MATING AND VEGETATIVE INCOMPATIBILITY

IN THE CELLULAR SLIME MOULD

DICTYOSTELIUM DISCOIDEUM

by

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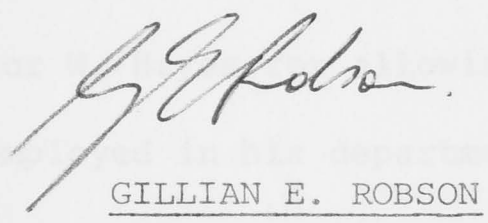
A thesis submitted to the Australian National University

for the degree of Master of Science

September, 1978

STATEMENT

The work reported in this thesis is my own except for the experiments involving the American wild isolates (Chapter 2) which were performed in collaboration with Dr. K. Williams.



GILLIAN E. ROBSON

ACKNOWLEDGEMENTS

Foremost I would like to thank my supervisor Dr Keith Williams for his inspiring enthusiasm and for his special PZAM! that have made working hours so much fun.

I am most grateful to Professor W. Hayes for allowing me to undertake a masters degree whilst employed in his department.

Many thanks to Liz Smith, Dennis Welker and Paul Fisher for their attentive and helpful criticisms of my thesis.

I am indebted to Professor K.B. Raper for his gift of *D. discoideum* wild isolates.

Thanks go to the Genetics Department for providing such an entertaining environment.

To Lyn Johnson I offer sincere thanks for her excellent job of typing this manuscript.

I would also like to thank Mary Williams for her care and good friendship.

Finally, special thanks go to Paul Whitington for his assistance with the photography and for his calming influence throughout the last few months.

Dr. Who to companion : Follow me! I've got a better idea.

Companion : Why? What is your idea?

Dr. Who : I don't know. That's the trouble with good ideas. They only come a bit at a time.

ABSTRACT

A quantitative technique for constructing sexual cysts (macrocysts), in D. discoideum is described. This technique was used to show that macrocysts can be formed with a single zygote and that sex hormone activity in D. discoideum is more complex than the simple one way system previously proposed.

Mating in D. discoideum was shown to be controlled by 2 alleles at a single locus (matA and mata). A system of vegetative incompatibility has been discovered in D. discoideum and was also shown to be controlled by only the mating-type locus. Amoebae of opposite mating-type (matA and mata) will form macrocysts but not parasexual (vegetative) diploids, whereas amoebae of the same mating-type (matA + matA or mata + mata) will form parasexual diploids but not macrocysts. This phenomenon was examined in a series of sexual and parasexual crosses between independent wild isolates of either mating-type and tester strains of NC4 (matA) and V12 (mata) origin. This finding was further supported by genetic analysis of three rare parasexual diploids ("illegitimate" diploids) which were formed between amoebae of opposite mating-type. These diploids were shown to be homozygous for vegetative incompatibility and mating-type.

The literature on vegetative incompatibility in the fungi, especially heterokaryon incompatibility in Neurospora crassa, is reviewed. Ways of overcoming vegetative incompatibility are discussed and suggestions are made towards constructing a system in which sexual and parasexual genetic analysis can be performed using strains derived from a single wild isolate.

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GENERAL INTRODUCTION

The cellular slime mould Dictyostelium discoideum has become a popular organism for studies of differentiation because of its well defined, yet simple, asexual cycle of development (Fig. 1). The short life-span and naked amoeboid state have made this lower eukaryote amenable to biochemical studies (Loomis, 1975). However, the sequence of biochemical events underlying developmental processes must always begin at the gene. A workable parasexual cycle has already provided a good foundation for genetic analysis in D. discoideum (Fig. 2), but sexual genetic analysis is not yet possible on a routine basis (Jacobson & Lodish, 1975). Access to both parasexual and sexual genetic analysis in D. discoideum would make this organism an even more attractive model system for the study of basic problems in cell biology.

D. discoideum has a haploid chromosome number of seven (Brody & Williams, 1974; Robson & Williams, 1977) and in the wild is predominantly a haploid organism. However, rare diploids are spontaneously formed at low frequency and in the laboratory can be selected and maintained indefinitely as stable diploid cultures (Loomis, 1969; Katz & Sussman, 1972; Welker & Deering, 1976; Newell et al., 1977; Williams, 1978). In a diploid population, haploids arise spontaneously at a low frequency (Brody & Williams, 1974) and these can be purified using selective methods (Katz & Sussman, 1972; Williams et al., 1974a). Alternatively haploidisation can be induced using the fungicide ben late (Williams & Barrand, 1978). This conversion from haploid to diploid and back again, plus mitotic crossing-over, represents a complete parasexual cycle (Fig. 2) (Pontecorvo, 1956). Since the haploid and diploid phases of the parasexual cycle of D. discoideum are both stable and can proceed through the whole asexual life cycle (Fig. 1), the allelism of mutations can be determined by complementation analysis (Williams & Newell, 1976).

