2	17	8	25		
		55	45	100		

For details of classification see text.

Parental genotypes:- APT1 mt_h;fusA2;apt-1$^-$
LU648 mt_1;fusA1;apt-1$^+$

Recombination between:-

mt and apt-1, recombinants 45 : parentals 55
apt-1 and fusA , recombinants 49 : parentals 51
mt and fusA , recombinants 52 : parentals 48

Allele ratios and recombinants : parentals do not differ significantly from 1:1 ($p > 0.05$).

Table 29. Analysis of progeny of the plasmodium (CL6111 x LU648)3

		npfA$^+$	npfA1$^-$	Totals			
mt_h	fusA1	9	18	27	47 mt_h	51 fusA1	47 fusA2
	fusA2	12	8	20			
mt_1	fusA1	(2)*	(2)	24	51 mt_1		
	fusA2	(2)	(2)	27			
		25	30	98			

* Figures in parentheses refer to a sample of only eight mt_1 clones classified for npfA.

For details of classification see text.

Parental genotypes:- CL6111 mt_h;fusA2;npfA1$^-$
LU648 mt_1;fusA1;npfA$^+$

Recombination between:-

mt	and npfA,	recombinants 25	: parentals 30
npfA	and fusA,	recombinants 34	: parentals 21
mt	and fusA,	recombinants 54	: parentals 44

Allele ratios and recombinants : parentals do not differ significantly from 1:1 ($p > 0.05$).

amoebal clones derived from each of eight hybrid plasmodia were isolated; mt_h clones from four plasmodia showed segregation of *npfA*$^+$ and *npfA1*$^-$, but all mt_h progeny of the remaining four plasmodia carried the *npfA1*$^-$ allele. Segregation of *fusA1* and *fusA2* was detected in the mt_h progeny of all eight plasmodia. It was concluded that four hybrid plasmodia had arisen from mt_1;*npfA*$^+$ clones and four from mt_1;*npfA1*$^-$ clones. Thus, although the *npfA1*$^-$ allele prevented clonal plasmodium formation, it did not prevent plasmodium formation in crosses between *npfA1*$^-$ strains.

The analysis of the cross CL6111 x LU648 showed no significant deviation from free recombination of *npfA*, *mt* and *fusA* ($p > 0.05$).

(vii) Test for allelism of *npfA1*$^-$ and *apt-1*$^-$ In order to test for allelism of *npfA1*$^-$ and *apt-1*$^-$, amoebae of strain LU867 (mt_h;*fusA1*; *npfA1*$^-$) were crossed with LU866 amoebae (LU866 was a mt_1;*fusA2*;*apt-1*$^-$ progeny of the cross APT1 x LU648) and plasmodia which fused with group III (*fusA1*/*fusA2*) tester plasmodia were recovered. Spores were obtained from one of these crossed plasmodia, (LU866 x LU867)1, and germinated. Two hundred amoebal progeny clones were isolated, plated on DSDM agar and incubated at 26°C for two weeks. It was expected that, if *npfA1*$^-$ and *apt-1*$^-$ were mutant alleles at two unlinked loci, approximately one eighth of progeny clones would form plasmodia in plaques (mt_h;*npfA*$^+$;*apt-1*$^+$ recombinants), one eighth would form a few "delayed" plasmodia per plate (mt_h;*npfA1*$^-$;*apt-1*$^+$) and the remainder would fail to form plasmodia. The absence of clones able to form plasmodia in plaques would indicate that *npfA1*$^-$ and *apt-1*$^-$ were alleles of the same locus, or of closely linked loci. Twenty-three clones formed plasmodia in plaques (11 *fusA1* : 12 *fusA2*) and 33 formed "delayed" plasmodia. Neither of these numbers differed significantly from the expectation for two unlinked loci ($p > 0.05$).

(viii) Analysis of anomalous clones In the crosses of NPF

strains with LU648 (see (i)) six anomalous clones were identified and excluded from the results of the appropriate analyses. These clones were redesignated LU890-LU895 (see Table 30). Three clones which formed plasmodia in plaques were shown definitely not to be haploid $\underline{mt}_h;\underline{npf}^+$ recombinants; plasmodia formed clonally from LU891-LU893 were found to have approximately diploid nuclear DNA values (i.e. about 1.2 pg DNA/nucleus instead of about 0.6 pg), and LU891 and LU892 gave rise to plasmodia of group III fusion behaviour (indicating heterozygosity at the fusA locus at least). A fourth clone, LU890, which formed plasmodia in plaques was found to give rise to a group V plasmodium with approximately haploid nuclear DNA content. This result did not exclude the possibility that LU890 was a haploid $\underline{mt}_h;\underline{npf}^+$ recombinant; however, attempts to cause sporulation of the plasmodium (LU890)1 failed and it was suspected that this might be due to some genetic abnormality. Two clones derived from the plasmodium (CL6111 x LU648)3 were initially classified as $\underline{mt}_h;\underline{npfA1}^-$ but were subsequently shown to give rise to group III plasmodia; LU894 amoebae and a clonally derived plasmodium were tested and found to contain nuclei of apparently haploid DNA content. The results of these preliminary investigations of LU890-LU895, which indicated that LU891-LU895 could not be normal haploid products of meiosis, are shown in Table 30 and Figure 24.

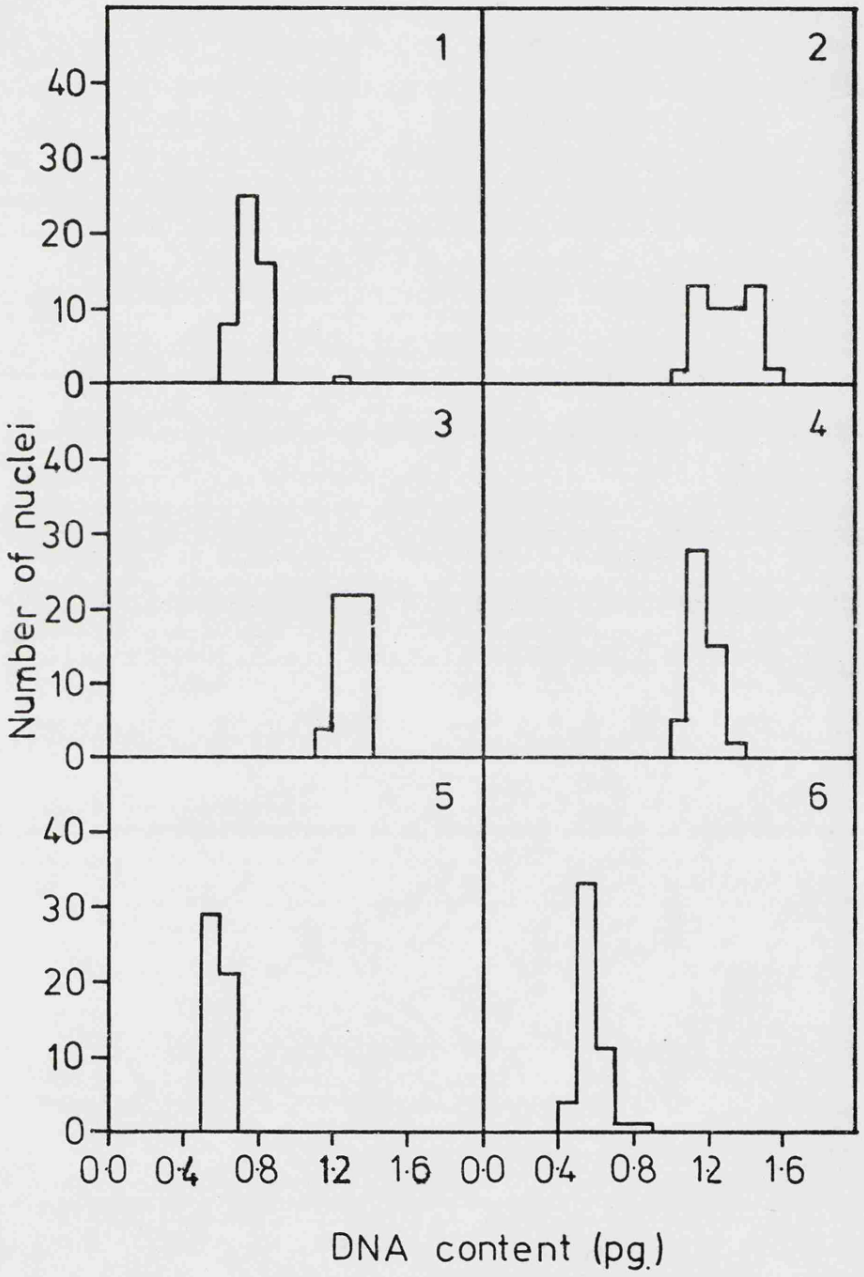
In order to obtain further insight into the anomalous behaviour reported above, progeny of some clonally formed plasmodia were analysed. Adler and Holt (1975) showed that diploid (or near diploid) amoebae heterozygous at the mating type locus gave rise to plasmodia in plaques without change in nuclear DNA content. Thus the likeliest explanation of the behaviour of LU891-LU893 was that these were diploid clones heterozygous at the mating type locus. In order to test this possibility,

Table 30. Preliminary analysis of anomalous clones derived from NPF x LU648 crosses

Progeny clone	Redesignated	Mean nuclear DNA content (pg)	Clonally derived plasmodium	Fusion type	Mean nuclear DNA content (pg)
(CL6049 x LU648)1:31	LU890	-	(LU890)1	V	0.78
(CL6130 x LU648)3:64	LU891	-	(LU891)1	III	1.29
(CL6136 x LU648)5:61	LU892	-	(LU892)1	III	1.33
(CL5001/8 x LU648)12:17	LU893	-	(LU893)*1	V	1.18
(CL6111 x LU648)3:14	LU894	0.59	(LU894)1	III	0.57
(CL6111 x LU648)3:82	LU895	-	(LU895)1	III	-

* Grew on DM-1 + leucine, died on DM-1. Thus leu-1$^-$, as CL5001.

Figure 24. Nuclear DNA contents of amoebae and plasmodia of anomalous clones identified among the progeny of NPF x LU648 crosses. 1 (LU890)1. 2 (LU891)1. 3 (LU892)1. 4 (LU893)1. 5 LU894 6 (LU894)1.

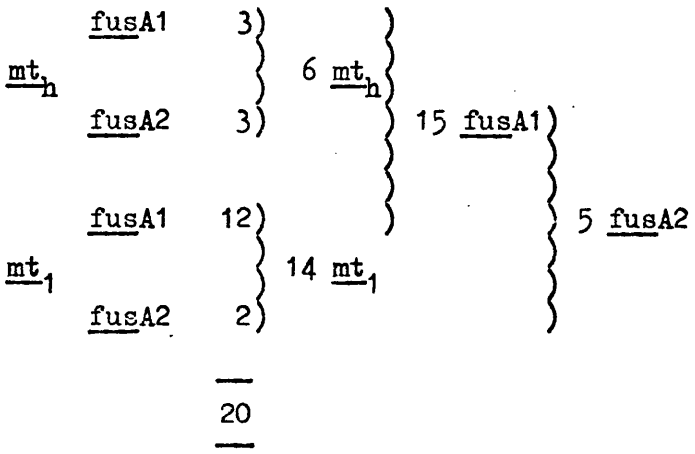

1
2
3
4
5
6
40
30
20
10
0
Number of nuclei
0·0
0·4
0·8
1·2
1·6
DNA content (pg.)

twenty amoebal progeny of (LU892)1 and (LU893)1 were isolated. All failed to form plasmodia in plaques. The clones were classified for their mating type alleles in the same way as described in (ii). Progeny derived from (LU892)1 were also classified for fusA alleles. Progeny derived from (LU893)1 were classified for leu-1 alleles. These analyses are shown in Tables 31 and 32. In each case both mt_h and mt_1 types were recovered and recombination of mt and fusA or leu-1 was detected. This was consistent with the view that these apparently mt_h;npf^+ recombinant clones were in fact diploid heterozygotes.

The preliminary analysis of LU890 failed to exclude the possibility that this was a mt_h;npf^+ recombinant clone, though the failure of the plasmodium (LU890)1 to sporulate suggested that it was defective in some way. If the formation of plasmodia in plaques was promoted by heterozygosity at the mating type locus, not all mating type heterozygotes would necessarily contain diploid nuclei; aneuploid nuclei might contain two chromosomes carrying different mating type alleles. Microdensitometric analyses of nuclear DNA contents were not sufficiently accurate to exclude this possibility.

If LU890 amoebae were heterozygous at the mating type locus due to aneuploidy, segregation of mating type alleles might occur in progeny of the plasmodium (LU890)1. Since the plasmodium failed to sporulate, it was fused with a CL5001 plasmodium (mt_h;leu-1^-) to form a heterokaryon. The heterokaryon was caused to sporulate and 50 amoebal progeny clones were isolated. They were plated on DSDM agar and incubated for two weeks. Forty-four clones formed plasmodia in plaques; all these plasmodia were shown to carry the leu-1^- allele and were thus presumably derived from CL5001 nuclei. The six clones which did not form plasmodia were inoculated into bacterial puddles with LU871 (a mt_h;fusA1 progeny clone

Table 31. Analysis of progeny of the plasmodium (LU892*)1



* LU892 = (CL6136 x LU648)5:61

For details of classification see (ii).

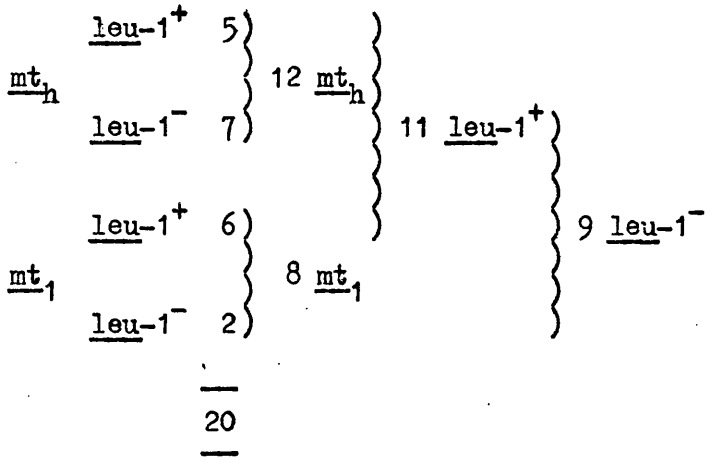
Allele ratios:-

mt_h:mt_1 not significantly different from 1:1 ($p>0.05$).

fusA1:fusA2 significantly different from 1:1 ($0.05>p>0.01$).