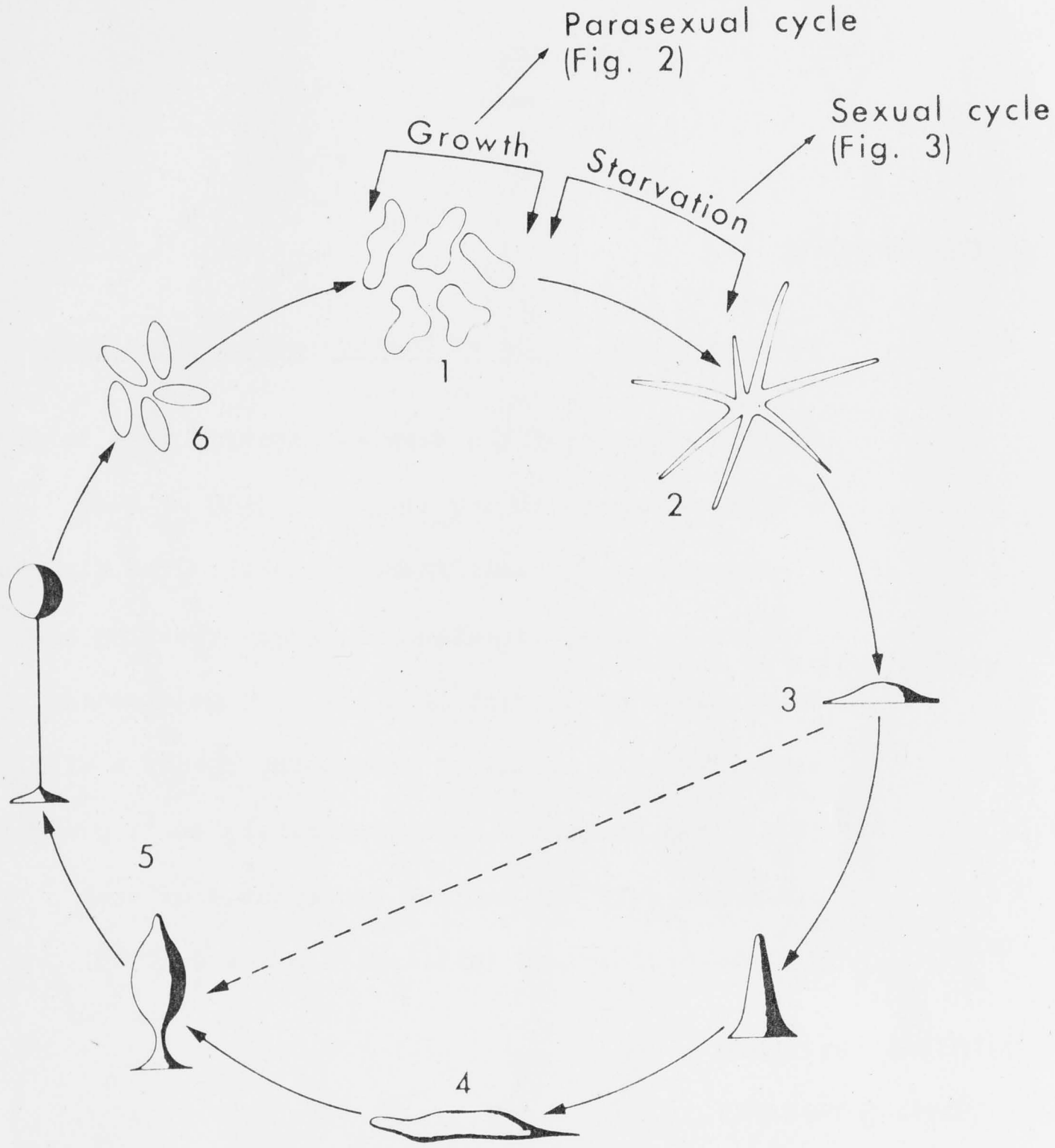


Fig. 1 Asexual life cycle of Dictyostelium discoideum

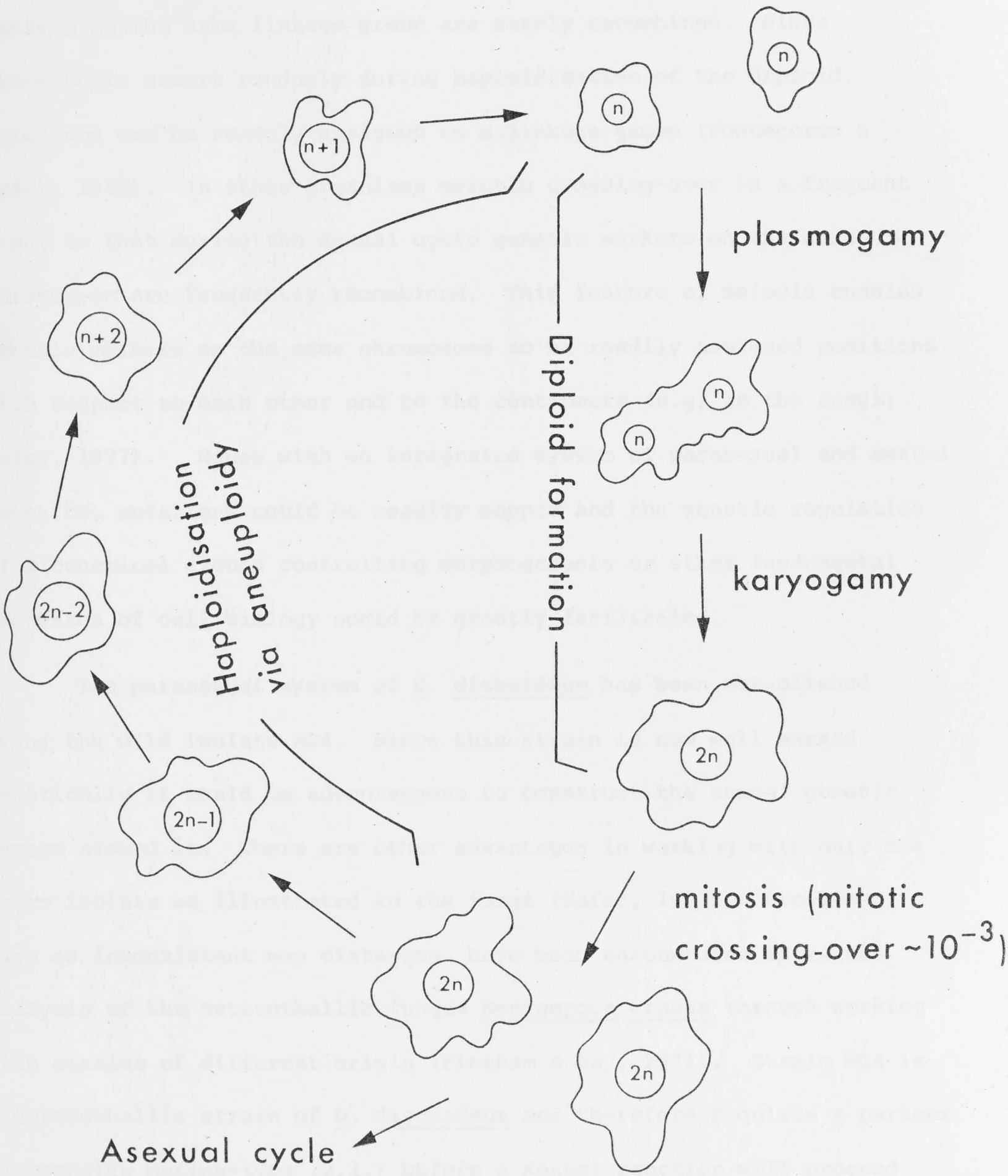


Fig. 2 Parasexual cycle of Dictyostelium discoideum

Mitotic crossing-over is a rare event (10^{-3} to 10^{-4} in D. discoideum, Mosses et al., 1975) and therefore in the parasexual cycle genetic markers on the same linkage group are rarely recombined. Since chromosomes assort randomly during haploidisation of the diploid, mutations can be readily assigned to a linkage group (Pontecorvo & Kafer, 1958). In other organisms meiotic crossing-over is a frequent event so that during the sexual cycle genetic markers on the same chromosome are frequently recombined. This feature of meiosis enables genetic markers on the same chromosome to be readily assigned positions with respect to each other and to the centromere (e.g. in the fungi; Kafer, 1977). Hence with an integrated system of parasexual and sexual genetics, mutations could be readily mapped and the genetic regulation of biochemical events controlling morphogenesis or other fundamental processes of cell biology could be greatly facilitated.

The parasexual system of D. discoideum has been established using the wild isolate NC4. Since this strain is now well marked genetically it would be advantageous to construct the sexual genetic system around it. There are other advantages in working with only one basic isolate as illustrated in the fungi (Kafer, 1977). Problems, such as inconsistent map distances, have been encountered in sexual analysis of the heterothallic fungus Neurospora crassa through working with strains of different origin (Fincham & Day, 1971). Strain NC4 is a heterothallic strain of D. discoideum and therefore requires a partner of opposite mating-type (2.1.) before a sexual reaction will proceed (Fig. 3). At this stage we are faced with the possibility of establishing a sexual system between the well marked strain NC4 and another, perhaps unrelated, strain of opposite mating-type. In view of the problems associated with basing a sexual system on crosses between different wild isolates (as in N. crassa) this situation would be best avoided. Indeed,

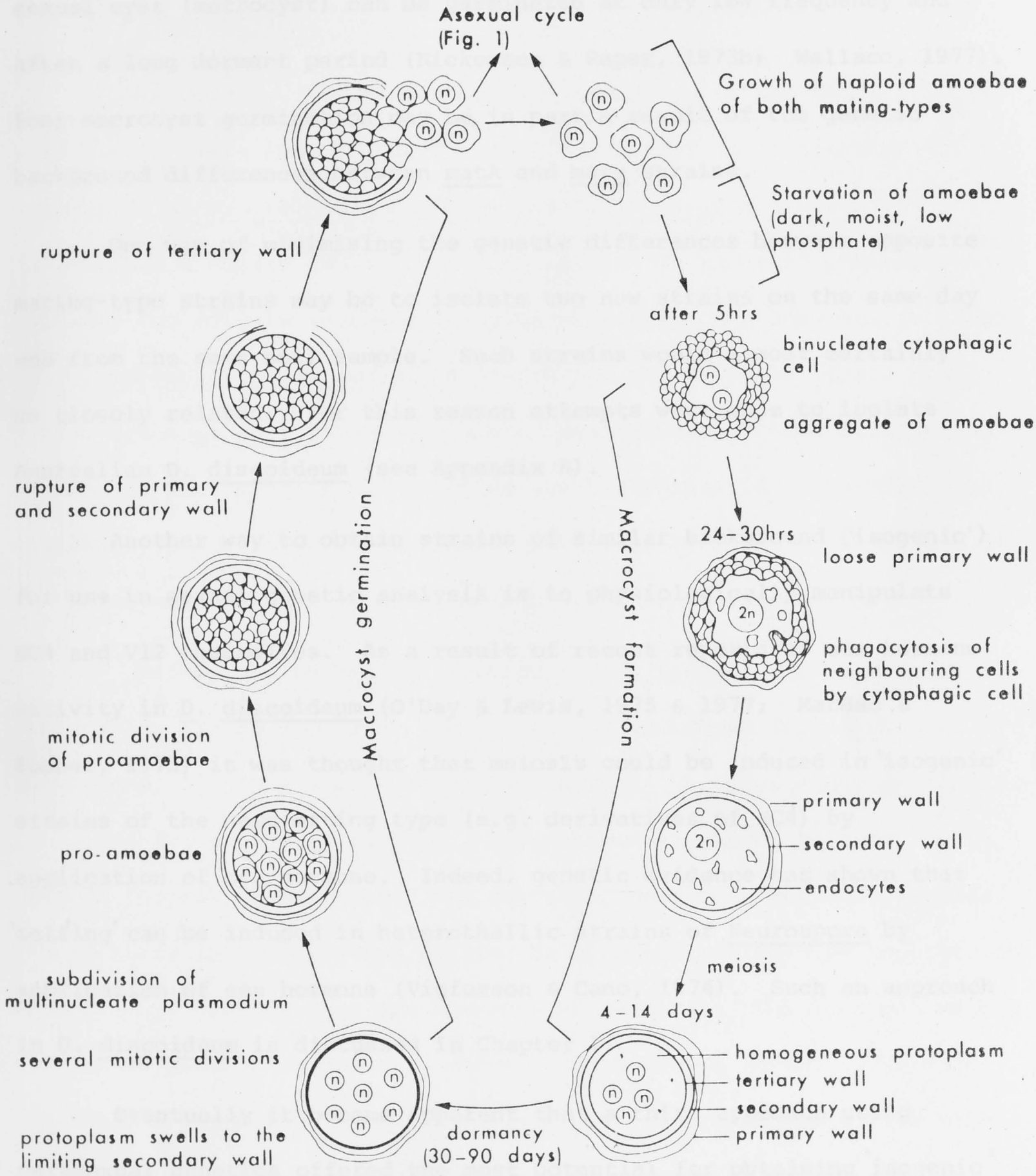


Fig. 3 Sexual life cycle of Dictyostelium discoideum

past attempts to cross strain NC4 sexually with a strain of opposite mating-type but of different origin (V12) have presented problems. The sexual cyst (macrocyst) can be germinated at only low frequency and after a long dormant period (Nickerson & Raper, 1973b; Wallace, 1977). Poor macrocyst germination may be, in part, a result of the genetic background differences between matA and mata strains.

One way of minimising the genetic differences between opposite mating-type strains may be to isolate two new strains on the same day and from the same soil sample. Such strains would almost certainly be closely related. For this reason attempts were made to isolate Australian D. discoideum (see Appendix A).

Another way to obtain strains of similar background ('isogenic') for use in sexual genetic analysis is to physiologically manipulate NC4 and V12 themselves. As a result of recent reports of sex hormone activity in D. discoideum (O'Day & Lewis, 1975 & 1977; MacHac & Bonner, 1975) it was thought that meiosis could be induced in 'isogenic' strains of the same mating type (e.g. derivatives of NC4) by application of sex hormone. Indeed, genetic evidence has shown that 'selfing' can be induced in heterothallic strains of Neurospora by application of sex hormone (Vigfusson & Cano, 1974). Such an approach in D. discoideum is discussed in Chapter 1.

Eventually it became apparent that a third approach using parasexual genetics offered the most potential for obtaining 'isogenic' strains of opposite mating-type. It was reasoned that if parasexual diploids could be constructed between NC4 and V12, the mating-type locus could be mapped using parasexual genetics. By manipulating the segregation of the diploid, one could then construct an NC4 haploid of V12 mating-type. This approach is described in Chapter 2 and a way of integrating sexual and parasexual genetic analysis based on the well marked strain, NC4, is presented.

CHAPTER 1 QUANTITATIVE ANALYSIS OF MACROCYST FORMATION

1.1 INTRODUCTION

Conditions favouring macrocyst formation in the cellular slime moulds were first established for 'self-crossing' (i.e. homothallic) strains of Dictyostelium mucoroides and Dictyostelium minutum (Blaskovics & Raper, 1957). Erdos et al. (1972) reported presumptive evidence for meiosis in the developing macrocyst of a homothallic strain of Polysphondylium violaceum. A search for mating-types (Clark et al., 1973; Erdos et al., 1973b) has since then shown that many strains and species of dictyostelid slime moulds are heterothallic. Therefore the macrocyst is now known as the sexual structure of cellular slime moulds and is commonly formed between amoebae of opposite mating-type.

Before any method for increasing the frequency of macrocyst germination can be explored, the process of macrocyst formation must be understood. To this end it would be desirable to establish a quantitative technique for making macrocysts. Various modifications to three basic methods for making macrocysts are currently used in other laboratories. Two of these involve growing strains of opposite mating-type together in the dark, on 0.1% LP-agar pre-spread with bacteria: One is a wet method (i.e. agar plates flooded with water : Nickerson & Raper, 1973a) and the other a dry method (Polysphondylium pallidum forms macrocysts more frequently when the plates are not flooded : Francis, 1975). The amoebae are left to eat the bacteria, starve and develop into macrocysts without being perturbed. A third method involves growing the amoebae in liquid culture, washing the amoebae by centrifugation, suspending them in saline and shaking on a reciprocal shaker (Filosa & Dengler, 1972).

None of these techniques have been used to quantify macrocyst

formation. In this chapter, I describe attempts to establish a quantitative technique of macrocyst formation in D. discoideum following upon the earlier study by Powell (1974) in this laboratory. With such a technique it may be possible to answer such questions as : 'Are all cells capable of zygote formation?' 'How many zygotes are required to make one macrocyst?' and 'What role if any do sex hormones (O'Day & Lewis, 1975 & 1977; MacHac & Bonner, 1975) play in D. discoideum?'

1.2 MATERIALS AND METHODS

1.2.A Materials

All media and chemicals are described in Appendix B.