Recombination between mt and fusA, (recombinants 5 : parentals 15) not significantly different from 1:1 ($0.05>p>0.01$).

Table 32. Analysis of progeny of the plasmodium (LU893*)1



*LU893 = (CL5001/8 x LU648)12:17. CL5001 was leu-1⁻

mt: classified as in (ii) except that tester clones were LU860 (mt_1;fusA1;leu-1⁻) and LU858 (mt_2;fusA1;leu-1⁻).

leu-1: group III plasmodia from crosses of progeny with LU860 or LU858 tested for growth on DM-1 agar and DM-1 + leucine (homozygous leu-1⁻ plasmodia die on DM-1 but grow on DM-1 + leucine; leu-1⁺ plasmodia grow on both).

Allele ratios:-

mt_h : mt_1 not significantly different from 1:1 ($p>0.05$)

leu-1⁺:leu-1⁻ not significantly different from 1:1 ($p>0.05$)

Recombination between mt and leu-1 (recombinants 13 : parentals 7) not significantly different from 1:1 ($p>0.05$).

of the cross CL6049 x LU648) and LU648 (*mt*$_1$;*fusA*1). Four clones formed group III plasmodia with LU871, indicating that they carried the *mt*$_1$ allele, and two clones formed group III plasmodia with LU648, indicating that they carried the *mt*$_h$ allele. This result (see Table 33) suggested that LU890 carried two different mating type alleles, and thus that the clone might by aneuploid. The results were consistent with the hypothesis that the formation of plasmodia in plaques was promoted by heterozygosity at the mating type locus.

The behaviour of the clone LU894 could also be explained by aneuploidy. Forty-seven amoebal progeny of the plasmodium (LU894)1 were isolated and plated on DSDM agar plates at 26°C. All clones formed "delayed" plasmodia within two weeks, indicating that they were of the genotype *mt*$_h$;*npfA*1$^-$. The plasmodia were tested for their fusion behaviour and were found to include representatives of groups I, III and V (see Table 34). The segregation of plasmodia of groups I (*fusA*1) and V (*fusA*2) was not significantly different from 1 : 1 ($p > 0.05$), and was thus consistent with heterozygosity of the *fusA* locus due to aneuploidy. The presence of group III (*fusA*1/*fusA*2) plasmodia indicated that, if aneuploidy was the correct explanation for the behaviour of LU894, this state could be maintained for many nuclear generations and through sporulation (though not necessarily through meiosis since the plasmodium was not diploid).

3.3g Screening of "delayed" strains for mutations at loci unlinked to the mating type locus

The results described in 3.3f indicated that 12 of the 13 NPF strains isolated in this study carried nuclear gene mutations at or closely linked to the mating type locus. NPF strains, rather than

Table 33. Analysis of progeny of the heterokaryotic plasmodium [(LU890**)1 + (CL5001)1]1

Progeny clones plated on DSDM agar and incubated for two weeks	Plasmodia in plaques	Growth on DM-1 and DM-1 + leucine of plasmodia formed from progeny clones:- DM-1	DM-1 + leucine	Deduced genotype	Number in class	Deduced origin
		-	+	leu-1⁻	44	(CL5001)1
	No plasmodia	Formation of hybrid* plasmodia with amoebae of strains:- LU648	LU871	Deduced genotype	Number in class	Deduced origin
		-	+	mt_1	4	(LU890)1
		+	-	mt_h	2	(LU890)1

*group III (fusA1/fusA2)

**LU890 = (CL6049 x LU648)1:31

Genotype of LU648 mt_1;fusA1

Genotype of LU871 mt_h;npf⁻;fusA1 (derived from the cross CL6049 x LU648)

+ = positive result. - = negative result.

Table 34. Analysis of progeny of the plasmodium (LU894*)1

Plasmodium formation on DSDM agar at 26°C	Fusion behaviour of clonally formed plasmodia with testers of groups:- I	III	V	Number in class	Deduced genotype[α]
All "delayed"**	F	NF	NF	7	$\underline{mt}_h$;$\underline{npf}A1^-$;$\underline{fus}A1$
	NF	F	NF	29	$\underline{mt}_h$;$\underline{npf}A1^-$;$\underline{fus}A1/\underline{fus}A2$
	NF	NF	F	11	$\underline{mt}_h$;$\underline{npf}A1^-$;$\underline{fus}A2$
			TOTAL	47	

* LU894 = (CL6111 x LU648)3:14

** "Delayed": 5–10 plasmodia per plate after 10–12 days (CL controls formed plasmodia in plaques after 4 days).

[α] See text for explanation.

F = fusion. NF = non-fusion.

"delayed" strains, were chosen for detailed genetic analysis because they could be handled as easily as heterothallic strains. However, in order to determine whether most of the "delayed" strains also carried mutations associated with the mating type locus, a number of such strains were analysed. Eight "delayed" strains isolated from different enrichment lines were inoculated into 0.2 ml bacterial puddles with LU862 amoebae (*mt*$_3$;*fusA1* - the derivation of LU862 is shown in Appendix 2). The puddles were incubated at 29-30°C. Two plasmodia were picked from each puddle and tested for their fusion behaviour with plasmodia of groups I, III and V. All plasmodia fused only with group III (*fusA1*/*fusA2*) testers, indicating the formation of hybrid plasmodia in the crosses. One hybrid plasmodium from each cross was caused to sporulate and 20 amoebal progeny clones were derived from each spore batch and anlysed (see Table 35).

In order to test for *mt* alleles, progeny clones were plated on DSDM agar with *E. coli* and incubated for two weeks. Clones which gave rise to plasmodia were classified as *mt*$_h$ and those which proliferated only as amoebae were designated *mt*$_1$. In each case the segregation of *mt*$_h$ and *mt*$_1$ was not significantly different from 1 : 1 ($p > 0.05$).

Two classes of plasmodium formation were recognised; some clones formed plasmodia in plaques at the same time as CL controls and were designated "CL-like", while some clones showed the "delayed" phenotype of the appropriate mutant parent. Seven crosses of "delayed" mutants with LU862 yielded progeny of both the above types, suggesting that the seven mutants involved might carry nuclear gene mutations unlinked to the mating type locus. The overall segregation of CL-like and "delayed" plasmodium formation in the *mt*$_h$ progeny of these seven crosses was not significantly different from 1 : 1 ($p > 0.05$). All the *mt*$_h$ progeny of a further two hybrid plasmodia formed plasmodia with the same "delayed"

Table 35. Analysis of progeny of "delayed" x LU862 crosses

Hybrid plasmodia	Plasmodium formation by progeny clones plated on DSDM agar*		
	CL-like	"delayed"	none
(CL6011 x LU862)1	0	6	14
(CL6058 x LU862)1	4	5	11
(CL6061 x LU862)1	6	6	8
(CL6062 x LU862)1	0	6	14
(CL6063 x LU862)1	4	7	9
(CL6066 x LU862)1	3	5	12
(CL6070 x LU862)1	5	3	12
(CL6081 x LU862)1	6	8	6
(CL6104 x LU862)1	2	9	9

* CL-like: plasmodia formed in plaques after 4 days, as in CL controls.

"Delayed": plasmodia formed as in control plates of appropriate mutant strains.

None: no plasmodia formed within 14 days.

phenotype as control plates of the corresponding mutant strains. Thus there was evidence that these two strains carried mutations linked to the mating type locus.

Plasmodia derived from all $\underline{mt}_h$ progeny clones were tested for their fusion behaviour with known plasmodia of groups I, III and V. In each case some $\underline{mt}_h$ progeny gave rise to group I (*fusA1*) plasmodia and some to group V (*fusA2*) plasmodia. No group III (*fusA1*/*fusA2*) plasmodia were identified. In each case individually testable, and overall, the ratio of *fusA1* to *fusA2* plasmodia was not significantly different from 1 : 1 ($p > 0.05$). From the 1 : 1 segregation of *fusA1* and *fusA2* alleles among the $\underline{mt}_h$ clones it was concluded that all the progeny were derived from heterozygous diploid plasmodia.

These results suggested that most "delayed" mutants did not carry mutations closely linked to the mating type locus. No attempt was made to analyse these strains further, so that it was not possible to determine whether few or many loci were involved, nor whether some mutants carried mutations of the *apt-1* or *npfA* loci.

3.3h Complementation of NPF strains

The analysis of crosses with LU648 (see 3.3f) indicated that 12 of the 13 NPF strains carried nuclear gene mutations in or closely linked to the mating type locus. In order to determine whether all 12 mutants were defective in the same gene, an attempt was made to test complementation between them. It was not evident at the outset that such an anlysis would succeed; it was not possible to carry out complementation tests using pairs of strains of different mating types and it was not known whether fusion of amoebae of the mutant strains could occur. (The cytological analysis of plasmodium formation in CL amoebae -

see Chapter 2 - had shown that amoebal fusions were not necessary for plasmodium formation in this strain but did not exclude the possibility that they sometimes occurred). CL6111 (mt_h;*npfA1*$^-$) and APT1 (mt_h;*apt-1*$^-$) were included in the analysis.

Amoebal clones of the class designated mt_h;*npf*$^-$;*fusA1* (or mt_h;*apt-1*$^-$;*fusA1*) were selected from progeny of the mutant x LU648 crosses (see 3.3f). Clones corresponding to each of the NPF mutants and APT1 were selected and designated LU864, LU867, and LU871-LU883. All possible mixtures of these derivative strains with the original mutants were set up in 0.2 ml puddles of bacterial suspension on DSDM agar plates. Each mixture of amoebal strains was inoculated into five puddles which were incubated at 26°C, or 28.5°C if temperature-sensitive strains were involved. Each pair of independently isolated mutations was thus crossed ten times (e.g. five CL6049 x CL6082-derivative puddles and five CL6082 x CL6049-derivative puddles), while each mutation was tested against itself only five times. Plasmodia which arose were tested for fusion with known plasmodia of groups I, III and V.

Sixty-four out of 196 combinations of strains were found to give rise to hybrid *fusA1*/*fusA2* plasmodia (i.e. group III). The results obtained for non-temperature-sensitive strains (see Table 36) were consistent with the interpretation that the strains carrying mutations associated with the mating type locus were defective in one or other of two genes, which were designated *npfB* and *npfC*. All *npfB*$^-$ x *npfC*$^-$ combinations gave hybrid plasmodia, but no *npfB*$^-$ x *npfB*$^-$ or *npfC*$^-$ x *npfC*$^-$ combinations did so. Both *npfB*$^-$ and *npfC*$^-$ strains crossed with *apt-1*$^-$ strains. While mixtures of strains carrying different mating type alleles routinely gave plasmodia in nearly 100% of puddles within two weeks, only about 40% of puddles of the complementing combinations shown

Table 36. Complementation of strains carrying non-temperature-sensitive mutations

CL6049	CL6082	CL6089	CL6129	CL6130	CL6134	CL6099	CL6136	CL6143	CL5001/8	APT1		Assigned mutant allele
0**	0	0	0	0	0	8*	5	6	5	7	CL6049	*npf*B1$^-$
	0	0	0	0	0	8	7	7	4	4	CL6082	*npf*B2$^-$
		0	0	0	0	3	5	1	5	5	CL6089	*npf*B3$^-$
			0	0	0	3	2	3	2	3	CL6129	*npf*B4$^-$
				0	0	3	4	4	2	4	CL6130	*npf*B5$^-$
					0	5	4	5	6	4	CL6134	*npf*B6$^-$
						0	0	0	0	3	CL6099	*npf*C1$^-$
							0	0	0	3	CL6136	*npf*C2$^-$
								0	0	2	CL6143	*npf*C3$^-$
									0	4	CL5001/8	*npf*C4$^-$
										0	APT1	*apt*-1$^-$

In crosses of mutants with one another, hybrid plasmodia could not be distinguished from rare clonal plasmodia. Thus each cross was between one of the original mutants (*fus*A2) and a derivative strain carrying the *fus*A1 allele.

* Number of puddles out of ten in which plasmodia of hybrid fusion type (group III) arose.

** Where mutants tested against themselves, only five puddles set up.

in Table 36 did so, and these plasmodia arose during the period 2-6 weeks after inoculation.

The efficiency of complementation was even lower with puddles involving temperature-sensitive strains at 28.5°C. Out of a total of 375 puddles incubated at 28.5°C, only three gave rise to plasmodia of hybrid fusion type. The combinations of strains were CL6100 x CL6099, CL6111 x CL6089, and CL6115 x CL5001/8, allowing the mutant alleles npfB7$^-$ and npfB8$^-$ to be tentatively assigned to the temperature-sensitive strains CL6100 and CL6115, respectively. Since it was known that CL6111 carried a mutation at the npfA locus, while CL6100 and CL6115 did not, it seemed likely that the low efficiency of complementation at 28.5°C was due to the elevated temperature, rather than particular characteristics of the temperature-sensitive strains. This interpretation was confirmed when 50 puddles of non-temperature-sensitive strains found to complement at 26°C were incubated at 28.5°C; no hybrid plasmodia were formed.

Nuclear DNA contents of 16 different hybrid plasmodia obtained in the complementation tests were estimated by scanning microdensitometry of Feulgen-stained preparations (see Table 37 and Figures 25 and 26). Mean values of 1.1-1.4 pg DNA/nucleus were obtained for each plasmodium. These were compared with values of 1.1-1.5 pg DNA/nucleus for the plasmodia formed by crossing the mutants with LU648 (Table 37 and Figure 26), and 0.5-0.8 pg DNA/nucleus for amoebae of the mutant strains, CL and LU648 (see Table 15 and Figure 22). Thus all the hybrid plasmodia tested appeared to be diploid rather than haploid.

3.3i Effects of npfB and npfC on the specificity of mating reactions

Since the genes npfB and npfC might be components of the mating type locus, attempts were made to determine whether npfB$^-$ or npfC$^-$ strains

Table 37. Mean nuclear DNA contents of plasmodia formed by mutant strains crossed with one another and LU648

No.*	Plasmodia	DNA (pg/nucleus)
1	(CL6049 x LU648)1	1.32
2	(CL6082 x LU648)3	1.23
3	(CL6089 x LU648)9	1.44
4	(CL6099 x LU648)3	1.20
5	(CL6100 x LU648)10	1.21
6	(CL6111 x LU648)3	1.33
7	(CL6115 x LU648)4	1.54
8	(CL6129 x LU648)3	1.26
9	(CL6130 x LU648)3	1.45
10	(CL6134 x LU648)2	1.14
11	(CL6136 x LU648)5	1.34
12	(CL6143 x LU648)11	1.48
13	(CL5001/8 x LU648)12	1.27
14	(APT1 x LU648)3	1.06

No.*	Plasmodia**	DNA (pg/nucleus)
15	CL6049 x CL6099	1.28
16	CL6049 x CL5001/8	1.25
17	CL6049 x APT1	1.24
18	CL6082 x CL6099	1.27
19	CL6082 x APT1	1.16
20	CL6089 x CL6143	1.38
21	CL6099 x CL6100	1.34
22	CL6099 x CL6129	1.15
23	CL6099 x CL6130	1.29
24	CL6115 x CL5001/8	1.11
25	CL6129 x CL6136	1.29
26	CL6129 x CL6143	1.32
27	CL6130 x CL6136	1.07
28	CL6130 x CL6143	1.16
29	CL6134 x CL6136	1.17
30	CL6134 x CL5001/8	1.21

* See Figures 25 and 26.

** Each of these plasmodia was the product of a cross between one of the original mutants and a derivative strain. Thus plasmodium no. 15 was in fact (CL6049 x LU879)1.

Figure 25. Nuclear DNA contents of crossed plasmodia isolated from mixtures of NPF and LU648 amoebae. See Table 37 for key to strains.

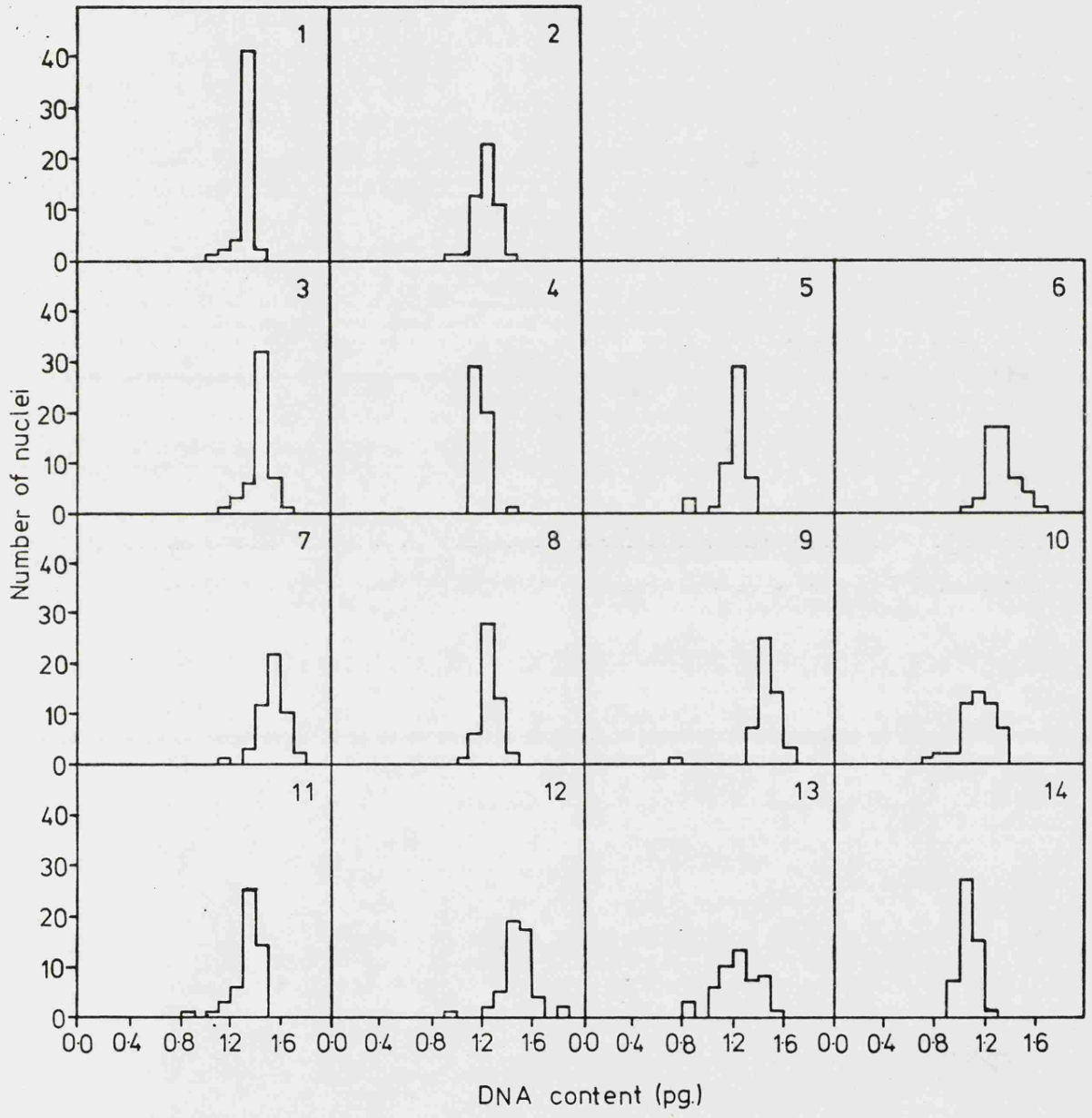
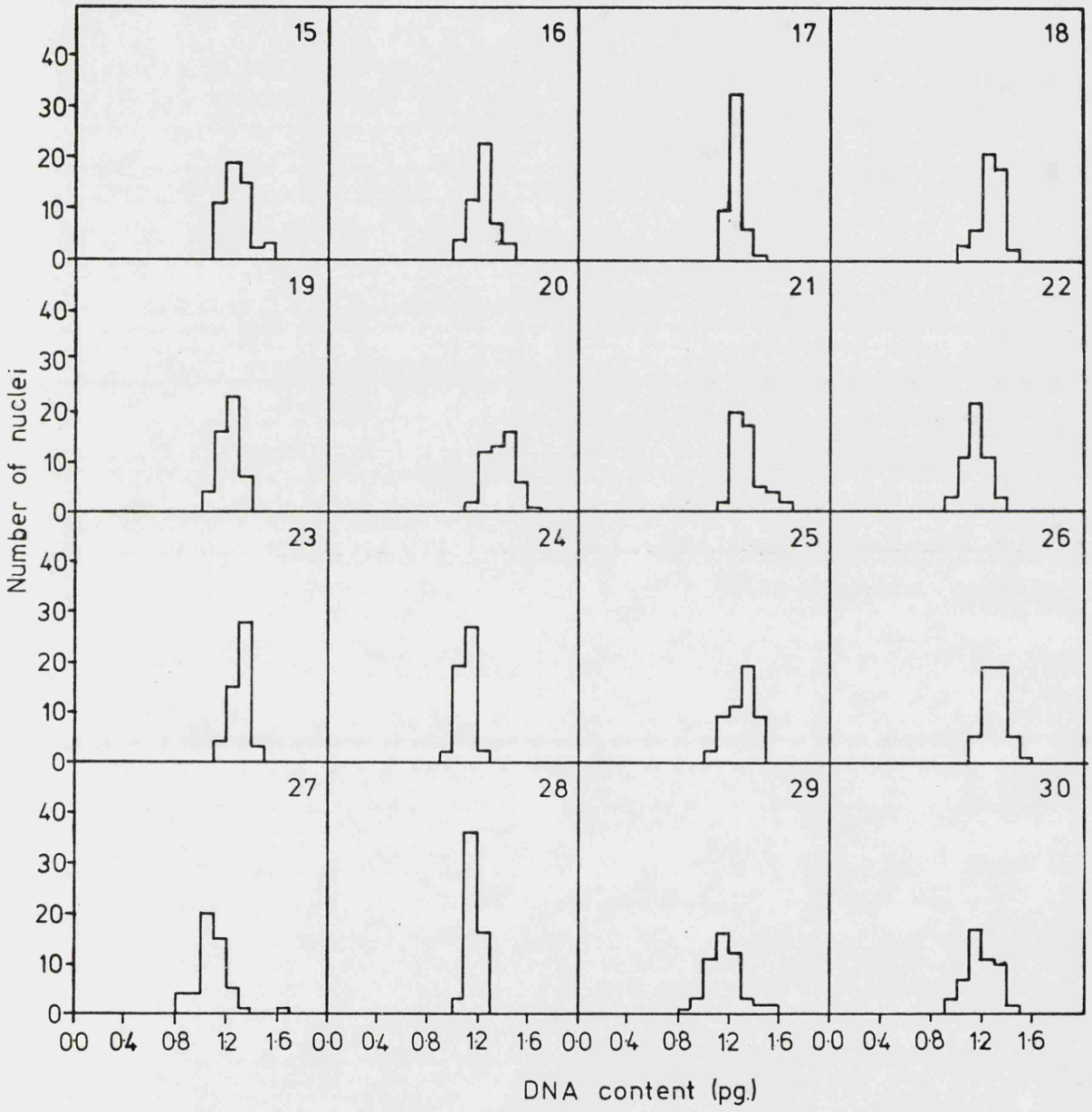

1
2
3
4
5
6
7
8
9
10
11
12
13
14
Number of nuclei
0
10
20
30
40
0·0 0·4 0·8 1·2 1·6
DNA content (pg.)

Figure 26. Nuclear DNA contents of plasmodia of hybrid fusion type isolated in the complementation analysis of NPF strains. See Table 37 for key to strains.


15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
Number of nuclei
0
10
20
30
40
0·0
0·4
0·8
1·2
1·6
DNA content (pg.)

were altered in their ability to cross with strains carrying various mating type alleles. The mutants carrying *npfB*$^-$ or *npfC*$^-$ alleles were inoculated into 0.2 ml puddles of bacterial suspension with amoebae of mt_1, mt_2, mt_3 and mt_4. All the mutants formed heterozygous diploid plasmodia when crossed with LU648 (mt_1), as already shown in 3.3f. Plasmodia of appropriate hybrid fusion behaviour (group III) were also obtained from all puddles with LU862 (mt_3;*fusA1*) and LU863 (mt_4;*fusA1*). LU688 (mt_2;*fusA1*) crossed very inefficiently, or not at all, with NPF strains. Wheals (1970) had also found that the efficiency of certain mt_n x mt_2 crosses was very low. Since Dee (1966) had noted that crosses between closely related strains were particularly prone to failure, a mt_2 strain which was not of Colonia genetic background was used to obtain the results shown in Table 38. This strain, i, was of the genotype mt_2;*fusA2*;*fusB2*. Thus, to allow identification of hybrid plasmodia by their fusion behaviour, amoebae of strain i were mixed with mt_n;*fusA1*;*fusB1* progeny of the mutant x LU648 crosses (the same clones, LU867-LU883, were used as in 3.3h). Five puddles of each test were set up. All puddles involving *npfB*$^+$;*npfC*$^-$ strains gave hybrid (group IV, *fusA1*/*fusA2*;*fusB2*) plasmodia but no hybrids were produced with *npfB*$^-$;*npfC*$^+$ strains.

3.4 DISCUSSION

The joint application of mutagenesis and enrichment procedures in this work yielded 13 independently isolated NPF strains. The number of mutants per viable cell after mutagenesis was about 7×10^{-6} and enrichment and screening procedures used a total of about 2×10^3 plates. No attempt was made to isolate mutants without enrichment but the probable

Table 38. Crosses of NPF strains with strains of various mating types

Mutant strains	Genotype	Formation of plasmodia of hybrid* fusion type with amoebae of:-			
		$\underline{mt}_1$	$\underline{mt}_2$	$\underline{mt}_3$	$\underline{mt}_4$
CL6049	$\underline{npfB1}^-;\underline{npfC}^+$	+	-	+	+
CL6082	$\underline{npfB2}^-;\underline{npfC}^+$	+	-	+	+
CL6089	$\underline{npfB3}^-;\underline{npfC}^+$	+	-	+	+
CL6129	$\underline{npfB4}^-;\underline{npfC}^+$	+	-	+	+
CL6130	$\underline{npfB5}^-;\underline{npfC}^+$	+	-	+	+
CL6134	$\underline{npfB6}^-;\underline{npfC}^+$	+	-	+	+
CL6100	$\underline{npfB7}^-;\underline{npfC}^+$	+	-	+	+
CL6115	$\underline{npfB8}^-;\underline{npfC}^+$	+	-	+	+
CL6099	$\underline{npfB}^+;\underline{npfC1}^-$	+	+	+	+
CL6136	$\underline{npfB}^+;\underline{npfC2}^-$	+	+	+	+
CL6143	$\underline{npfB}^+;\underline{npfC3}^-$	+	+	+	+
CL5001/8	$\underline{npfB}^+;\underline{npfC4}^-$	+	+	+	+

$\underline{mt}_1$ strain = LU648 ($\underline{fusA1};\underline{fusB1}$)
$\underline{mt}_2$ strain = i ($\underline{fusA2};\underline{fusB2}$)
$\underline{mt}_3$ strain = LU862 ($\underline{fusA1};\underline{fusB1}$)
$\underline{mt}_4$ strain = LU863 ($\underline{fusA1};\underline{fusB1}$)

* Mutant strains were crossed with $\underline{mt}_1$, $\underline{mt}_3$ and $\underline{mt}_4$ strains to give plasmodia of group III fusion type. Derivative strains LU871-LU883 ($\underline{fusA1};\underline{fusB1}$) were crossed with amoebae of strain i to give plasmodia of group IV.