1.2.B Maintenance of stocks

(i) Bacteria

Klebsiella aerogenes is used as a food source for growth of D. discoideum. It is grown for 2 days at 21°C and then stored at 4°C clonally on SM-agar or in bulk supply as mass plates on SM-agar. K. aerogenes is scraped from a mass plate (stored in the cold for less than 3 weeks) into S.S. and 0.1 ml aliquots are used as inocula for nutrient agar plates.

(ii) D. discoideum

All strains are described in Appendix B.

Current stocks of D. discoideum are maintained as streak clones at 21±1°C on SM-agar, prespread with K. aerogenes; they are restreaked weekly. The stocks are stored in opaque plastic boxes loosely covered with aluminium foil in a lighted room (∴ semi-light). For long term storage of D. discoideum, ~3 x 10^8 - 10^9 clonally derived spores are suspended in approximately 0.2 ml of normal horse serum (CSL Australia) and then dried on to washed, dry-sterilised, silica gel (6-20 mesh). When these silica gel stocks are stored at 4°C the spores remain viable

for several years.

1.2.C 'Quantitative-technique' of macrocyst formation

Amoebae of the strains to be crossed were grown separately on either LP-agar or SM-agar together with K. aerogenes, and were incubated in the dark (petri plates were wrapped in a double layer of aluminium foil) or in the light (unwrapped petri-plates were placed upside down about 2 feet beneath a daylight fluorescent light). The plates were inoculated with the required number of amoebae (10^4 - 4×10^5 depending on the strain) to produce 'clearing' plates in 48±1hr. Plates are said to be 'clearing' when the amoebae have almost completely eaten the available bacteria but are not yet aggregation competent.

The 'clearing' amoebae were harvested from the agar by dislodging them with a sterile bent glass rod spreader into 20 ml ice cold sterile distilled water. They were washed 3 times by centrifugation (Sorvall SS34 head) at 1.5K for 2 minutes in sterile distilled water. The final pellet was resuspended at 5×10^7 amoebae/ml in sterile distilled water. The amoebal density was determined by haemacytometer counts.

Clear plastic, flat bottomed, Linbro tissue culture dishes (FB-16-24-TC) were prepared in advance with sterile 20mM $CaCl_2$ (final concentration in 1 ml). The washed amoebae were added to the wells at the required concentration; either 5×10^6 amoebae of each strain per well (e.g. Table 8 - Chapter 2) or a series of dilutions ranging from 5×10^6 to 5×10^1 amoebae of each strain (e.g. Table 1 - Chapter 1).

The viability of the washed amoebae was tested at this stage by plating them clonally on to SM-agar with K. aerogenes and incubating at 21±1°C. The number of colonies could be counted after 4 or 5 days and hence the percentage viability calculated.

The culture dishes were wrapped in a double layer of foil and incubated, without shaking, at 21±1°C. After 7 days of incubation, the dishes were unwrapped and the number of macrocysts were counted using an Olympus CK inverted microscope at 40 x magnification.

1.3 RESULTS

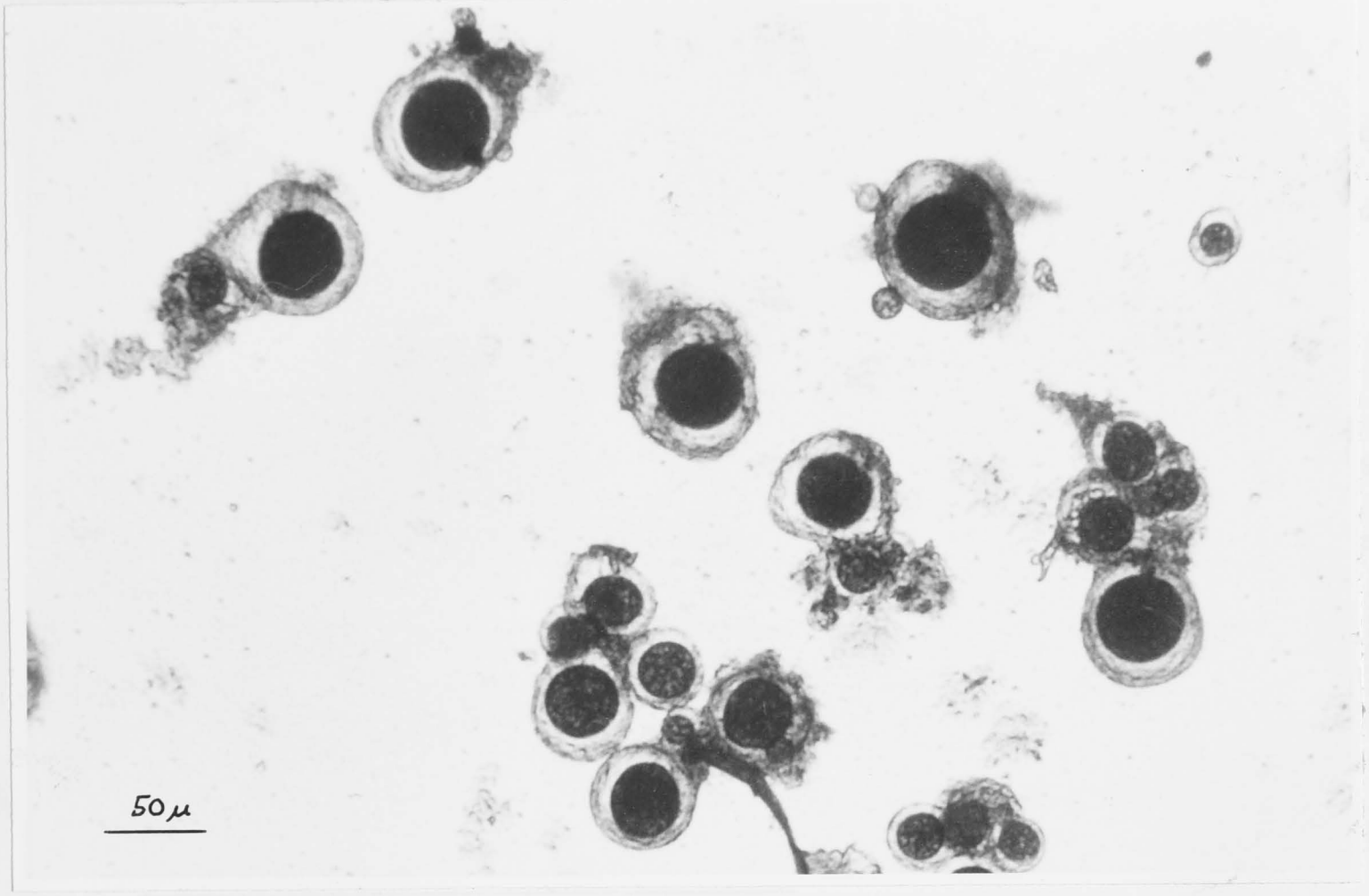
1.3.A Quantitative analysis of macrocyst formation in D. discoideum

(i) Characterisation of macrocysts

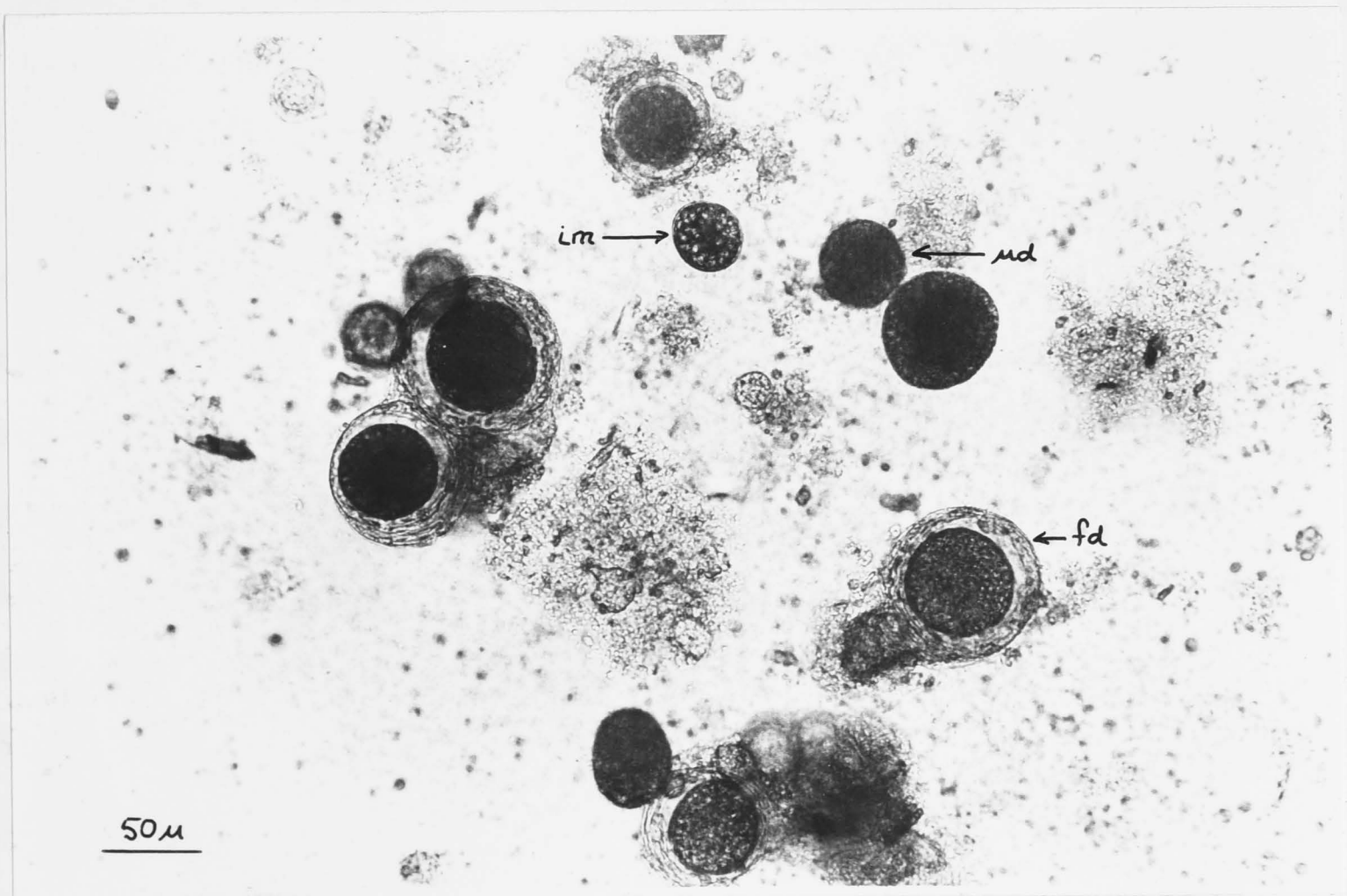
A macrocyst is a spherical structure with a loose multi-layered primary wall, a more compact rigid secondary wall and a triple layered pliable tertiary wall (Erdos et al., 1972). In this work macrocysts of D. discoideum have been classified as either fully developed (in which all three walls are clearly visible) or poorly developed (in which only the primary or primary and secondary walls are visible); they vary in size from 20µ - 80µ (Fig.4). The poorly developed macrocysts include both immature macrocysts that have the potential to become fully developed, and under-developed macrocysts that never proceed past the development of the secondary wall. The number of under-developed macrocysts produced after 7 days in a cross varies from 0% to 95% of the total number of macrocysts present, depending to some extent on the strain used. For example in tests performed in 2.3.A, homothallic strain AC4 consistently formed about 90% fully developed macrocysts, whereas homothallic strain Za-3a, when 'selfed', consistently formed less than 10% fully developed macrocysts. Heterothallic crosses invariably fall between these two extremes. No attempt has been made to determine why poorly developed macrocysts are so commonly formed.

(ii) Effect of amoebal growth conditions on macrocyst formation

The 'quantitative-technique' (1.2.C) separates macrocyst formation into two stages: growth of amoebae and incubation of amoebae under conditions favouring macrocyst formation. In previous attempts to



Fully developed macrocysts of homothallic strain, AC4



Poorly developed macrocysts include both immature macrocysts (im) and underdeveloped macrocysts (ud). Fully developed macrocysts (fd) are also shown: Macrocysts were formed between strains WS582 & HU299.

Fig. 4 Macrocysts of Dictyostelium discoideum

quantify macrocyst formation, Powell (1974) established that incubation of amoebae in the dark in 20mM $CaCl_2$ resulted in good macrocyst production. These incubation conditions have been used in all of the studies reported here.

I have investigated the effect of different growth conditions on subsequent macrocyst formation. In four experiments I crossed strains of opposite mating-type, TS12 (matA1) and V12 (mata2), in various pairwise combinations to determine the effect of light vs dark or of different growth media on macrocyst formation.

(a) The effect on macrocyst production of using different growth media prior to macrocyst formation

Amoebae of TS12 (matA1) and V12 (mata2) were grown separately in the dark on either SM-agar or LP-agar. SM-agar is the rich nutrient medium normally used for maintenance of stocks (1.2.B(ii)). LP-agar is a low nutrient medium which is unbuffered and therefore low in phosphate. Amoebae were harvested separately and incubated together in the dark in 20mM $CaCl_2$ (1.2.C). The effect of prior growth medium on macrocyst production is shown in Fig. 5. Qualitatively all four experiments gave the same results and these were consistent with the findings of Nickerson & Raper (1973a); i.e. a medium low in phosphate (LP) favours macrocyst production. Quantitatively there was considerable variation from one experiment to the next (see error bars : Fig. 5).

(b) The effect on macrocyst production of growth in the light vs the dark prior to macrocyst formation

Amoebae were grown separately on LP-agar in either the light or the dark (1.2.C) prior to harvesting for macrocyst formation. The effect of 'prior' light conditions is shown in Fig. 6. The four experiments all gave the same qualitative results, and these agreed with the findings of

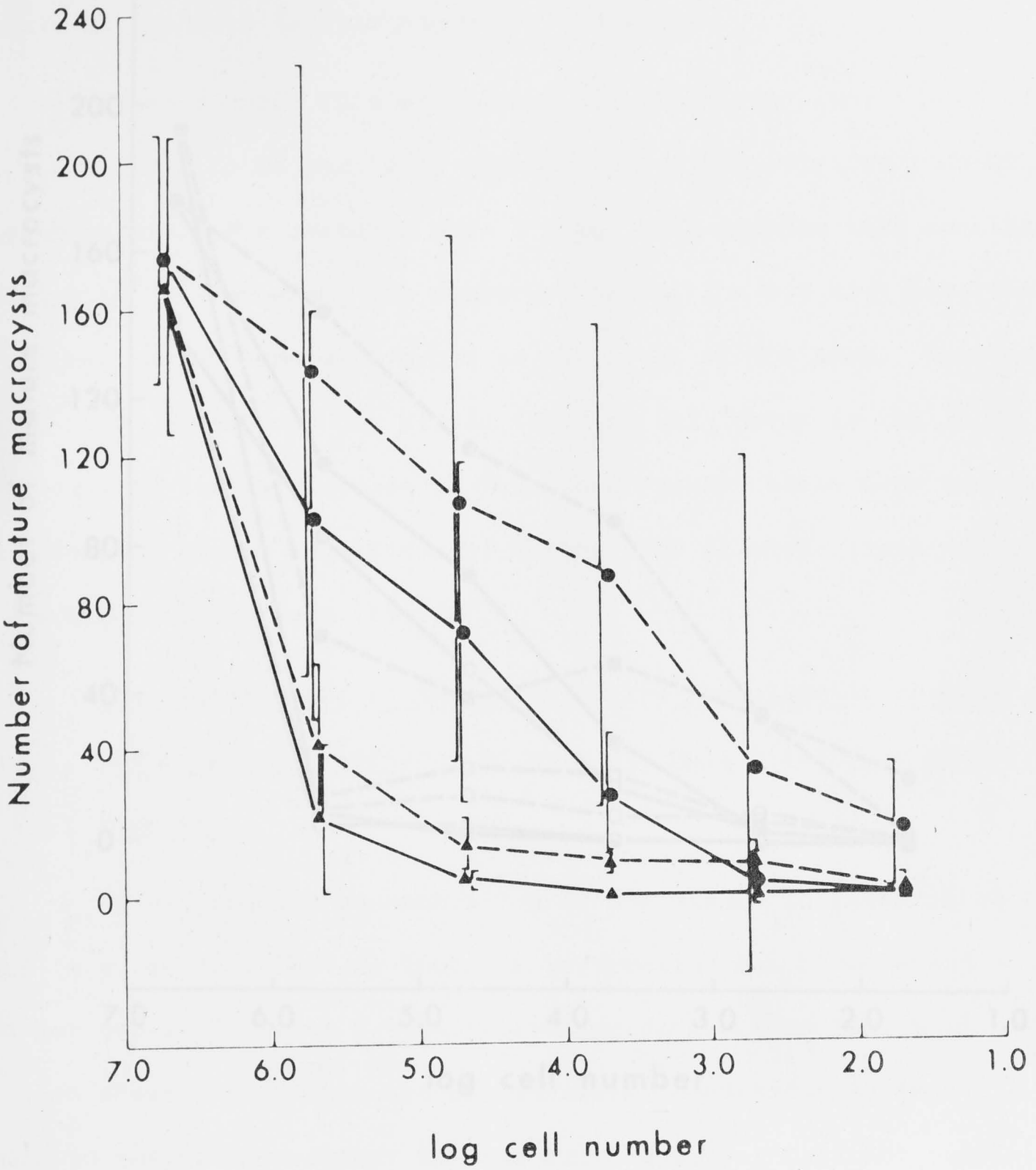


Fig. 5 The effect on macrocyst production of using different growth media prior to macrocyst formation.

● Amoebae were pregrown in the dark on LP-agar.

▲ Amoebae were pregrown in the dark on SM-agar.

— — — — Strain TS12 constant at 5×10^6 amoebae; strain V12 diluted from 5×10^6 amoebae to 5×10^1 amoebae.

———— Strain V12 constant at 5×10^6 amoebae; strain TS12 diluted from 5×10^6 amoebae to 5×10^1 amoebae.

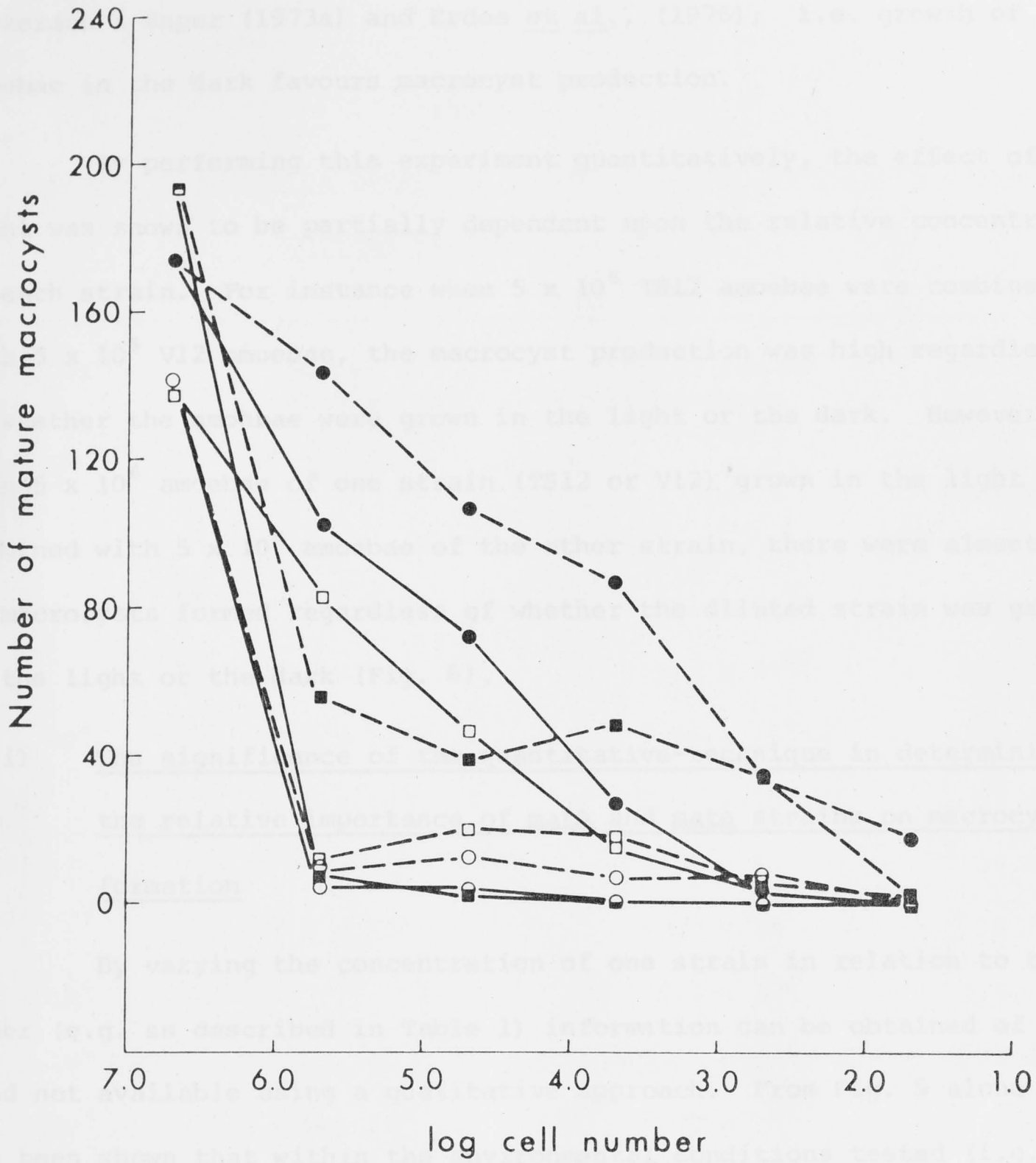


Fig. 6 The effect on macrocyst production of growth in the light vs the dark prior to macrocyst formation.