+ = hybrid plasmodium formation. - = no hybrid plasmodia.

effort involved may be calculated. When mutagenised cultures were plated out to give separate plaques (this was done to obtain viable counts) the development of different colonies on each plate was very asynchronous; when the first large plasmodia were migrating outwards from the plaques in which they had formed, other amoebal colonies were barely large enough to be visible. Given this degree of asynchrony, screening of no more than twenty plaques per plate for plasmodium formation would have been possible. Thus, to screen 2×10^6 clones, it would have been necessary to examine about 10^5 plates. The number of plates used in the isolation procedure described in this chapter was only 2% of the number which would have been necessary to screen the same number of cells without enrichment. The enrichment therefore gave significant savings of effort and materials provided the use of this method led to recovery of at least 2% of the mutants which would have been isolated without enrichment. If less than 2% of mutants were recovered, this would imply a mutation rate of about 1×10^{-3} or better. Poulter (personal communication) failed to isolate NPF strains by NMG mutagenesis without enrichment but would probably have succeeded if the mutation rate had been as high as 1×10^{-3}. Wheals (1973), using UV + caffeine mutagenesis but no enrichment, recovered APT (i.e. NPF) mutants at a rate of about 1×10^{-5} per survivor.

Although the enrichment procedure proved effective in isolating NPF strains, it clearly restricted the mutant classes recoverable to those in which amoebae, or small multinucleate cells, retained the ability to proliferate at nearly the same rate as wild-type amoebae. It was thus likely that at least the majority of mutants would be defective in the initiation of plasmodium formation, and the fact that all the NPF strains grew as uninucleate cells indistinguishable from amoebae was

consistent with this view. Dispensing with the enrichment might allow mutants with defects of later stages in plasmodium formation to be isolated. One of the strains which Wheals (1973) isolated without enrichment was APT1, yet the reconstruction experiment with mixtures of APT1 and CL amoebae indicated that enrichment for APT1 cells was readily achieved.

NMG was used as mutagen because it was already known to be effective in P. polycephalum (e.g. Cooke and Dee, 1975). However, it was also known that NMG caused high frequencies of double mutants in some prokaryotes (Guerola et al., 1971). The analysis of crosses with LU648 suggested that none of the mutant strains carried unlinked double npf$^-$ mutations. All the NPF strains grew well as amoebae and occasionally gave rise to viable plasmodia. In addition, when progeny of the revertant plasmodia (CL6099)9 and (CL6129)1 were examined, it was found in both cases that reversion was probably due to mutations linked to or at the sites of the original npfB$^-$ and npfC$^-$ mutations. This was consistent with the occurrence of back mutations and implied that the NPF phenotypes were due to single nuclear gene mutations.

Adler and Holt (1975) suggested that heterozygosity at the mating type locus in diploid amoebae promoted the formation of plasmodia within plaques. The results obtained in the analysis of anomalous clones derived from crosses with LU648 were consistent with their interpretation. From work reported in this chapter it seemed that plasmodium formation was also promoted by mating type heterozygosity probably due to aneuploidy. In addition, one clone was shown to be heterozygous for fusA alleles but not for mating type. This clone did not form plasmodia in plaques but behaved as expected for a strain of the genotype mt_h;npfA1$^-$.

The formation of plasmodia in plaques was frequently used to

indicate that amoebal clones were of the genotype $\underline{mt_n};\underline{npf}^+$. The possibility that many clones might be heterozygous at the mating type locus cast some doubt on this assumption. However, there were two arguments against the formation of mating type heterozygotes leading to misinterpretation of data. (1) The work of Adler and Holt (1975) suggested that amoebae heterozygous at the mating type locus were mainly generated in crosses of amoebal strains which were diploid or polyploid. In the current work it was shown that the NPF mutants and other strains were haploid rather than diploid. (2) Adler and Holt tested the fusion behaviour of nine mating type heterozygotes clonally derived from the spores of a cross between strains differing in their plasmodial fusion genotypes. Four strains retained the hybrid fusion type of the crossed plasmodium and it might thus be expected that, among the progeny of crosses of fusA1 and fusA2 strains, a proportion of amoebae heterozygous at the mating type locus would be of the genotype fusA1/fusA2. This expectation was strengthened when it was found that two of the four $\underline{mt_n}/\underline{mt_1}$ amoebal clones analysed in this work were also heterozygous for fusA alleles. Plasmodia formed in plaques were routinely checked for fusion type and were never (except in the two cases mentioned above) found to be of group III (fusA1/fusA2) behaviour.

The analysis of the temperature-sensitive mutant CL6111 indicated that a mutation at a locus (npfA) unlinked to the mating type locus affected the ability of $\underline{mt_n}$ amoebae to form plasmodia in plaques, but did not affect crossing, even when both strains involved in a cross carried the same mutant allele. This may indicate that the formation of plasmodia in plaques requires the function of some product not necessary in the formation of crossed plasmodia, though it seems likely that most functions involved in plasmodium formation are the same for both "selfing"

and crossing.

In addition to its importance as an amoebal-plasmodial transition mutant, CL6111 has several features of value in routine genetic analyses. (1) Dee *et al.* (1973) demonstrated that APT1 could be used to identify recessive mutations carried by heterothallic strains and expressed only in the plasmodial phase. Laborious backcrossing would be required to reveal such mutations with only heterothallic strains. Dee *et al.* crossed amoebae of a heterothallic strain with APT1 amoebae and isolated progeny clones. One quarter of the amoebal progeny formed plasmodia within plaques (mt_h;*apt*-1^+ recombinants) and some of these showed a new phenotype, plasmodial growth requirement for valine. The details of the analysis of this valine requirement were complex, but in principle any plasmodial character due to a nuclear mutation unlinked to the mating type locus should have been identifiable in plasmodia formed within plaques. CL6111 and other strains carrying the *npfA*1^- allele would be of particular value in this kind of analysis, since at 26°C *all* the mt_h progeny of crosses with heterothallic strains would form plasmodia within clones, including half which would form plasmodia in plaques (mt_h;*npfA*$^+$). (2) Cooke and Dee (1975) used a "delayed" strain (CLd) as wild type in mutant isolation. The use of CLd allows large amoebal populations to be obtained (an advantage for studying amoebal mutants) but readily yields clonal plasmodia which can be scored for plasmodial characters. However, Cooke and Dee found that most plasmodia isolated from mixtures of CLd and mt_1 strains appeared to have arisen by "selfing" of CLd. The use of CL6111 as a wild type strain in the isolation of amoebal mutants would be preferable to the use of CLd; at 26°C clonal plasmodia would be formed as readily as by CLd, while at 28.5°C mixtures of CL6111 and mt_1 amoebae would give rise only to crossed plasmodia.

Twelve of the thirteen NPF mutants carried mutations at or closely linked to the mating type locus, and the crosses between these mutants defined two complementation groups. However, more than two genes could have been involved, since cell fusion was not demonstrated in those combinations of strains which did not complement; if cells failed to fuse there would have been no possibility of intracellular complementation.

The results of crosses with LU648 were pooled for all npfB$^-$ strains and for all npfC$^-$ strains. The pooled data indicated that the probability that all mt_h progeny would be npf$^-$ (as found) was equal to or greater than 5% if npfB was 0.4 map units or less from mt and if npfC was 0.8 map units or less from mt.

The complementation of strains designated npfB$^-$ and npfC$^-$ gave rise to diploid plasmodia in every case tested, rather than haploid heterokaryons. Since this was the normal behaviour of strains carrying different mating type alleles, the behaviour of these strains was consistent with their carrying mutations at the mating type locus. This view was not contradicted by the analysis of clonal plasmodium formation; NPF strains gave rise to clonal plasmodia at about the same frequency as did heterothallic strains, and progeny of some plasmodia formed in both NPF and heterothallic clones gave rise to plasmodia in plaques.

One possible explanation for the existence of two complementation groups associated with the mating type locus was that these did not represent mutations in two genes, but that the mt_h allele mutated to generate heterothallic strains which were able to cross with one another (npfB$^-$ might then be assumed to be similar to mt_2). However, it seemed unlikely that twelve independent mutational events would generate only two heterothallic mating types (i.e. npfB$^-$ and npfC$^-$), since Collins (1975) had demonstrated that there were ten different mating type alleles in the

five heterothallic isolates of P. polycephalum he studied. It was concluded that npfB$^-$ and npfC$^-$ strains probably carried mutant alleles of two genes.

When Adler and Holt (1975) concluded that the presence of two different mating type alleles within the same cell promoted plasmodium formation, they also suggested that mt_h might consist of two different mating type alleles closely linked on the same chromosome. The results obtained in the complementation analysis of npfB$^-$ and npfC$^-$ strains were consistent with this suggestion. If the mt_h strains from which the mutants were derived each contained two mating type alleles, mutation causing loss of expression of one allele would result in a strain expressing only the other mating type. Two complementation groups would be expected, as was found. The failure of npfB$^-$;npfC$^+$ strains to cross with a mt_2 tester strain would suggest that the npfC$^+$ allele might be identical to the mt_2 allele.

The nature and role of the mating type locus are discussed further in Chapter 6.

C H A P T E R 4

STIMULATION OF "SELFED" PLASMODIUM FORMATION

CHAPTER 4 STIMULATION OF "SELFED" PLASMODIUM FORMATION

4.1 INTRODUCTION

As was noted in Section 3.3f, some crosses of the NPF mutants (*fusA2*) with LU648 (*fusA1*) yielded not only plasmodia of group III (*fusA1*/*fusA2*) behaviour, but also plasmodia of group V (*fusA2*). For example, out of ten plasmodia picked from crosses of CL6049 with LU648, four were of group V. If, as seemed likely, the group V plasmodia were derived from amoebae of the NPF strains, the frequency of such plasmodia was higher than would be predicted by puddle tests of the NPF strains alone (see 3.3e).

This chapter presents a brief account of preliminary work carried out to investigate this stimulation of "selfed" plasmodium formation.

4.2 MATERIALS AND METHODS

Methods were as described in Chapter 1. All cultures were incubated at 26°C.

Strains	Amoebae:		
		CL6049	mt_h;*npfB1*$^-$;*fusA2*
		CL6082	mt_h;*npfB2*$^-$;*fusA2*
		CL6089	mt_h;*npfB3*$^-$;*fusA2*
		CL6099	mt_h;*npfC1*;*fusA2*
		CL6143	mt_h;*npfC3*$^-$;*fusA2*
		LU648	mt_1;*fusA1*
		LU688	mt_2;*fusA1*
		LU861	mt_2;*fusA2*
		LU871	mt_h;*npfB1*$^-$;*fusA1*

Plasmodia:	Fusion testers of groups
I	fusA1
III	fusA1/fusA2
V	fusA2

All strains carried the fusB1 and fusC1 alleles.

4.3 RESULTS

4.3a Frequency of "selfed" plasmodium formation

In order to estimate the frequency with which "selfed" plasmodia arose within mixtures of strains, attempts were made to determine the proportion of plasmodia formed which were "selfed" and the total number of plasmodia formed within each puddle.

To determine the proportions of "selfed" plasmodia, mixtures of five NPF strains and LU861 (mt_2) with LU648 (mt_1) were inoculated into 0.2 ml bacterial puddles on DSDM agar plates. Two small (less than 1 mm^2), well separated plasmodia were picked from each puddle and inoculated on to separate SDM agar plates; the plasmodia were clearly of independent origin. About 50 plasmodia were picked for each mixture of strains and tested for fusion with known plasmodia of groups I, III and V. The results of this analysis are shown in Table 39.

The total number of plasmodia formed per plate was estimated by scanning puddles daily, scoring plasmodia as they became visible (i.e. when they were 0.5-1.0 mm^2) and removing them from the puddle by cutting out small blocks of agar. This procedure prevented the puddle tests becoming overgrown by a few large plasmodia. About 25 puddles of each of the crosses CL6143 x LU648 and LU861 x LU648 were treated in this way and the average number of plasmodia picked per puddle was about fifteen. This was

Table 39. Proportion of fusA2 plasmodia among plasmodia derived from mixtures of fusA1 and fusA2 amoebal strains

Cross**	Fusion type* of resulting plasmodia I	III	V	% fusA2 plasmodia
CL6049 x LU648	0	31	16	34
CL6082 x LU648	0	49	5	9
CL6089 x LU648	0	50	10	17
CL6099 x LU648	0	54	6	10
CL6143 x LU648	1	49	0	0
LU861 x LU648	2	43	2	4

* Fusion groups:- I fusA1
III fusA1/fusA2
V fusA2

** The first strain shown in each cross carried the fusA2 allele, the second strain fusA1.

a minimum estimate, since when this number of plasmodia had been cut out of a puddle, most or all of the puddle had been removed. It was thus assumed that about twenty plasmodia formed in each puddle, with the qualification that the real figure was probably much higher.

The number of "selfed" plasmodia formed per puddle in each cross was calculated by multiplying the total number of plasmodia assumed to form on each plate (20) by the proportion of plasmodia which were of group V fusion behaviour. The numbers of plasmodia formed per puddle of the NPF strains and LU861 alone were calculated from the data shown in Table 14. As shown in Table 40, the frequencies of "selfing" by the three *npfB*$^-$ strains in crosses with LU648 were 30-300 times higher than the frequencies of plasmodium formation in puddles of the *npfB*$^-$ strains alone. For the two *npfC*$^-$ strains and LU861 there was no clear difference between behaviour alone and with LU648.