● DK TS12/DKV12 ; ○ LT TS12/LTV12

■ DK TS12/LTV12 ; □ LT TS12/DKV12

In all of these experiments the amoebae were grown on LP-agar. DK represents growth of amoebae in the dark; LT represents growth of amoebae in the light.

— — — Strain TS12 constant at 5×10^6 amoebae; strain V12 diluted from 5×10^6 amoebae to 5×10^1 amoebae.

——— Strain V12 constant at 5×10^6 amoebae; strain TS12 diluted from 5×10^6 amoebae to 5×10^1 amoebae.

Nickerson & Raper (1973a) and Erdos et al., (1976); i.e. growth of amoebae in the dark favours macrocyst production.

By performing this experiment quantitatively, the effect of light was shown to be partially dependent upon the relative concentration of each strain. For instance when 5×10^6 TS12 amoebae were combined with 5×10^6 V12 amoebae, the macrocyst production was high regardless of whether the amoebae were grown in the light or the dark. However, when 5×10^6 amoebae of one strain (TS12 or V12) grown in the light were combined with 5×10^5 amoebae of the other strain, there were almost no macrocysts formed regardless of whether the diluted strain was grown in the light or the dark (Fig. 6).

(iii) The significance of the quantitative-technique in determining the relative importance of matA and mata strains on macrocyst formation

By varying the concentration of one strain in relation to the other (e.g. as described in Table 1) information can be obtained of a kind not available using a qualitative approach. From Fig. 5 alone it has been shown that within the environmental conditions tested (i.e. growth in the dark on either SM-agar or LP-agar), a constant supply of excess TS12 amoebae favoured macrocyst formation more so than a constant supply of excess V12 amoebae. This effect was shown to be related to the mating-type of the strain rather than to the strain itself in a series of experiments outlined in Table 2. The conclusion from these experiments is that the matA1 strain plays an important role in macrocyst formation and that when excess matA1 amoebae (5×10^6) are available, any individual mata2 amoeba can form a macrocyst. The reverse is not true (see Table 2). In fact no macrocysts were formed until about 100 matA1 amoebae were added to a large excess (5×10^6) of mata2 amoebae (Table 1). However, that the mata2 amoebae are not passive during macrocyst formation

TABLE 1

Quantitative analysis of macrocyst formation under favourable conditions between strains TS12 and V12

Number of amoebae of strain TS12 (*matA1*)	Number of amoebae of strain V12 (*mata2*)	Total number of amoebae in cross	Number of fully developed macrocysts formed (± SE)*
↕	5×10^6	1×10^7	174 ± 34
	5×10^5	5.5×10^6	144 ± 83
5×10^6	5×10^4	5.05×10^6	107 ± 72
↕	5×10^3	5.01×10^6	87 ± 64
	5×10^2	5.00×10^6	34 ± 87
	5×10^1	5.00×10^6	18 ± 17
5×10^5	↕	5.5×10^6	106 ± 56
5×10^4		5.05×10^6	72 ± 46
5×10^3	5×10^6	5.01×10^6	27 ± 16
5×10^2	↑	5.00×10^6	3 ± 6
5×10^1	↓	5.00×10^6	0

These results are the average of 4 experiments in which TS12 and V12 were grown on LP-agar in the dark (Fig. 5) and then crossed using the 'quantitative-technique' of macrocyst formation (1.2.C).

* Only fully developed macrocysts were scored in these experiments.

SE = Standard error of mean

TABLE 2

The relative importance of matA and mata on macrocyst formation

Numbers of amoebae		Crosses*			Average number of macrocysts formed
5×10^6	5×10^1				
matA1	mata2	HU1	x	NP158	7
		X9	x	NP158	20
		TS12	x	V12	24
mata2	matA1	NP158	x	HU1	0
		NP158	x	X9	0
		V12	x	TS12	0

Strains derived from the matA1 strain NC4 (HU1, X9 and TS12) or the mata2 strain V12 (NP158 and V12) were crossed ('quantitative-technique' 1.2.C) so that 5×10^6 amoebae of one strain were combined with 5×10^1 amoebae of the other strain in the combinations shown.

* Two types of crosses were performed: (i) 5×10^6 matA1 amoebae + 5×10^1 mata2 amoebae OR (ii) 5×10^6 mata2 amoebae + 5×10^1 matA1 amoebae.

The HU1 x NP158 crosses were performed twice. HU1 and NP158 were grown on SM-agar in semi-light. They were harvested, washed and diluted as for the 'quantitative-technique' (1.2.C). The X9 x NP158 crosses were performed 3 times. X9 and NP158 were grown on SM-agar in semi-light and then used as for the 'quantitative-technique'. The TS12 x V12 crosses were performed 4 times. TS12 and V12 were grown on LP-agar in the dark and were then used as for the 'quantitative-technique'.

The number of macrocysts includes poorly developed macrocysts as well as fully developed macrocysts.

is apparent from experiments in which amoebae of matA1 or mata2 were each grown under different environmental conditions (e.g. in the light vs the dark). The key experiments illustrating this point are shown in Fig. 6. Consider the experiments where excess amoebae of the mata2 strain, V12, were combined with dilutions of the matA1 strain, TS12. When both strains were grown in the light (————o————) almost no macrocysts were formed. However, when V12 was grown in the dark and TS12 in the light (————□————) a high number of macrocysts were formed. This shows that the dark grown mata2 strain is contributing to macrocyst formation.

Thus, both the matA1 strain and the mata2 strain play an active role in macrocyst formation.

1.4 DISCUSSION

1.4.A Quantitative analysis of macrocyst formation

By quantifying macrocyst formation in D. discoideum, I have sought to increase the understanding of macrocyst formation beyond the limitations of qualitative studies. The 'quantitative-technique' described herein (1.2.C), separates the growth phase of amoebae from the phase of macrocyst development. (The amoebae of each strain are grown separately before they are combined for macrocyst development). Therefore one can examine separately the effect on macrocyst production of altering the growth conditions of amoebae or the environmental conditions of macrocyst development. In this Chapter, I describe experiments in which only the growth phase was varied. Qualitatively my results support previous reports in which LP-agar and the absence of light were shown to favour macrocyst production (Nickerson & Raper, 1973a; Erdos et al., 1976). The reports of Nickerson & Raper (1973a) and Erdos et al. (1976) did not distinguish between the growth of amoebae vs. macrocyst development. Powell (1974) showed that the absence of light and phosphate were essential during macrocyst development. My results show that a low phosphate medium and darkness are also critical during the growth of amoebae prior to macrocyst development.

(i) Are the progeny of one macrocyst the product of one meiosis?

At germination, over one hundred amoebae can emerge from a single macrocyst (Wallace, 1977). Therefore a macrocyst could conceivably have more than one meiosis. Either a single zygote could divide mitotically prior to mitosis (thus producing in effect several zygotes) or several zygotes could be trapped in the same macrocyst at the time of its formation. For reasons outlined in 2.4.D and 2.4.F(iii), mitotic division of a zygote (i.e. a diploid containing both mating-types) is

unlikely. Wallace (1977) has presented genetic evidence that in some circumstances more than one zygote is present in macrocysts of D. discoideum. However, since he formed macrocysts after growing high numbers of amoebae of each mating-type together, it was to be expected that, by chance, some macrocysts would include more than one zygote. I have shown that by mixing 50 mata2 amoebae with excess matA1 amoebae, an average of 18 fully developed macrocysts were formed (Table 1). Assuming a random distribution of the 50 mata2 amoebae it is unlikely that more than one zygote would be included in any macrocyst. This is an important advantage for future sexual genetic analysis in D. discoideum, since it means that we have the potential to analyse the products of a single meiosis.

(ii) A suggested improvement to the quantitative-technique

A major problem with the 'quantitative-technique' of macrocyst formation was the high degree of quantitative variation from one experiment to the next, even when environmental parameters were controlled (Figs. 5 & 6). It is possible to exclude cell viability as a cause of the fluctuation, since in all of the four experiments reported in Fig.6, the cell viability was consistently high. The greatest variable factor of the quantitative technique was the growth stage of the amoebae when harvested. The growth of amoebae was not uniform on a clearing plate and varied depending on how well the amoebae had been spread on to the agar. Therefore it is possible that the high degree of quantitative variation encountered in these experiments was due to differences of amoebal 'age'.

A change of approach is needed. It is suggested that amoebae be grown in liquid culture in the future so that a synchronous population of amoebae can be harvested at a precise stage of amoebal growth or starvation.

1.4.B The role of sex hormone activity in D. discoideum

From the experiments described in this Chapter certain conclusions can be made about the mechanisms involved in macrocyst formation. It was shown in 1.3.A(iii) that the matA1 strain is actively involved in macrocyst formation (when excess mata2 amoebae were available, no macrocysts were formed until about 100 matA1 amoebae were present). This result indicated that a system of recognition was operating between the amoebae of opposite mating-type during macrocyst formation. Since the mata2 strain also played an active role during macrocyst formation (1.3.A(iii)), any recognition system operating between the two strains must be a feedback system rather than a simple one way recognition system. This is contrary to current reports in the literature in which a simple one way system of sex hormone activity in D. discoideum has been proposed (O'Day & Lewis, 1975 & 1977; MacHac & Bonner, 1975). These authors described a system in which NC4 was the secreter of hormone and V12 the responder. However, recent attempts to repeat the findings of O'Day & Lewis (1975) using the same isolates and techniques have been unsuccessful (Wallace, 1977).

In early experiments I attempted to demonstrate a one way system of sex hormone activity by adding supernatant from washed amoebae of one strain back to amoebae of a strain of opposite mating-type. This approach was also unsuccessful. However, in view of my evidence for a more complex system of recognition, the failure of these experiments is perhaps understandable. It is helpful to note that similar problems were encountered during initial attempts to demonstrate sex hormone activity in the fungi. Banbury (1954) was unable to induce zygophore production in the Phycomycete, Mucor mucedo by exposing the mycelium of one strain to a filtrate of the other. However, when a filtrate of a combined (+) and (-) culture was added back to the (+)

strain, zygophores were produced. Hence, Banbury (1954) indicated the requirement for two sequential steps in the initial sexual expression of Mucor mucedo. Sequential feedback systems have now been shown to initiate and regulate sexual interaction in many different fungi (e.g. the most thoroughly researched are Ascobolus, Achlya, Mucor and and Neurospora: Machlis, 1966; Raper, 1967; Barksdale, 1969; Vigfusson & Cano, 1974). Therefore it is possible that by searching for a sequential system of sex hormone activity in D. discoideum, a more reproducible system will be demonstrated.

Before sex hormones can be used to induce 'selfing' between strains derived from the same basic isolate (General Introduction) it will be necessary firstly to develop a more reliable technique for quantitative analysis and secondly to establish the balance of hormones required to induce macrocyst formation.