Evidence from other mixtures indicated that the stimulation of "selfing" only occurred in mixtures of strains which were able to cross with one another. Puddle tests of CL6049, CL6082 and CL6089 were set up with LU688 (*mt*$_2$;*fusA1*). As shown in 3.3i, the three *npfB*$^-$ strains did not cross with *mt*$_2$ strains. Eight puddles of each attempted cross were set up. The frequency of group V plasmodium formation (i.e. "selfing") observed in puddles with LU648 was 1.9-6.8 per plate, yet the 24 puddles with LU688 yielded only one group V plasmodium during six weeks incubation (this plasmodium arose in the cross CL6082 x LU688). The result was not significantly different from that expected for CL6049, CL6082, and CL6089 incubated in puddles alone.

4.3b Analysis of plasmodia apparently derived from CL6049

The stimulation of "selfing" in mixtures with LU648 was greatest

Table 40. Comparison of "selfing" frequencies of amoebal strains in puddles alone and with LU648

Amoebal strain*	Genotype	No. of fusA2 plasmodia per puddle:- with LU648**	alone
CL6049	mt_h;npfB1$^-$	6.8	0.02
CL6082	mt_h;npfB2$^-$	1.9	0.06
CL6089	mt_h;npfB3$^-$	3.3	0.08
CL6099	mt_h;npfC1$^-$	2.0	0.76
CL6143	mt_h;npfC3$^-$	0.0	0.20
LU861	mt_2	0.9	0.82

* All carried the fusA2 allele.

** fusA1

for CL6049 amoebae ($\underline{mt}_n$;$\underline{npfB1}^-$) and it could be argued that, for any group V plasmodium arising in the cross CL6049 x LU648, the probability that it was formed as a result of the presence of LU648 was 99.7%.

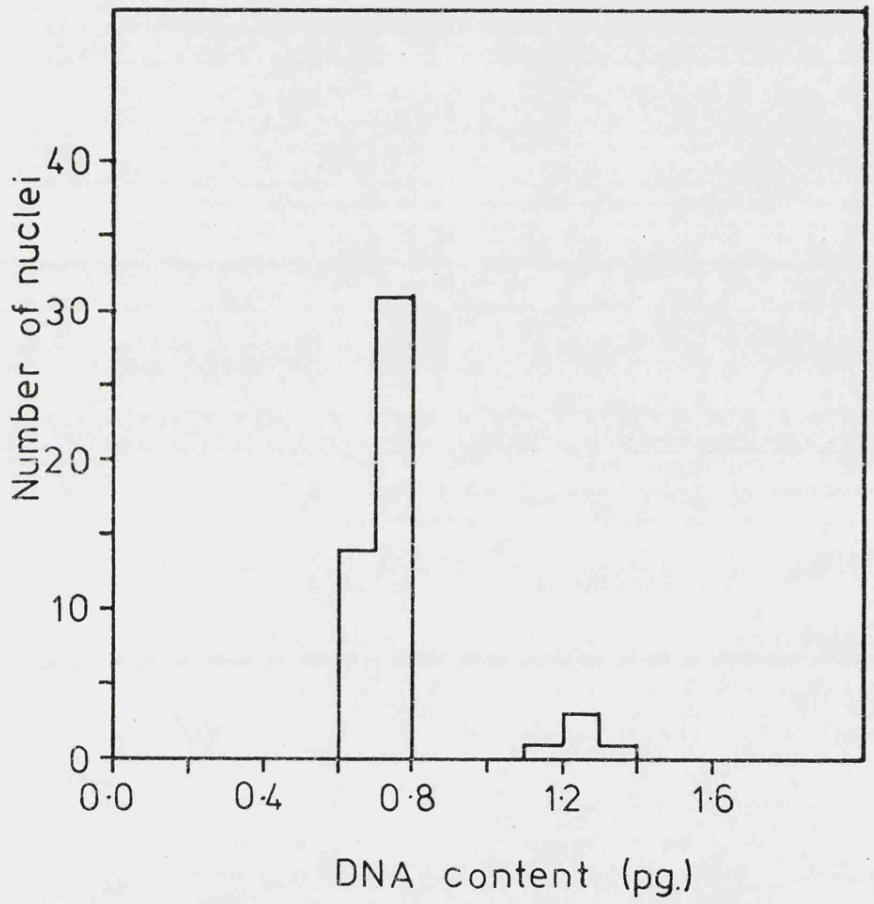
Two "selfed" plasmodia, (CL6049 x LU648)8 and (CL6049 x LU648)9, were analysed in an attempt to determine whether any progeny were derived from LU648 nuclei. The plasmodia were first caused to sporulate and about 100 progeny clones were derived from the spores of each (188 clones in all). These amoebal progeny were inoculated into bacterial puddles on DSDM agar with $\underline{mt}_n$;$\underline{npfB1}^-$ strains carrying $\underline{fusA1}$ and $\underline{fusA2}$ alleles (LU871 and CL6049, respectively). It was expected that any $\underline{mt}_1$ progeny clones (which must be derived from LU648 nuclei) would give rise to crossed plasmodia of hybrid fusion type (group III) when mixed with one or other of LU871 and CL6049. The puddles were incubated for six weeks. About ten plasmodia arose and were tested for their fusion behaviour; no plasmodia of group III fusion behaviour were recovered. Since no evidence was obtained to indicate that any of the progeny clones carried the $\underline{mt}_1$ allele, these results were consistent with the interpretation that group V plasmodia arising in the cross CL6049 x LU648 were derived only from CL6049 amoebae.

Nuclei were isolated from the plasmodium (CL6049 x LU648)8 and their DNA contents estimated. The mean value was 0.76 pg DNA/nucleus and the majority of values fell in a single peak corresponding to the haploid DNA content (see Figure 27).

4.4 DISCUSSION

The results presented in this chapter showed that at least some of the NPF mutants gave rise to "selfed" plasmodia at higher frequencies in

Figure 27. Nuclear DNA contents of the plasmodium (CL6049 x LU648)8.


Number of nuclei
40
30
20
10
0
0·0
0·4
0·8
1·2
1·6
DNA content (pg.)

mixed cultures with LU648 ($\underline{mt}_1$) than when cultured alone or with LU688 ($\underline{mt}_2$). This stimulation of "selfing" seemed to be restricted to $\underline{npfB}^-$ strains, suggesting that $\underline{npfB}$ and $\underline{npfC}$ might have different functions. Since the stimulation of "selfed" plasmodium formation was noted only in mixtures of strains which were able to cross, it seemed likely that it was in some way dependent upon crossing. Thus an understanding of the mechanism of stimulation might lead to greater knowledge of the nature of crossing itself.

Three types of explanation could account for the stimulation of "selfing". (1) Diffusible factors were released into the medium and in some way stimulated apogamic plasmodium formation. (2) Interaction of cell surfaces of the two strains led to the initiation of plasmodium formation without cell or nuclear fusion. (3) Cells of different strains fused with one another and initiated plasmodium formation but did not undergo nuclear fusion.

Explanation (3) implies that heterokaryons formed by fusion of NPF and LU648 amoebae were unstable. LU648 nuclei might have been eliminated in some way; Cooke (1974) showed that elimination of one nuclear type could occur in heterokaryons. Alternatively, cell division in very small heterokaryons (e.g. fission of binucleate cells, as described in Chapter 2) could yield uninucleate cells which might sometimes develop into plasmodia.

Preliminary experiments have been carried out in an attempt to distinguish between explanations (1)-(3). Amoebae of strains CL6049 and LU648 were cultured on opposite sides of thin (10 μm) permeable membranes (Uni-Pore polycarbonate membranes, Bio-Rad Laboratories, U.S.A.). It was hoped that any diffusible factors with a stimulatory effect on plasmodium formation would act through the membranes, while the two cell types would

be kept apart. This type of experiment is subject to the criticism that contact of cell processes may occur through the pores of the membranes. Control experiments demonstrated that whole amoebal cells were able to pass through the membranes initially used (0.8 μm pore size). Further work is in progress, using smaller pore sizes.

If trans-filter stimulation of clonal plasmodium formation can be demonstrated it will suggest that diffusible factors may be involved. It will also provide a sensitive test for stimulation. The experiment described in Section 4.3a was insensitive, due to the large number of crossed plasmodia formed in each puddle; trans-filter experiments would be free of this "noise". It would thus be simple to test many more combinations of strains and determine whether, as suggested by the work described in this chapter, the stimulation of "selfing" is restricted to mixtures of *npfB*$^-$ strains and strains with which they are able to cross.

Therrien and Collins (1976) have recently reported that crosses between a haploid amoebal strain of *D. iridis* and a polyploid strain yielded only haploid plasmodia. The two amoebal strains carried different mating type alleles and progeny of the resulting plasmodia all carried the same mating type allele as the haploid parent clone. Therrien and Collins favour the idea of a "plasmodial inducing substance", which might be bound to amoebal surfaces, but also suggest that cell fusion without nuclear fusion might be involved. Their results are very similar to those reported in this chapter and it seems likely that the same mechanism is responsible for stimulating the formation of "selfed" plasmodia in both cases.

C H A P T E R 5

CHARACTERISATION OF THE EXTERNAL PROTEINS OF AMOEBAE AND MICROPLASMODIA

CHAPTER 5 CHARACTERISATION OF THE EXTERNAL PROTEINS OF AMOEBAE AND MICROPLASMODIA

5.1 INTRODUCTION

If the mating types of amoebal cells are expressed at the cell surface (see Chapter 2) the necessary mating type specificity might reside in protein molecules. This chapter reports preliminary experiments which were mainly aimed at determining whether differences in external proteins could be detected between amoebae carrying different mating type and *npf* alleles. Microplasmodia of different fusion types were also studied and a comparison of the external proteins of amoebae and plasmodia was made.

The technique employed to study external proteins was enzymatic radio-iodination, using the lactoperoxidase system (Phillips and Morrison, 1970; Hynes, 1973). Intact cells were treated with ^{125}I and lactoperoxidase. In the presence of hydrogen peroxide, lactoperoxidase catalyses the reaction of iodide with proteins:

$$\text{Protein} + H_2O_2 + {}^{125}I \xrightarrow{\text{lactoperoxidase}} \text{Protein} - {}^{125}I$$

Iodination occurs specifically at tyrosine and (to a limited extent) histidine residues. Hydrogen peroxide was generated by the reaction of glucose and molecular oxygen, catalysed by glucose oxidase:

$$\beta\text{-D-Glucose} + O_2 \longrightarrow \text{D-Glucose-}\delta\text{-Lactone} + H_2O_2$$

Since lactoperoxidase has a molecular weight of 84,000, brief periods of incubation label only proteins external to the plasma membrane. Labelled cells were solubilised and electrophoresis was carried out on sodium dodecyl

sulphate-polyacrylamide gels. Autoradiography of the gels allowed proteins labelled with ^{125}I to be identified and compared on the basis of their molecular weights.

This work was possible through the kindness of Dr. D. Critchley, who was responsible for the radio-iodination and electrophoresis of samples supplied to him.

5.2 MATERIALS AND METHODS

Methods were as described in Chapter 1, with additional methods as described below. All cultures were incubated at 26°C.

5.2a Strains

Amoebae:	CL	$\underline{mt}_h$
	LU648	$\underline{mt}_1$
	LU688	$\underline{mt}_2$
	LU862	$\underline{mt}_3$
	LU863	$\underline{mt}_4$
	APT1	$\underline{mt}_h;\underline{apt}\text{-}1^-$
	CL6111	$\underline{mt}_h;\underline{npfA}1^-$
	CL6129	$\underline{mt}_h;\underline{npfB}4^-$
	CL6143	$\underline{mt}_h;\underline{npfC}3^-$
Plasmodia:	testers of groups:	
	I	fusA1;fusB1
	II	fusA1;fusB2
	III	fusA1/fusA2;fusB1
	IV	fusA1/fusA2;fusB2
	V	fusA2;fusB1

All strains carried the fusC1 allele.

5.2b Preparation of cells for radio-iodination

Amoebal cultures were grown as described in Chapter 1 for the isolation of nuclei. The cultures were harvested in 40 ml of phosphate-buffered saline A (containing, by weight, 1% NaCl, 0.025%KCl, 0.144% Na_2HPO_4, 0.025% NaH_2PO_4; pH 7.2) and centrifuged for 5 minutes at 500 g. Cells were resuspended and centrifuged a total of three times.

Plasmodia were grown as microplasmodia in shaken liquid culture (Daniel and Rusch, 1961). To set up each culture a block carrying a piece of plasmodium was cut from an SDM agar plate and transferred to 25 ml of liquid SDM in a 250 ml conical flask. The flask was placed on a reciprocating shaker operating at 120 strokes/minute, the stroke length being approximately 7 cm. The plasmodium broke into small fragments (microplasmodia) which proliferated. Subculturing was carried out every 3-4 days by transferring about 2.5 ml of microplasmodial suspension to a flask containing fresh medium. For radio-iodination, about 10 ml of microplasmodial suspension was taken after about 24 hours incubation and centrifuged for 30 seconds in a bench centrifuge. Microplasmodia were resuspended in phosphate-buffered saline A and centrifuged again. Two further washes were carried out.

5.2c Radio-iodination

To each amoebal or plasmodial pellet was added 0.5 ml of phosphate-buffered saline (containing, by weight, 0.833% NaCl, 0.021% KCl, 0.12% Na_2HPO_4, 0.021% NaH_2PO_4, 0.011% $CaCl_2.2H_2O$, 0.008% $MgCl_2.6H_2O$) + 5 mM glucose + $Na^{125}I$ (200 µCi/ml; Radiochemical Centre, Amersham, Bucks). The reaction was initiated by addition of 10 µl of a mixture of lacto-peroxidase (Calbiochem) and glucose oxidase (Worthington Biochemicals) to give final concentrations of 20 µg/ml and 0.1 units/ml, respectively.

The tubes were incubated, with occasional shaking, for 15 minutes at room temperature. The reaction was stopped by addition to each tube of 3 ml of phosphate-buffered iodide (phosphate-buffered saline A with the NaCl replaced by NaI). The cells were centrifuged as described in Section 5.2b and the supernatant was removed by suction. The cells were resuspended in 3 ml of phosphate-buffered iodide and centrifuged, twice more. Each pellet was resuspended in 360 µl of 0.1M Tris containing 15% glycerol and 2 mM phenyl methyl sulphonylfluoride (to inhibit proteases). Sodium dodecyl sulphate (40 µl of a 20% solution in water) was added to each tube whilst it was vortexed on a Whirlimixer. The tubes were placed in a boiling water bath for 4 minutes. This treatment caused a viscous precipitate to form in amoebal samples, which were thus centrifuged at 500 g for 5 minutes. Radio-iodine labelling was restricted to the supernatant, which was used for further procedures.

5.2d Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Samples were reduced by boiling in 0.1M dithiothreitol for 2 minutes. Electrophoresis was carried out on slab gels, essentially as described by Laemmli (1970). Gels containing 4% ("stacking gel") or 7% ("running gel") acrylamide plus 0.1% sodium dodecyl sulphate were prepared using a stock solution of 30% by weight of acrylamide (Eastman) and 0.8% by weight of bis-acrylamide (Eastman). In addition, the stacking gel contained 0.125 M Tris-HCl (pH 6.8) and the running gel 0.375 M Tris-HCl (pH 8.8). Stacking gels were polymerised by addition of 0.05% by weight of ammonium persulphate and 0.2% by volume of tetramethyl ethylene diamine; corresponding percentages for running gels were 0.03 and 0.067, respectively. Samples (50-100 µl), containing bromophenol blue, were loaded on to the stacking gel and electrophoresis was carried out at 50

volts. When the indicator dye entered the running gel the potential difference was increased to 100 volts. Electrophoresis through the running gel took about 5 hours. The gel was removed from the apparatus and stained overnight in Coomassie blue stain containing 125 mg Coomassie blue per litre of a mixture comprising, by volume, 41% methanol, 52% water and 7% glacial acetic acid. Destaining of the background was achieved by soaking for about one day in the above mixture, without Coomassie blue. The gel was washed in water and dried down under vacuum. Autoradiographs were made on Kodirex X-ray film, using 5-30 days exposure.

5.3 RESULTS

Amoebal cultures were harvested, radio-iodinated, run on gels and autoradiographed. The banding patterns obtained by staining the gels with Coomassie blue were essentially the same for all amoebal strains tested (listed in Section 5.2a). Similarly, banding patterns obtained on autoradiography of these gels were essentially all the same. However, as shown in Figure 28, fewer bands were visible in the autoradiographs than in the stained gels. The difference in patterns suggested that only a fraction of total cell proteins was iodinated, as expected if only external proteins were available for iodination.

In order to determine whether the banding patterns obtained were due to *E. coli* proteins, bacteria were inoculated on to LIA plates, incubated for five days at 26°C, harvested and iodinated. Harvesting and iodination were carried out as for amoebal samples, except that centrifugations were at 1500 g for 15 minutes and only one was was carried out prior to iodination. The banding patterns obtained differed from those of amoebal preparations (see Figure 28), indicating that the proteins

Figure 28. Autoradiograph (top) of slab gel shown below. The gel was run downwards and shows bands in the molecular weight range 45,000 (bottom of gel) - 250,000 (top of gel). Autoradiograph and gel both show, from right to left, radio-iodinated preparations from:

1. Fusion group I plasmodium.

2. Fusion group III plasmodium.

3. Fusion group V plasmodium.

4. CL6129 amoebae.

5. CL6143 amoebae.

6. APT1 amoebae.

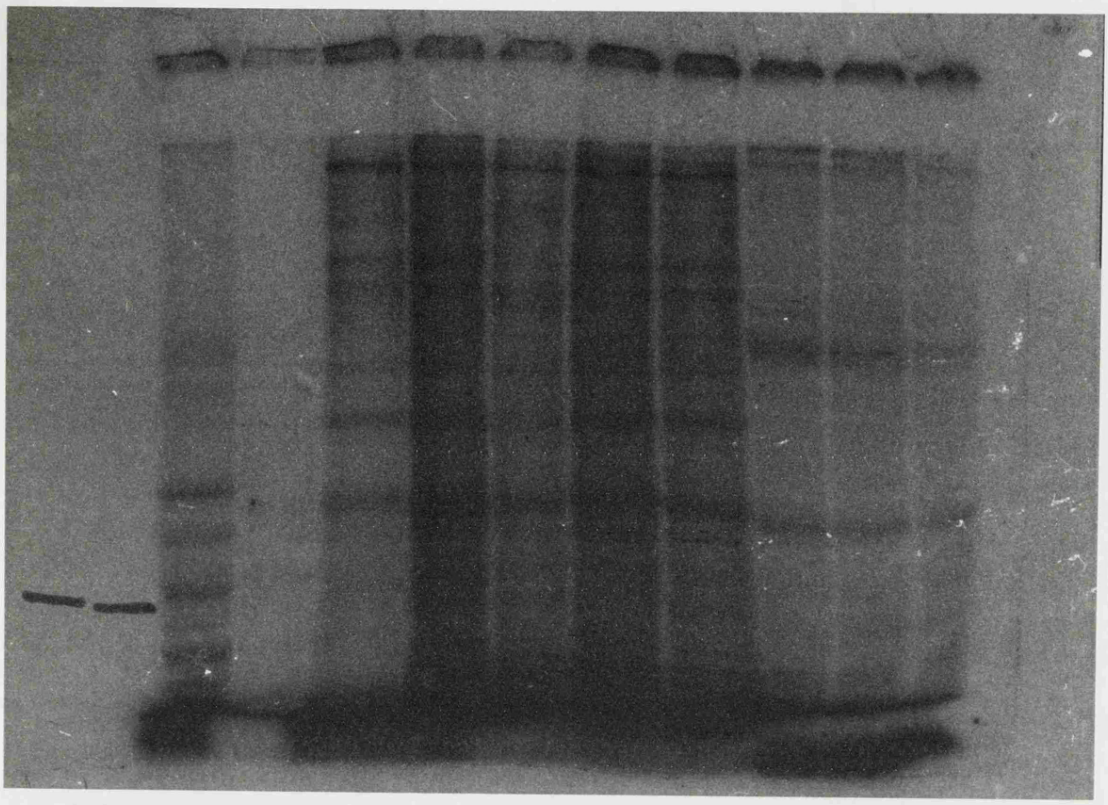
7. LU648 amoebae.

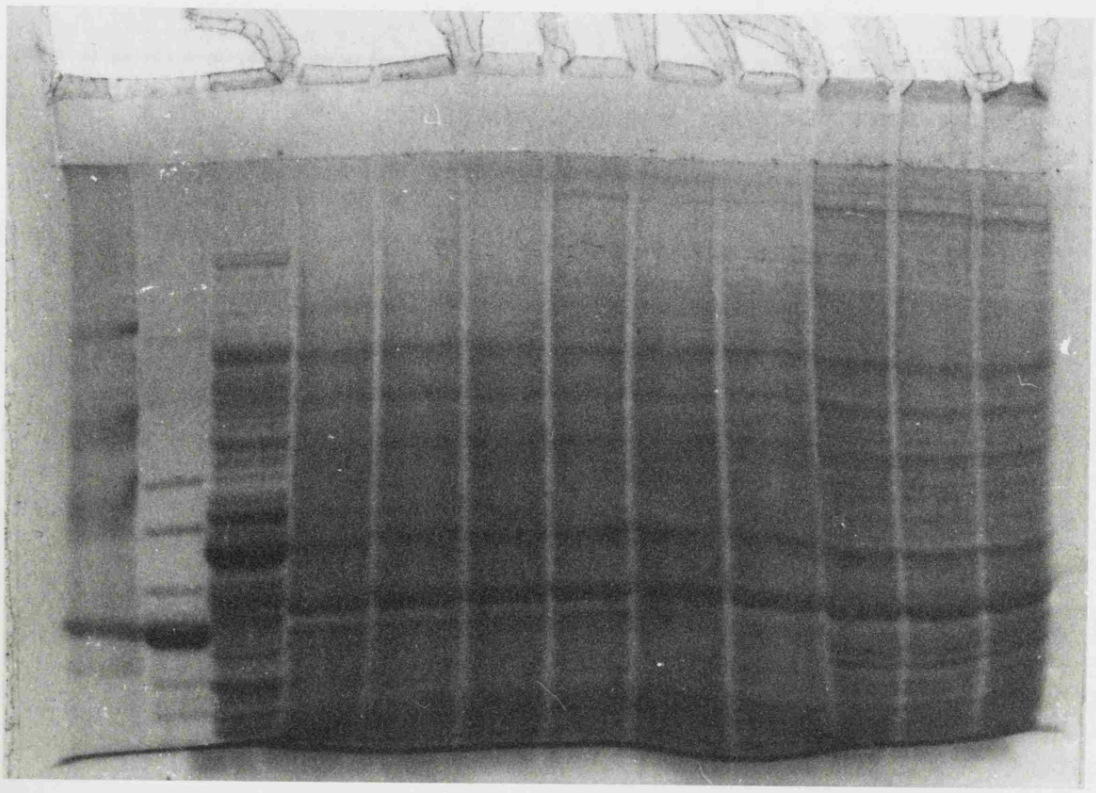
8. CL6129 amoebae plus trypsin.

9. CL6143 amoebae minus lactoperoxidase.

10. E.coli.

11 and 12. Bovine serum albumin standard mix (non-iodinated). The main band (m.wt. = 68,000) has been marked on the autoradiograph.





obtained from amoebal cultures were primarily of amoebal origin.

Plasmodial cultures were harvested, radio-iodinated, run on gels and autoradiographed. All strains tested (listed in Section 5.2a) gave the same Coomassie blue banding pattern and the same banding pattern on autoradiographs. As with amoebal preparations, autoradiographs showed fewer bands than visible in stained gels. The Coomassie blue staining patterns obtained for plasmodia and amoebae showed some differences and autoradiographs showed fewer bands in plasmodial preparations than in amoebal ones (see Figure 28).

In order to show that iodination was dependent upon lactoperoxidase, amoebal and plasmodial preparations were treated as described in Sections 5.2b and 5.2c, except that lactoperoxidase was omitted. Coomassie blue staining patterns obtained from these samples were identical to lactoperoxidase-treated preparations, but no banding pattern was obtained on autoradiographs. Thus iodination was dependent upon the presence of lactoperoxidase.

In order to obtain further evidence suggesting the external location of the iodinated proteins, amoebal and plasmodial preparations were obtained as previously described, with the modification that after iodination and washing in phosphate-buffered iodide, the cells were treated with trypsin. Very mild treatment was employed (20 μg/ml of trypsin for 10 minutes, followed by addition of trypsin inhibitor), so that removal of labelling would imply that iodination probably affected external proteins. The intensity of bands obtained on autoradiographs was slightly reduced for both amoebal and plasmodial preparations, but all bands could still be seen. No firm conclusion could be drawn from this result.

5.4 DISCUSSION

The preliminary studies reported here failed to detect any protein differences between the amoebal strains tested, either on the basis of banding patterns on autoradiographs (to show external proteins) or on the basis of Coomassie blue staining patterns (to show total cell proteins). Among the group of amoebal strains studied, different members carried five mating type alleles ($\underline{mt}_h$, $\underline{mt}_1$, $\underline{mt}_2$, $\underline{mt}_3$, and $\underline{mt}_4$) and two different alleles at each of four other loci known to be expressed in the amoebal phase (*apt*-1^+ and *apt*-1^-, *npfA*$^+$ and *npfA*1^-, *npfB*$^+$ and *npfB*4^-, *npfC*$^+$ and *npfC*3^-).

Similarly, no differences were detected between the plasmodial strains tested, even though they differed in their fusion types, which are likely to be expressed at the cell surface. The strains used carried various combinations of *fusA*1, *fusA*2, *fusB*1 and *fusB*2.

Since no protein differences were detected within the group of amoebal strains or within the group of plasmodial strains, there was no evidence that expression of different alleles of the loci mentioned above was connected with the presence of different external proteins on the cell surface. However, the failure to detect such differences did not indicate that they did not exist. If different alleles of a locus specified different external proteins which did not differ in their molecular weights, the electrophoresis technique used would not have distinguished between them. Alternatively, the harvesting and washing procedure might have removed proteins prior to iodination or caused cells to stop exposing them, or the cells might have been cultured in such a way that the relevant proteins were never expressed.

The results for amoebae and plasmodia showed considerable differences. This was not surprising, since amoebae and plasmodia show

a number of differences in appearance and behaviour. However, the possibility was not excluded that the differences observed were due to the different growth conditions used in the preparation of samples, or to the presence of residual slime adhering to plasmodial surfaces after washing.

Juliano and Behar-Bannelier (1975) showed that techniques depending on surface labelling of intact cells may be subject to error due to internal labelling of inviable cells. Further work is required to demonstrate that iodination in the present work was restricted to proteins external to the plasma membrane. However, the results reported here constitute suggestive evidence that the external proteins of amoebae and plasmodia may be susceptible to analysis by surface labelling techniques. Several further studies are thus possible. Labelling under different growth conditions may reveal differences in the external proteins of cells carrying different alleles of various loci. In addition, changes in external proteins may allow a number of stages to be recognised in the amoebal-plasmodial transition. Several other surface labelling techniques are available (Juliano and Behar-Bannellier, 1975) and may show differences between external molecules not distinguishable using the lactoperoxidase system, such as glycoproteins and glycolipids. Mating types in the yeast Hansenual wingei, for example, are known to be specified by external glycoproteins (Crandall and Brock, 1968; Crandal et al., 1974).

C H A P T E R 6

GENERAL DISCUSSION

CHAPTER 6 GENERAL DISCUSSION

There is probably no need to justify the assertion that an understanding of the complex molecular events which must regulate differentiation in eukaryotes will not come from the study of any one system, but will require a variety of approaches in a range of organisms. Thus the kind of preliminary analysis described in this thesis must be made in many systems in order to assess their potential value. It is the aim of this chapter to show that the results obtained in this and other studies indicate that the amoebal-plasmodial transition of Physarum polycephalum is a developmental process which is a suitable subject for further analysis.