CHAPTER 2 VEGETATIVE INCOMPATIBILITY AT THE MATING-TYPE LOCUS IN D. DISCOIDEUM

2.1. INTRODUCTION

Attempts to form parasexual diploids between strains of opposite mating-type in D. discoideum have generally been unsuccessful (Williams unpublished). Only one such diploid, DP72, has been reported in the literature (Mosses et al., 1975). This lack of opposite mating-type diploids suggests that a system of 'self' 'not-self' incompatibility is operating in D. discoideum as it does in a wide range of organisms from fungi through to man (2.4.H). It was therefore considered likely that an attempt to construct 'isogenic' strains of opposite mating-type using a parasexual genetic approach (General Introduction) would present problems.

The literature on incompatibility systems in the lower eukaryotes has been examined in the hope of exposing the nature of these problems. There are two well documented systems of incompatibility operating in the lower eukaryotes: sexual and asexual. To avoid confusion, the former will be referred to in the context of mating and mating-types, and the latter will be referred to as vegetative incompatibility.

2.1.A Mating-type systems in the lower eukaryotes

In the fungi and other lower eukaryotes, mating is controlled by mating-type genes, which operate regardless of whether or not there are any morphological sex structures (Raper, 1964; Bergman, et al. 1969; Carlile 1973). Heterothallism, in which successful mating occurs only between strains of different mating-type, is a common system of sexual reproduction for these organisms. Homothallism, in which mating occurs between genetically identical organisms is also widespread through-

out the lower eukaryotes (Carlile, 1973).

The genetic basis of mating has been well documented in the fungi. It ranges in complexity from a simple one locus-2 allele system to a highly evolved two factor-multi allelic system (e.g. Two mating type alleles at a single locus, in the Phycomycetes and Ascomycetes: for general reviews see Whitehouse, 1949 ; Raper, 1966; Fincham & Day, 1971; Esser & Blaich, 1973. A number of alleles at two different complex loci, in the Basidiomycetes: see Fincham & Day, 1971; Papazian, 1951). In the Myxomycetes (acellular slime moulds) two amoebae fuse to form a plasmodium, the first step in the sexual cycle. This is governed by a one locus-multiple allele mating system (i.e. a number of alleles at a single locus; e.g. in Physarum: Henney & Henney, 1968; Dee, 1966; and in Didymium iridis: Collins, 1963).

Few details of mating in the cellular slime moulds are yet known. Mating-types were first recognised in the cellular slime moulds by Clark et al. (1973) and Erdos et al. (1973b) and since then the mating-type systems of a number of species have been examined. It has been shown, however, that in the eight species examined for mating competence (macrocyst formation), both heterothallism and homothallism are found. Two mating-types have been defined in Polysphondylium pallidum (Francis, 1975; Eisenberg & Francis, 1977), although non-mating and homothallic strains were also reported (Eisenberg & Francis, 1977). Clark (1974) proposed, from a study in which forty nine wild isolates of Polysphondylium violaceum were tested for macrocyst formation, that P. violaceum has two syngens each with two mating-types. She reports some overlap of the two groups however, and so it is possible that there are in fact only two mating-types in this species. Ten of the forty-nine strains tested in this study were apparently asexual (Clark, 1974). In a study on mating in the species Dictyostelium giganteum, Erdos et al. (1975) claim that there

are four mating-types expressed. Of the forty isolates tested, seventeen expressed one or another of the proposed four mating types, sixteen produced no macrocysts in any of the tests performed and seven of the isolates gave inconsistent mating reactions. No homothallic strains have been reported for D. giganteum. A report on mating in Dictyostelium purpureum (Clark et al. 1973), indicates that of the eight isolates tested, four express one of two mating-types and four are apparently asexual. Dictyostelium rosarium expresses three mating-types (Chang, 1976) and only homothallic strains of Dictyostelium mucoroides and Dictyostelium minutum have so far been reported (Clark et al. 1973; Nickerson & Raper, 1973a). Of particular importance to this thesis are the mating reactions in the cellular slime mould Dictyostelium discoideum. Erdos et al. (1973b) and Clark et al. (1973) define a system with two mating-types in this species, although both groups also describe some apparently asexual strains. In addition, Erdos et al. (1973b) describe one homothallic strain (selfer) and two bisexual strains (i.e. non selfing, but successfully make macrocysts when paired with strains of each mating-type). Erdos et al. (1973b) and Wallace (1977) suggest that these bisexual strains are expressing a third mating-type allele in D. discoideum.

While the literature on mating in the cellular slime moulds is, as yet, essentially restricted to the discovery of mating-types, it is plausible that many of the species operate on a simple one locus-2 allele genetic basis. In particular the evidence for a one locus-2 allele mating system in D. discoideum is quite strong, since most isolates express one of two mating-types. Strains which do not fit this simple scheme (e.g. asexual, homothallic and bisexual strains) are re-examined here. The evidence for a third mating-type allele (Erdos et al., 1973b; Wallace, 1977) is thus reassessed critically (2.4.C).

2.1.B Vegetative Incompatibility

In the fungi, vegetative fusion in the form of hyphal anastomosis, results in heterokaryon formation. Failure to form stable heterokaryons is a common phenomenon, but details of the genetic control are in general unknown (Carlile, 1973). An exception is found in the Ascomycetes, Aspergillus and Neurospora in which extensive studies on vegetative incompatibility reveal that stable heterokaryon formation depends upon identity at a number of different heterokaryon incompatibility (het) loci (Garnjobst & Wilson, 1956; Jinks & Grindle, 1963; Kwon & Raper, 1967; Mylyk, 1975).

In N. crassa one of the het loci is shown to be coincident with or very closely linked to the mating-type locus, which is a single locus with 2 alleles (Newmeyer, 1970). Hence strains of opposite mating-type, that are identical at all other het loci, will still not make stable heterokaryons.

A similar system of intra-specific somatic fusion has also been reported for the Myxomycetes. Stable plasmodial fusion only occurs between plasmodia with identical 'f' factors (Carlile, 1973). As in N. crassa the vegetative incompatibility system of the Myxomycetes is polygenic (e.g. in Physarum polycephalum and Didymium iridis: see Carlile, 1973; Clark & Collins, 1973 ; Physarum cinereum: see Clark, 1977).

Cytotoxic reactions have been observed to occur in both N. crassa and the Myxomycetes as a result of vegetative incompatibility. Such reactions are often lethal and generally follow after plasmogamy (Garnjobst & Wilson, 1956; Clark & Collins, 1973). Pre-plasmogamy blocks to stable heterokaryon formation have not been ruled out as alternative responses to vegetative incompatibility (Carlile, 1973).

Systems of vegetative incompatibility have not been reported for any of the cellular slime moulds, even though vegetative fusion of amoebae (the first step in parasexual diploid formation) is extensively used for parasexual genetic analysis of D. discoideum. However, the studies on D. discoideum have almost exclusively employed parasexual diploid formation in strains derived from a single wild isolate (NC4).

Parasexuality is not well documented in the heterothallic fungi, even though it may be prevalent (Van Tuyl, 1977). Only in the homothallic species Aspergillus nidulans and species lacking a sexual cycle (e.g. Penicillium chrysogenum and Aspergillus niger) has it been described in any detail (Pontecorvo, 1956; Fincham & Day, 1971). While N. crassa has a similar mating system to the cellular slime mould, D. discoideum, it has no stable diploid phase (Perkins & Barry, 1977) and so a complete parasexual cycle involving diploid formation and haploidisation via aneuploidy has not been described. For these reasons, direct parallels concerning vegetative incompatibility in parasexual systems cannot be made between the fungi and D. discoideum. However, in the light of the studies on control of heterokaryosis in N. crassa it is perhaps likely that parasexual diploid formation in D. discoideum is controlled by a system of vegetative incompatibility. This chapter describes a detailed investigation of the genetic control on parasexual diploid formation in D. discoideum and looks for possible links between vegetative incompatibility (i.e. failure to form vegetative diploids between two haploid strains) and the mating-type locus.

2.2. MATERIALS AND METHODS

2.2.A Materials

All media and chemicals are described in Appendix B.

2.2.B Maintenance of stocks

All strains are described in Appendix B. Details of strain maintenance are described in 1.2.B.

2.2.C Macrocyst formation

(i) Quantitative technique

See 1.2.C.

(ii) Toothpick technique

This technique was used for routine mating-type tests. One ml of sterile 20mM $CaCl_2$ was dispensed into each of 24 flat bottomed clear plastic wells (capacity ~3.2ml) of a sterile Linbro FB-16-24TC multi-well dish. Amoebae of two strains, one of known mating-type, were picked into each well from stock streak clones on SM-agar using a sterilised conically pointed wooden toothpick. In 'selfing' experiments, only one strain was picked into each well. For good macrocyst production large picks of cells were required (approximately 10^6 to $2x10^6$ amoebae per pick). The multi-well dish was swirled briefly and gently before covering with foil for dark incubation. The dish was not shaken during incubation and was stored at $21\pm1^{o}C$ for seven days. The multi-well dish was then unwrapped and scored for macrocysts, using an Olympus CK inverted microscope at 40 x magnification. Several attempts to use the 96 well Linbro tissue culture dish FB-96-TC or Falcon 3040 with lid 3041 (0.1ml 20mM $CaCl_2$ per well) proved unsuccessful since macrocysts were rarely formed even in mating-type tests between sexually competent strains. Each well in these dishes has a capacity of only 0.35ml and so perhaps the failure was related to the smaller size of the well.

2.2.D Parasexual diploid formation

(i) Co-aggregation of amoebae

Vegetative amoebae of the same mating-type, taken from clearing plates and co-aggregated on filters (Loomis, 1969) or in liquid (Williams unpublished) for less than 2 hours, form parasexual diploids at a frequency of approximately 10^{-7} . However, when they are co-aggregated for more than 15 hours, the frequency of parasexual diploid formation plateaus at approximately 10^{-5} (Williams unpublished). Therefore, to increase the frequency of parasexual diploid formation, the amoebae to be crossed were always co-aggregated.

(a) Washed cell technique

This technique is a quantitative technique for parasexual diploid formation (Williams et al., 1974b). Linbro FB-16-24-TC multi-well dishes were prepared in advance with sterile incubation solutions: distilled H_2O, 20mM K/K phosphate pH7.5, 20mM K/K phosphate pH6.5, 20mM $CaCl_2.2H_2O$, 30% Bonners salt solution (30% S.S.) and 20mM KCl (final concentration in 1 ml). The strains to be crossed were harvested in sterile distilled water, from 'semi-light' grown clearing SM-agar plates as for the 'quantitative-technique' of macrocyst production (1.2.C) and diluted to a concentration of 5×10^7 amoebae per ml. 5×10^6 amoebae of each of the two strains to be crossed were added to each well and the volume adjusted to 1 ml (i.e. 10^7 amoebae per ml final concentration). The tissue culture dish was shaken at 21±1°C on an orbital shaker at 150 cycles per minute for 17-18 hours. The shaken suspensions of amoebae were transferred, using sterile pasteur pipettes, into sterile test tubes and whirlimixed. The starving amoebae often aggregate into 'balls' characteristic of the strain (Powell, 1974) and whirlimixing is generally required to disrupt them. A known volume of each suspension and an 0.1 ml

suspension of K. aerogenes were plated on to SM-agar using a sterile bent glass rod spreader.