P. polycephalum is a "simple" eukaryote which can be easily cultured and manipulated by standard microbial techniques. The analysis of differentiation in the cellular slime mould Dictyostelium discoideum has well illustrated the value of one such "simple" system in the study of development (Jacobson and Lodish, 1975). It must be emphasised that D. discoideum and P. polycephalum have dissimilar life cycles, so that different studies may be undertaken in these two slime mould species. At present, genetic analysis can be performed relatively more easily in P. polycephalum but biochemical studies on the amoebal-plasmodial transition are as yet almost absent; biochemical studies of development are well established in D. discoideum.

Early interest in the amoebal-plasmodial transition was mainly concerned with mating. Horenstein and Cantino (1969) suggested that the Myxomycetes (in particular P. polycephalum) were "perhaps even the choicest of fungi for studies of gamete physiology and fertilization

mechanisms", pointing to the fact that the gametes (i.e. amoebae) could propagate themselves apart from parental attachments and influence. The existence of heterothallic mating type systems in some Myxomycetes identified one locus (the mating type locus) which had an important role in the mating process, though the nature of that role was not clear. Formally, Myxomycete mating type systems were similar to the multi-allelic bipolar mating type systems of some fungi (Raper, 1966) and self-incompatibility systems in some plants (Arasu, 1968); these were all homogenic incompatibility mechanisms (Esser, 1971; Esser and Blaich, 1973) which allowed mating only of individuals carrying different incompatibility alleles.

Ross and Cummings (1970) proposed a model of plasmodium formation in D. iridis. They suggested that a crucial event in plasmodium formation was amoebal fusion, which was under the control of the mating type locus; fusion triggered plasmodial differentiation, which then followed as a more or less orderly sequence of events. As already noted, plasmodium formation in P. polycephalum and D. iridis is very similar, so that the model proposed by Ross and Cummings will be discussed with reference to both species:

(1) The first step in plasmodium formation postulated by Ross and Cummings was the induction of competence to mate. They pointed out that fusions in D. iridis did not occur until several hours after mingling of amoebae of different mating types and suggested that mating competence was attained in response to chemical inducers released into the medium. It is similarly found in P. polycephalum that plasmodium formation in crosses does not occur immediately upon plating. Cooke (1974) showed that plasmodium formation in mt_n cultures occurred after an initial proliferative phase. He inoculated a number of agar cultures

of CL amoebae and harvested these in water after different periods of incubation. The suspensions obtained were diluted and plated. Amoebal cells gave rise to visible amoebal plaques but cells committed to plasmodium formation gave rise to plasmodia without first proliferating only as amoebae. Only amoebal plaques appeared on plates inoculated from cultures harvested during the early stages of growth. Holt and Adler (1976) used essentially the same method as Cooke to carry out a fuller study of the kinetics of clonal plasmodium formation in a number of strains. They concluded that CL amoebae did not undergo the amoebal-plasmodial transition until a particular cell density had been attained. They suggested that amoebae might release a substance (or substances) during growth and that plasmodium formation would be stimulated by a critical concentration of this substance. The results obtained by Holt and Adler were not compatible with the idea of a "cellular clock" that was set at the time of plating and which allowed plasmodium formation only after an interval.

(2) Ross and Cummings suggested that induction of mating competence occurred by new gene activity (particularly of the mating type genes) which resulted in cell surface changes. Fusion was only possible between competent cells expressing different mating types. Ross et al. (1973) provided strong evidence that the mating type locus was involved in the control of amoebal fusions in D. iridis; they showed that fusions did not occur within clones under conditions in which fusions were detectable in mixed mating type cultures. However, as discussed in Chapter 2, this type of evidence does not exclude the possibility that amoebal fusions are random, but only occur within mixed mating type cultures. Cytological and biochemical analyses described in this thesis did not provide clear evidence for or against the involvement of the mating

type locus of P. polycephalum in the control of amoebal fusions. However, it seems likely that the mating type locus will be found to have similar roles in both P. polycephalum and D. iridis.

(3) Ross and Cummings suggested that plasmodium formation was triggered by the act of fusion, or cell surface changes occurring as a result of fusion. There is no direct experimental evidence to support this view and work described in this thesis shows that mt_h amoebae of P. polycephalum can give rise to plasmodia without fusion; in this case, at least, fusion is clearly not the trigger for plasmodium formation. However, it is possible to reconcile the observations of mt_h plasmodium formation with the model by assuming that the cell surfaces of mt_h amoebae in some way mimic those of the products of fusion of sexually compatible amoebae. For example, plasmodium formation might be triggered by the interaction of mating-type-specified products at the cell surface. If, as suggested in Chapter 3, the mt_h allele is in fact two closely linked heterothallic mating type alleles, the cell surfaces of mt_h amoebae might be able to trigger plasmodium formation without the occurrence of fusion.

(4) Ross and Cummings assumed that plasmodial differentiation, once triggered, proceeded through the activation of new genes, including those responsible for plasmodial characters. They did not indicate precisely how this activation was to be achieved, though it is possible to envisage a mechanism of the sort proposed by Britten and Davidson (1969), in which "activator" molecules (RNA or protein) bind to "receptor genes" and thus allow transcription of genes under the control of the receptors.

The model proposed by Ross and Cummings provides a useful basis for discussion since it attempts to explain the whole process of plasmodium formation. Other workers have tended to restrict their consideration to the role of the mating type locus. Twelve of the thirteen NPF strains

whose isolation is described in this thesis carried mutations in or closely linked to the mating type locus; thus the role of the mating type locus will be considered at some length.

Therrien (1966a) proposed a model of the mating type locus in D. iridis. He envisaged mating as a process of complementation, suggesting that the mating type locus was "a linear segment on a chromosome that is composed of adjacent sites:

$$\text{i.e.} \quad \underline{a^+ b^+ c^+ d^+}$$

With all (+) "alleles" (as above) the organism is homothallic. If, however, one site is in the (-) state, then a genome with the (+) state for that site is required for plasmodium formation". Therrien apparently assumed that amoebal fusion was necessary for plasmodium formation, but in order to account for the behaviour of $\underline{mt_h}$ strains in P. polycephalum it is only necessary to substitute "apogamic" for "homothallic" in the above description.

If, as suggested by Therrien, the mating type locus is a cluster of genes, this model can account for both "selfing" and crossing in a system with only a few alleles. However, there are at least ten heterothallic mating type alleles in P. polycephalum (Collins, 1975), each compatible with all the others. Thus there would have to be at least this number of genes within the mating type locus. Presumably at least ten possible classes of complementing NPF mutants could then be generated from $\underline{mt_h}$ strains by mutations abolishing the activity of individual mating type genes. However, the study reported in Chapter 3 identified only two classes of complementing npf mutations in the mating type locus.

This problem can be overcome by assuming that the mating type locus contains a single gene and that mating occurs not by inter-genic

complementation but by intra-genic complementation. This is possibly the simplest kind of mechanism which can successfuly explain the action of the mating type system at the molecular level. Polypeptides specified by different mating type alleles and located within the cell or at its surface might form functioning multimers which would trigger plasmodium formation; polypeptides specified by the same mating type allele would not form a functioning product. The observations of Adler and Holt (1975) and in Chapter 3, that amoebae heterozygous for mating type alleles form plasmodia within plaques, are consistent with this model. In addition, as shown in Chapter 3, the behaviour of $\underline{mt}_h$ strains and the generation of $\underline{npfB}^-$ and $\underline{npfC}^-$ mutants can be explained by assuming that the $\underline{mt}_h$ "allele" is in fact two closely linked heterothallic mating type alleles. Although mating can be explained as a kind of intra-genic complementation, this model is not entirely satisfactory since it is difficult to imagine how all combinations of different mating type proteins would interact; intra-genic complementation normally occurs in only a fraction of the possible combinations of different alleles (Fincham and Day, 1963).

Poulter (personal communication) has proposed another type of model for the control of plasmodium formation. He suggests that the mating type locus is essentially an operon whose structural gene product(s) trigger(s) plasmodium formation. Transcription of the structural gene(s) is prevented by the binding to the operator of a repressor protein. Both operator and repressor are mating type specific and the regulator gene (which produces the repressor protein) is closely linked to the operator. Poulter proposes that plasmodium formation is triggered when the operator is no longer binding a repressor molecule. This normally occurs in a cross when two amoebae of different mating type fuse, since the consequent halving of the repressor concentration is sufficient to

prevent efficient repression. Clonal plasmodia will occasionally arise in heterothallic strains if the repressor concentration is low enough to allow occasional transcription of the structural gene(s). Poulter accounts for $\underline{mt}_h$ strains by assuming that they lack repression, due to mutation in the regulator or operator genes. NPF strains may be generated by the restoration of repression or by structural gene mutation and more than two classes of complementing mutations might occur at the mating type locus.

It is implicit in Poulter's model that fusion of amoebae is not under the control of the mating type locus. A demonstration that the mating type locus was involved in amoebal fusion would thus be strong evidence against this theory.

A further prediction of Poulter's model which can be easily tested is concerned with the generation from heterothallic strains of mutants with the ability to give rise to plasmodia within plaques; the intra-genic complementation model of plasmodium formation makes a different prediction. Adler and Holt assumed that, if the $\underline{mt}_h$ allele was in fact two heterothallic alleles, it would have arisen through non-reciprocal recombination in a mating type heterozygote. Yet a clone of the $\underline{mt}_2$ strain LU688 gave rise to a spontaneous mutant which was able to form plasmodia within plaques, and this strain carried a mutation in or linked to the mating type locus. It seems likely that this "CL-like" mutant forms plasmodia in the same way as $\underline{mt}_h$ amoebae, yet it was not generated by non-reciprocal recombination in a mating type heterozygote. One possible explanation of the origin of this mutant is based upon the observed failure of $\underline{mt}_2$ strains to cross with $\underline{npfB}^-$ strains. Possibly $\underline{mt}_2$ is a mutant $\underline{mt}_h$ "allele" of the genotype $\underline{npfB}^-;\underline{npfC}^+$, so that "CL-like" strains can readily arise from $\underline{mt}_2$ strains

by reversion to *npfB*$^+$;*npfC*$^+$. This explanation implies that strains carrying other (single) heterothallic mating type alleles will probably not be found to give rise to spontaneous mutants able to form plasmodia in plaques. According to Poulter's model, mutations abolishing repression of the plasmodial differentiation genes could occur at the mating type locus in any heterothallic strain to generate "CL-like" mutants.

The small amount of experimental evidence at present available makes it easy to construct a variety of models for the control of plasmodium formation. As shown above, simple tests of the type used in this thesis should yield further useful information. However, these alone would not merit sustained interest in *P. polycephalum*. It is, for example, highly desirable to complement the genetical studies with biochemical analyses. As discussed in Chapter 1, the plasmodial phase of *P. polycephalum* is a well established system for biochemical analysis and great progress has been made in the axenic liquid culture and biochemical analysis of amoebae. However, the main requirement for physiological and biochemical analysis of the amoebal-plasmodial transition is not at present available; that is, the synchronous formation of plasmodia in a homogeneous amoebal population. The comments made earlier in this chapter on the possibility of chemical induction of plasmodium formation suggest that plasmodium formation might be synchronous within shaken liquid cultures of mt_n amoebae. However, preliminary attempts to set up such cultures have failed (Anderson and McCullough, unpublished), due to the occurrence of plasmodium formation before proliferating cultures could be established. It is hoped that this problem will be solved shortly.

There seem to be no insurmountable problems in the further

genetic analysis of the amoebal-plasmodial transition, though it would be foolish to suppose that genetic analysis in P. polycephalum is either quick or easy. The possibility of isolating large numbers of mutants specifically defective in the amoebal-plasmodial transition, including some temperature-sensitive mutants, has been demonstrated. Although the thirteen NPF strains analysed carried mutations in only three genes, the preliminary analysis of "delayed" mutants suggests that a number of other genes affecting the amoebal-plasmodial transition may be identified (particularly if different isolation procedures are employed). These results provide an interesting comparison with those obtained on conidiation in Aspergillus nidulans; Clutterbuck (1969) initially identified only two genes essential for the later stages of conidiation, yet Martinelli and Clutterbuck (1971) were able to show that the number of loci specifically involved in conidiation might be 45-150.

In conclusion, it seems undeniable that further investigation is justified into the nature of the amoebal-plasmodial transition of P. polycephalum. It is to be hoped that some insight into cell differentiation in this eukaryotic system may be obtained from these studies.