(b) Multi-well pick technique

This technique is used for routine parasexual diploid formation (Williams & Newell, 1976). 0.1 ml of sterile 20mM $CaCl_2$ was dispensed into each of 96 flat bottomed clear plastic wells (capacity ~0.35ml) of a sterile Linbro tissue culture dish FB-96-TC. Experiments using this 96 well tissue culture dish gave good results and so it was used in preference to the tissue culture dish Falcon FB-16-24TC in which only twenty four crosses can be performed per dish.

Amoebae of the strains to be crossed were picked using a sterile toothpick from the growing edge of streak clonal plates and swirled into the designated well. Small picks were used (about 5×10^5 amoebae per pick). The multi-well dish was shaken at 150 cycles/min at 21±1°C on an orbital shaker for 17-18 hours. The contents of each well were sucked up and down several times using a 100µl Oxford sampler with sterile tip to disaggregate the amoebae. The entire contents of the well were then plated onto either SM-agar or SM-agar containing cobaltous chloride (2.2.D(ii)).

(ii) Parasexual diploid selection

Parasexual diploid formation is a rare event (about 10^{-5} in co-aggregated amoebae of the same mating-type) and so techniques have been developed to selectively kill the parental haploids, whilst allowing the diploids to grow (Loomis, 1969; Katz & Sussman, 1972; Welker & Deering, 1976; Newell et al., 1977; Williams, 1978). Two selective techniques have been used in this study and are referred to as the 'complementing ts technique' and 'ts dominant cobalt technique'.

(a) Complementing ts technique

This technique can only be used between strains each carrying recessive complementing growth temperature sensitive (ts) mutations. After incubation, the co-aggregated amoebae (2.2.D(i)) were transferred to a growth environment (SM-agar spread with K. aerogenes) at the restrictive temperature, 26.8±0.3°C.

The diploids were heterozygous at both ts loci and therefore grew at 26.8° (Loomis, 1969; Williams et al., 1974b). If the ts mutations in the parental haploids are at all leaky they can cause problems of leaky growth at the restrictive temperature. However, diploids are usually 'punchy' whereas leaky growth is diffuse and therefore diploids can be easily distinguished in most cases. Since diploids have a larger spore size than haploids (Sussman & Sussman, 1962; Sinha & Ashworth, 1969; Katz & Sussman, 1972), 'punchy' haploids caused by reversion of the ts mutation were excluded by examining the spore size of all colonies that grew at the restrictive temperature. All diploids were then checked for parental markers (e.g. spore shape, fruiting body pigment, drug markers) to exclude rare isogenic diploids. Diploids heterozygous for parental markers were purified by clonal passage at the permissive temperature on SM-agar and dried on to silica gel for storage (1.2.B(ii)).

(b) Ts dominant cobalt technique

The 'complementing ts technique' has the disadvantage that both parental haploids must carry recessive, non allelic, ts mutations. The 'ts dominant cobalt technique' provides a means of selecting parasexual diploids in which only one parental haploid has to be genetically marked (Williams, 1978). This enabled selection of parasexual diploids between genetically marked haploids and unmarked wild isolates (2.3.B).

A strain carrying a ts mutation and a dominant mutation which

allows growth on SM-agar containing cobaltous chloride was co-aggregated with a wild type strain in the usual way (2.2.D(i)). The amoebae were then transferred to SM-$CoCl_2 \cdot 6H_2O$ (300 or 350 µg/ml) agar spread with a cobalt resistant strain of K. aerogenes and incubated at the restrictive temperature, 26.8±0.3°C. The wild type parent is sensitive to cobaltous chloride and the cobalt resistant parent is ts. The diploid, however, is tr (ts/+) and is resistant to cobaltous chloride (e.g. cob-354/+ where cob-354 is dominant). Therefore the parental haploids die and the hybrid diploids survive under these selective conditions. Diploid colonies were checked for genotype and dried on to silica gel as described in (a) above.

2.2.E Haploidisation of parasexual diploids

(i) Selection of haploid segregants using recessive drug resistance mutations

In most experiments haploids bearing a recessive drug resistance mutation were selected from diploids heterozygous for that drug resistance mutation by plating the diploid on SM-agar containing the drug (Katz & Sussman, 1972). Between 5×10^3 and 5×10^4 diploid amoebae (depending on the diploid) were plated on to SM-agar containing either methanol 2% (v/v), cycloheximide (500 µg/ml) or cobaltous chloride (350 µg/ml). Drug resistant haploids and diploids homozygous for the mutation began to appear between 4 and 7 days. Haploid segregants were used for further genetic analysis only if they were of independent origin. To avoid selecting multiples of the same haploidisation event (multiplication of haploids in a diploid population could occur before exposure to the drug), haploids were assumed to be of independent origin only if they were genotypically different or if they were derived from separate platings of different diploid clones. Haploid and diploid colonies were distinguished on the basis of spore size (Sussman & Sussman, 1962;

Sinha & Ashworth, 1969; Katz & Sussman, 1972). Haploid colonies were purified by clonal passage on SM-agar before checking the genotype, except when a large number of haploids was to be characterised (e.g. analysis of crossing-over on linkage group II of DU260 - 2.3.D(iii)). In this case amoebae from the desired clone were picked on to the appropriate tester plates (drug plates, SM-agar at 21^{o} and SM-agar at 26.8^{o}) directly from the original selective plate. Haploids were always purified by clonal passage before drying on to silica gel for storage.

The same principle of haploidisation applies to selection of haploids in liquid axenic medium (Williams et al., 1974b). Axenic growth in NC4 derived strains is a recessive character. Therefore diploids heterozygous for axenic growth can be selectively haploidised in axenic medium, since only the axenic haploids will grow. Axenic medium was used to select haploids from diploid DU260 (2.3.D(i)). Strain NP158, the mata2 parent of DU260 is an axenic grower but the other parent, HU1, is non-axenic and so the diploid was expected to be heterozygous for axenic growth. Hence eight individual 125ml sterile flasks each with 25ml axenic medium were incubated with between 10^{4} and 10^{5} amoebae/ml from separate clones of DU260. The flasks were shaken at $21\pm1^{o}C$ on an orbital shaker at 150 cycles/min. Amoebae and spores were counted periodically. Representative haploid segregants of the different phenotypes from each flask were purified by clonal passage on SM-agar. They were characterised for parental markers and dried on to silica gel for storage.

(ii) Selection of haploid segregants using 20µg/ml ben late - SM-agar

Recently a technique for haploidisation of diploids has been described in which selective markers are not required (Williams &

Barrand, 1978). When diploids are plated clonally on nutrient agar containing low levels (between 20 µg/ml and 50 µg/ml) of the fungicide ben late, essentially pure haploid segregants of one genotype arise from each clone. The advantage of this technique is that many haploids of independent clonal origin can be obtained from one 20 µg/ml ben late - SM-agar plate. This technique is used for some of the more recent work described in this thesis (e.g. haploidisation of diploid DU454; see 2.3.F).

2.2.F Chromosome staining

Amoebae were washed in distilled H_2O, fixed in methanol : acetic acid (3:1 v/v) and air dried on to clean slides. They were stained with 10% Gurr's improved R66 Giemsa stain in M/15 Sorensen's phosphate buffer, pH6.8. (Brody & Williams, 1974; Robson & Williams, 1977).

2.2.G Photography

Spores on a haemacytometer slide were photographed using an Olympus BHT microscope with Polaroid Large Format Camera Back: PM-10-M with 3¼" x 4¼" Polaroid Back. Colonies of D. discoideum on SM-agar plates were photographed using a Polaroid MP-4 Land Camera. Polaroid Type 665 negative/positive land film, 8.3 x 10.3 cm was used.

2.3. RESULTS

To transfer the mating-type locus (mat) into another genetic background firstly requires a knowledge of its position in the genome. In the parasexual cycle there is a low frequency of recombination so each chromosome segregates as a single linkage group (Pontecorvo & Kafer, 1958). Therefore using a diploid heterozygous for mating-type, one should be able to assign mat to a linkage group using parasexual genetics. Attempts to cross strains of opposite mating-types NC4 (matA1) and V12 (mata2) have been largely unsuccessful (Williams unpublished), either because of a vegetative incompatibility at the mating-type locus, (as in Neurospora: Newmeyer et al., 1973; Griffiths & Delange, 1978) or because of problems peculiar to that cross. To investigate the nature of the incompatibility, parasexual crosses were performed on a number of independent wild isolates, each belonging to one of four mating classes; heterothallic, homothallic, asexual or bisexual. D. discoideum has not been isolated in Australia (see Appendix A) and so a range of American wild isolates of known mating competence were used (received from Professor K.B. Raper, see: Erdos et al., 1973b). All strains were tested for both mating competence and parasexual diploid formation in crosses with tester strains of each mating-type.

2.3.A Mating competence of independent wild isolates of D. discoideum

A quick and reliable technique for making macrocysts, referred to as the 'toothpick technique', was devised to simplify mating-type tests (see Methods 2.2.C(ii)). The reliability of this technique was determined by using it to check the mating competence of the strains obtained from Professor Raper. The results (Table 3) show that all strains, except WS380b, comply to the mating classification of Erdos

TABLE 3

Mating competence of independent wild-isolates of D. discoideum

Mating class*	Wild isolate	Mating-type of tester-strain matA	mata
Heterothallic	WS472	-	+
	WS195-6	-	+
	WS205-4	-	+
	WS583	-	+
	WS5-1	-	+
	†HU188 (WS10)	-	+
	†HU182 (WS585)	-	(+)
	WS655	-	+
	WS7	+	-
	WS582	+	-
	WS656	+	-
Homothallic	AC4	+	+
	Za-3a	+	+
Asexual	WS57-6	-	-
	WS269a	-	-
	WS380b	-	(+)
	WS584	-	-
	WS526	-	-
Bisexual	WS216-2	+	+
	WS112b	+	+

Independent wild isolates were tested for their ability to make macrocysts when paired with tester strains of either mating-type, using the 'toothpick technique' (2.2.C(ii)). The tester strains used were : matA, WS583, HU235, HU1 or TS12; mata, WS582, HU89. +; macrocysts formed: -; no macrocysts formed: (+); poorly developed macrocysts formed infrequently.

* The wild isolates are grouped into mating classes according to the classification by Erdos et al. (1973b). Exceptions are strains WS655, WS656 & Za-3a, which have not been classified in the literature.

† Strains WS10 and WS585 were diploids when received from Professor Raper; they were haploidised by plating clonally on ben late (20 µg/ml)-SM-agar (Williams & Barrand, 1978).

et al., (1973b). Strain WS380b formed poorly developed macrocysts in one out of three tests with strains of mata mating-type. Therefore, this strain could tentatively be called a heterothallic strain of low matA mating competence, rather than an asexual strain, as classified by Erdos et al. (1973b). Further evidence presented in section 2.3.B supports this reclassification. Strains WS655, WS656 and Za-3a have not yet been referred to in the literature. Strain WS655 was matA and WS656 was mata (confirming Professor Raper's unpublished observations). Using the 'toothpick technique' Za-3a was found to 'self' and is therefore homothallic.