APPENDICES

APPENDIX 1

ANALYSIS OF A SPONTANEOUS MUTATION CONFERRING WHITE PLASMODIAL COLOUR

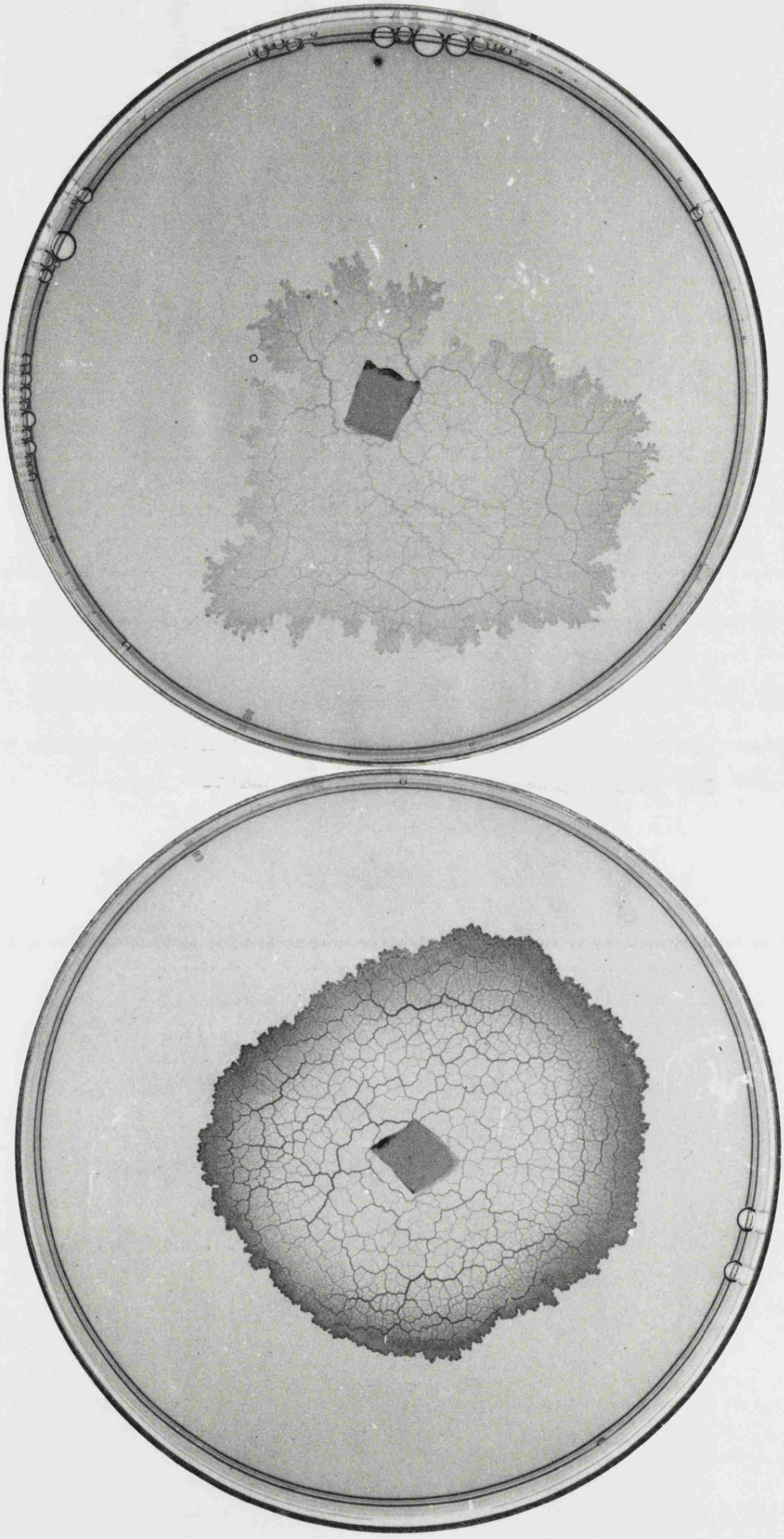
As noted in Chapter 3, CL6082 did not cross with mt_2 strains. However, from one attempted cross between CL6082 and the mt_2 strain LU688, a group I (*fusA1*) plasmodium was recovered and found to be white in colour instead of the normal yellow (see Figure 29). The white colour was easily scored under all conditions of plasmodial growth and was thus potentially suitable for use as a genetic marker. No other plasmodial colour markers were available in *P. polycephalum*, though two unlinked mutations conferring cream plasmodial colour (instead of brown) had been reported in *D. iridis* (Collins and Clark, 1966; Collins, 1969).

When the white plasmodium,(CL6082 x LU688)1 grew into contact and fused with a yellow group I tester plasmodium, the resulting heterokaryon was yellow. This suggested that white plasmodial colour was recessive to yellow.

The normal way to analyse recessive plasmodial characters in heterothallic strains would be to cross with APT1 or CL6111 and score plasmodia formed in plaques by mt_h;*apt*-1^+ or mt_h;*npfA*$^+$ progeny (see Chapter 3). The use of APT1 was avoided as there was a small chance that this strain carried a translocation (Dee *et al.*, 1973). Since the white plasmodium was of fusion group I it presumably arose from LU688 amoebae. Unfortunately, LU688 amoebae do not cross with CL6111 and it was thus not surprising that 40 bacterial puddles inoculated with CL6111 and progeny of the white plasmodium failed to give rise to any plasmodia of hybrid fusion type when incubated at 28.5°C.

Since it was not possible to use CL6111 or APT1 in the analysis

Figure 29. White and yellow plasmodia, photographed using transmitted light. The white plasmodium (top) appears pale. x 1.3



of white plasmodial colour, LU887 (a progeny clone of the white plasmodium) was crossed with amoebae of a heterothallic strain and the colours of plasmodia produced by backcrossing progeny of this cross to LU887 were scored. LU887 amoebae were of the genotype $\underline{mt}_2;\underline{fusA1}$ and were crossed with LU853 amoebae ($\underline{mt}_1;\underline{fusA2};\underline{leu}\text{-}1^-$). A group III plasmodium, (LU853 x LU887)1, was obtained and caused to sporulate. Ninety six progeny amoebal clones were derived from the spores and backcrossed to LU887. Progeny of the genotype $\underline{mt}_1;\underline{fusA2}$ could be positively identified in these backcrosses as they gave rise to group III plasmodia. The number in this class was 22, not significantly different from the expected one quarter ($p > 0.05$).

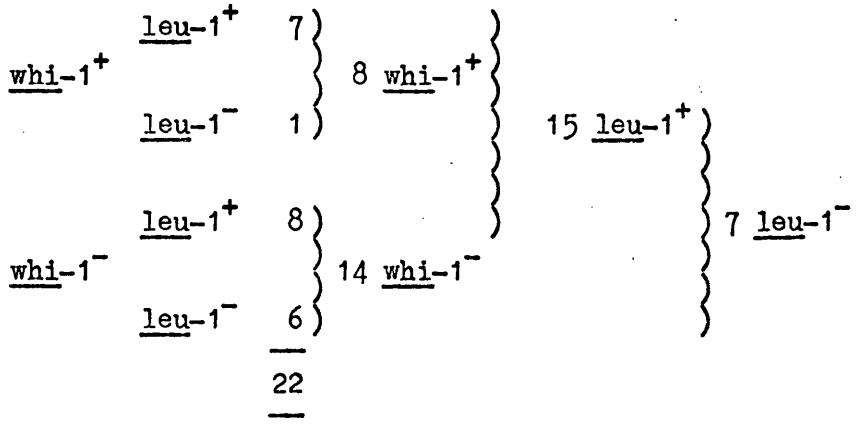
Group III plasmodia produced in backcrosses of $\underline{mt}_1;\underline{fusA2}$ progeny to LU887 were scored for their colour; 14 plasmodia were white and 8 yellow. The expected segregation if white plasmodial colour was conferred by a recessive allele ($\underline{whi}\text{-}1^-$) of a single gene unlinked to $\underline{mt}$ or $\underline{fusA}$ was 1 : 1. Linkage to either $\underline{mt}$ or $\underline{fusA}$ would lead to a shortage of clones of the genotype $\underline{mt}_1;\underline{fusA2};\underline{whi}\text{-}1^-$. The observed ratio was not significantly different from 1 : 1 ($p > 0.05$).

The plasmodial marker most frequently used in this laboratory (excluding fusion markers) is leucine requirement ($\underline{leu}$-1; Cooke and Dee, 1975). LU688 carried the $\underline{leu}\text{-}1^+$ allele and the white plasmodium was found to grow on DM-1 agar, showing that it did not require leucine for growth. Since LU853 carried the $\underline{leu}\text{-}1^-$ allele it was possible to test recombination between $\underline{whi}$-1 and $\underline{leu}$-1; $\underline{mt}_1;\underline{fusA2}$ progeny of the plasmodium (LU853 x LU887)1 were crossed with LU858 amoebae ($\underline{mt}_2;\underline{fusA1};\underline{leu}\text{-}1^-$). Resulting group III plasmodia were tested for growth on DM-1 and DM-1 + leucine agar plates; 7 plasmodia died on DM-1 but grew on DM-1 + leucine and 15 plasmodia grew on both media. Thus the segregation of $\underline{leu}\text{-}1^+$

and *leu*-1$^-$ in the *mt*$_1$;*fusA*2 progeny was not significantly different from 1 : 1 ($p > 0.05$). Table 41 summarises the results of the analysis of *mt*$_1$;*fusA*2 progeny for *whi*-1 and *leu*-1 alleles. There was no significant deviation from free recombination between *whi*-1 and *leu*-1 ($p > 0.05$).

Further work is in progress to analyse the inheritance of the *whi*-1$^-$ allele more fully and it is being crossed into *mt*$_h$ and *mt*$_3$ strains. Cooke (personal communication) has recently isolated a recessive nuclear mutation conferring pale yellow plasmodial colour. Attempts are in progress to test the complementation of this mutation with *whi*-1$^-$.

Table 41. Analysis of mt_1;fusA2 progeny of the plasmodium (LU853 x LU887)1



Parental genotypes:-

LU853 mt_1;fusA2;leu-1^-;whi-1^+

LU887 mt_2;fusA1;leu-1^+;whi-1^-

Allele ratios:-

whi-1^+ : whi-1^- not significantly different from 1:1 ($p>0.05$).

leu-1^+ : leu-1^- not significantly different from 1:1 ($p>0.05$).

Recombination between whi-1 and leu-1 (recombinants 13 : parentals 9) not significantly different from 1:1 ($p>0.05$).

APPENDIX 2

DERIVATION OF STRAINS LU862 AND LU863

Adler and Holt (1974b) derived mt_3 and mt_4 strains partially isogenic with the Colonia strain CL. They supplied two strains, CH188 (mt_3) and CH207 (mt_4) to this laboratory. These strains are the result of backcrossing to CL amoebae for six and five generations, respectively. They are both of the fusion genotype *fusA2;fusB1;fusC2* (Adler and Holt did not present conclusive evidence that the fusion allele they designated *fusC2* was not in fact a new allele of *fusA* or *fusB*). Strains used in this laboratory are normally *fusA1;fusB1;fusC1* or *fusA2;fusB1;fusC1*, so that only plasmodial fusion testers of groups I, III and V are routinely required. In order to cross mutants derived from CL (*fusA2;fusB1;fusC1*) with mt_3 and mt_4 strains it was desirable to use heterothallic *fusA1;fusB1;fusC1* strains. Thus mt_3 and mt_4 strains of this fusion genotype were selected from among the progeny of crosses of CH188 and CH207 with LU640 (mt_h;*fusA1;fusB1;fusC1*).

The crosses were carried out at 29-30°C to reduce the frequency of plasmodia derived only from LU640. Plasmodia were picked from the cross puddles and tested for their fusion behaviour. No hybrid (group IX) plasmodia were available as testers; thus plasmodia were tested only for group I fusion behaviour, since plasmodia arising directly from LU640 would be of this type. Two plasmodia which were not of group I behaviour were selected, (CH188 x LU640)2 and (CH207 x LU640)1. These plasmodia were caused to sporulate and progeny amoebal clones were isolated from the spores of each.

Progeny clones of the two presumed crossed plasmodia were plated

on DSDM agar plates with *E. coli* and incubated for two weeks. In each case about half the progeny formed plasmodia in plaques (mt_h) and the remainder did not form plasmodia (mt_3). The segregation mt_h : mt_3 was 1 : 1 (20 : 20) while the segregation mt_h : mt_4 was not significantly different from 1 : 1 (12 : 15; $p>0.05$). Thus the progeny were derived from hybrid plasmodia.

The mt_3 and mt_4 progeny clones were crossed with LU860 amoebae (mt_1;*fusA1*;*fusB1*;*fusC1*;*leu*-1^-) and resulting plasmodia were tested for fusion with a group I tester plasmodium. Thirty-one plasmodia were tested in this way; 10 fused with a group I plasmodium and 21 did not. Clones which gave rise to group I plasmodia in crosses with LU860 were designated mt_3;*fusA1*;*fusB1*;*fusC1* and mt_4;*fusA1*;*fusB1*;*fusC1* (the plasmodia were found to grow on DM-1 agar so that they could not have arisen by "selfing" of LU860, which was *leu*-1^-). A mt_4 clone was designated LU863.

CH188 carries a mutant allele (*eme*^{r}) conferring amoebal resistance to emetine chloride (Adler and Holt, 1974b). Clones of the genotype mt_3;*fusA1*;*fusB1*;*fusC1* were tested for growth on LIA plates containing 100 µg/ml emetine chloride. A resistant clone was selected and designated LU862.

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SUMMARY

ANALYSIS OF THE AMOEBAL-PLASMODIAL TRANSITION IN PHYSARUM POLYCEPHALUM

by

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This thesis describes work carried out to analyse a developmental process in a eukaryote, the amoebal-plasmodial transition in the Myxomycete Physarum polycephalum. Macroscopic, multinucleate plasmodia are normally formed by fusion of microscopic, uninucleate amoebae carrying different alleles of the mating type (mt) locus; haploid amoebae give rise to diploid plasmodia. Amoebae carrying the mt_h allele are capable of undergoing the amoebal-plasmodial transition with high efficiency within clones to give haploid plasmodia.

Chapter 1 contains an introduction to the life cycle of P.polycephalum, followed by a review of previous work on the amoebal-plasmodial transition in P.polycephalum and some other Myxomycetes. The work described in this thesis is briefly introduced and details of materials and methods are given.

Chapter 2 describes studies undertaken to determine whether amoebal fusions occur within clonal cultures. These studies mainly concern mt_h strains; no clear conclusions emerge from genetical analyses but a cinematographic analysis indicates that plasmodium formation in mt_h strains occurs without amoebal fusion.

Chapter 3 describes the isolation and genetical analysis of mutants defective in clonal plasmodium formation. The results of detailed analysis of 13 mutants allow three new genes to be identified. Two of these genes (npfB and npfC) are closely linked within the mating

type locus. Strains carrying *npfB*⁻ alleles mimic mt_2 strains. A third gene (*npfA*) is unlinked to *mt* and a locus (*apt-1*) previously shown to be involved in plasmodium formation. A mutant allele of *npfA* appears to have no effect on crossed plasmodium formation, though it renders clonal plasmodium formation temperature-sensitive.

Chapters 4 and 5 describe preliminary attempts to characterise physiologically and biochemically the mutant strains whose isolation and genetical analysis are described in Chapter 3.

Chapter 6 contains a discussion of the work described in this thesis, with particular reference to the possible roles of the mating type locus.

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