The 'toothpick technique' was used in a study of comparative mating strengths of homothallic and bisexual strains (Table 4). These strains were tested for their capacity to 'self' and their ability to make macrocysts when crossed with tester strains of either mating-type. AC4 has a strong mating reaction in all combinations. Strain Za-3a is a weak 'selfer' and expresses the matA mating-type more strongly than the mata mating-type. Strains WS112b and WS216-2 do not self, but express the mata mating-type more strongly than the matA mating-type.

2.3.B Parasexual diploid formation between independent wild isolates of D. discoideum and tester strains of either mating-type

A quick technique for routinely making parasexual diploids was devised by Williams & Newell (1976) and in this thesis is termed the 'multi-well pick technique' (see Methods 2.2.D(i)b). The same independent wild isolates used in 2.3.A were tested for parasexual diploid formation in crosses with tester strains of either mating-type (Table 5). Since wild isolates grow at the restrictive temperature, it was necessary to use a fusion technique other than the commonly used 'complementing ts technique' (see Methods 2.2.D(ii)a). The 'ts dominant cobalt technique'

TABLE 4

Mating strengths of homothallic and bisexual wild isolates when 'selfed' and when paired with tester strains of either mating-type

Wild isolate	'Selfing' reaction	Mating-type of tester strain	
		matA1	mata2
AC4	+++	+++	+++
Za-3a	+	++	+++
WS112b	-	++	+
WS216-2	-	++	+

Homothallic and bisexual wild isolates were 'selfed' or paired with tester strains of each mating-type using the 'toothpick technique' (see Methods 2.2.C(ii)). Three experiments gave the same results.

Tester strains were: matA1 : HU235 or TS12; mata2 : HU89 or WS582.

+++: Strong mating reaction (more than 50 fully developed macrocysts formed);

++: Moderately strong mating reaction (10-50 fully developed macrocysts formed);

+: Weak mating reaction (less than 10 fully developed macrocysts formed);

-: No mating reaction (no macrocysts formed).

TABLE 5 (legend)

Independent wild isolates were tested in 2 experiments for their ability to make parasexual diploids when crossed with tester strains of each mating-type. The 'multi-well pick technique' (see Methods 2.2.D(i)b) was combined with the 'ts dominant cobalt technique' (see Methods 2.2.D(ii)b).

Tester strains were: matA1 : HU32; mata2 : HU156 or HU184.

+: at least one parasexual diploid obtained (all diploids were shown to be heterozygous for parental genetic markers);

-: no diploids obtained in either experiment;

(-): no diploids obtained, but inconclusive because WS584 and WS526 are somewhat resistant to cobaltous chloride.

* Wild isolates are grouped into mating classes according to the classification of Erdos et al. (1973b) and Table 1.

TABLE 5

Parasexual diploid formation between independent wild isolates of *D. discoideum* and tester strains of each mating-type

Mating class*		Wild isolate	Parasexual Diploid formation Mating-type of tester strain	
			*matA*1	*mata*2
Heterothallic	*matA*	WS472	+	-
		WS195-6	+	-
		WS205-4	+	-
		WS583	+	-
		WS5-1	+	-
		HU188	+	-
		HU182	+	-
		WS655	+	-
	mata	WS7	-	+
		WS582	-	+
		WS656	-	+
Homothallic		AC4	-	-
		Za-3a	-	-
Asexual		WS57-6	+	-
		WS269a	+	-
		WS380b	+	-
		WS584	(-)	(-)
		WS526	(-)	(-)
Bisexual		WS216-2	-	-
		WS112b	-	-

(see Methods 2.2.D(ii)b) was therefore used. The results (presented in Table 5) are as follows:

(i) Heterothallic wild isolates

Wild isolates of mating-type matA make stable parasexual diploids when crossed with the matA1 tester strains, HU32, but not when crossed with the mata2 tester strain, HU156 or HU184. Conversely wild isolates of mata mating-type make parasexual diploids when crossed with the mata2 tester strains, HU156 or HU184 but not when crossed with the matA1 tester strain, HU32. Hence parasexual diploids are only detected at an appreciable frequency in crosses between strains of the same mating-type. It is significant that all heterothallic strains formed parasexual diploids with haploids of the same mating-type.

(ii) Homothallic and bisexual wild isolates

Repeated attempts were made to form parasexual diploids in crosses (additional to those shown in Table 5) between homothallic or bisexual strains and testers of either mating-type. No diploids were detected in any of the tests.

(iii) Asexual wild isolates

Three of the asexual haploids WS57-6, WS269a, and WS380b made parasexual diploids successfully when crossed with the matA1 tester strain, HU32, but not when crossed with the mata2 tester strains, HU156 or HU184. The other two asexual wild isolates received from Professor Raper, WS584 and WS526, were both resistant to cobaltous chloride. Therefore, the crosses employing these strains were hindered by 'leaky' growth on the cobaltous chloride SM-agar 'fusion' plate.

2.3.C Illegitimate diploid formation in D. discoideum

The tests performed on independent wild isolates (see 2.3.B) suggested that vegetative incompatibility in D. discoideum is common

and may be controlled by 2 alleles at a single locus associated with the mating-type locus. With the knowledge that the diploid, DP72, was formed "illegitimately" between two strains of opposite mating-type (Mosses et al., 1975), attempts were made to construct another illegitimate diploid with better genetic markers than DP72. It was hoped that in so doing, vegetative incompatibility and its mating-type association could be investigated still further. The matA1 strains HU1 and X9 were each crossed with mata2 strain NP158 (Table 6). In three experiments of this kind a total of approximately 10^8 amoebae were plated on to growth medium. Two illegitimate diploids were detected in crosses between HU1 and NP158 but none in crosses between strains X9 and NP158. Therefore from these experiments, the estimated frequency of illegitimate diploid formation between strains of opposite mating-type was 2×10^{-8}.

The first diploid detected was not isolated successfully, but a haploid sector, HU89, which was recombinant for genetic markers of the parents HU1 and NP158, was isolated and purified. A second diploid, DU260, was detected as a small spotty-edged colony 12 days after plating the amoebae on to growth plates. Controls were performed in crosses between strains of the same mating-type, i.e. mata x mata: HU89 x HM3; matA x matA: M28 x TS12. Parasexual diploids were detected at a frequency of approximately 7×10^{-6}, with some variation depending upon the incubation solution (Table 6). This frequency is close to the average frequency (approximately 10^{-5}) of a good cross between strains of the same mating-type (Table 15). The results of the control crosses (Table 6) support the finding by Powell (1974) that 20mM $CaCl_2$ is the most favourable incubation solution.

A third illegitimate diploid, DU454, has been constructed recently between the mata2 - V12/NC4 recombinant haploid, HU89 and the

Crosses were performed between haploids of opposite mating-type or between haploids of the same mating-type ('washed cell technique' in combination with the 'complementing ts technique' 2.2.D(i)a and (ii)a) and the frequency of parasexual diploid formation determined.

* This number assumes 100% recovery of amoebae from each incubation well. Haemacytometer counts indicate that less than 100% amoebae are recovered (e.g. in the cross M28 x TS12 haemacytometer counts showed that about 40% of amoebae were recovered after incubation. Therefore, based on haemacytometer counts, the frequency of parasexual diploid formation for M28 x TS12 would be approximately 1.8×10^{-5}). As is clear from the difference between the "Total number of amoebae plated" and the "Total number of amoebae crossed", not all amoebae from each cross were plated at the restrictive temperature.

TABLE 6

arasexual diploid formation between strains of opposite mating-type and between strains of the same mating-type in *D. discoideum*

rosses	Total number of amoebae crossed	Number of diploids detected (Incubation solutions)						Total number of amoebae plated*	Total number of diploids detected	Frequency of parasexual diploid formation
		H_2O	20mM K/K Phos pH 7.5	20mM K/K Phos pH 6.5	20mM $CaCl_2$	30% S.S.	20mM KCl			
pposite mating types										
X9 x NP158 matA1) (mata2)	2.4×10^8	0	0	0	0	0	0	6×10^7	0	2×10^{-8}
HU1 x NP158 matA1) (mata2)	1.8×10^8	0	0	0	1	1	0	4.8×10^7	2	
Same mating types										
HM3 x HU89 mata2) (mata2)	6×10^7	4	3	6	20	4	3	6×10^6	40	6.6×10^{-6}
M28 x TS12 matA1) (matA1)	5×10^7	6	8	3	7	12	N.D.	5×10^6	36	7×10^{-6}

well marked matA1 - NC4 derived haploid, HU227. DU454 was formed using the 'multi-well pick technique' (see Methods 2.2.D(i)b). Sixty crosses were performed between HU89 and five well marked haploids: HU227, HU226, HU225, HU235 and HU234. In this experiment more cells were picked than usual (~10^6/strain instead of ~5 x 10^5/strain - see Methods 2.2.D(i)b). Approximately 10^8 amoebae were examined and thus, at the estimated frequency of illegitimate diploid formation (~2 x 10^{-8}) two diploids were expected. In fact, one diploid named DU454 was detected in the cross between HU89 and HU227. Therefore, when this result is taken into account the approximate frequency of illegitimate diploid formation is in the range of 10^{-8} to 2 x 10^{-8}.

From this low frequency of diploidisation the questions arise: "How is it that illegitimate diploids can be formed at all? Do they escape from vegetative incompatibility? Is the mating-type locus involved?" Haploid segregants of the three illegitimate diploids were characterised to help answer these questions.

2.3.D Analysis of illegitimate diploid DU260 by characterisation of haploid segregants

Diploid DU260 was haploidised on meth 2% - SM-agar. The haploid segregants were characterised into phenotypic groups (Table 7 and Appendix B) and were tested for mating-type using the 'toothpick technique'.

(i) Mating-type analysis of haploid segregants

The haploids expressed phenotypic characteristics of both parents, HU1 and NP158, but were found to express only one functional mating-type, matA1 (Table 7). DU260 is heterozygous for linkage group II;

Mating-type tests ('toothpick technique' 2.2.C(ii)) were conducted between haploid segregants of DU260, each derived from an independent haploidisation event, and tester strains of each mating-type. The tests were scored for macrocysts after 7 days.

-: no macrocysts formed; +: macrocysts formed; (+): poorly developed macrocysts formed rarely.

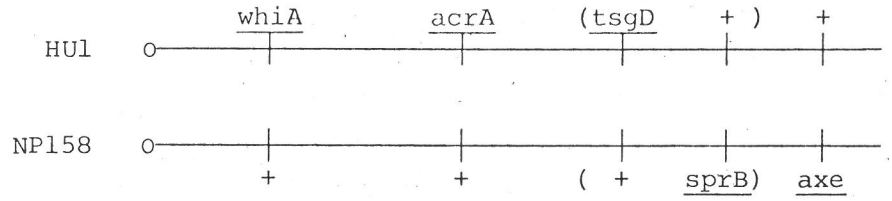
* Phenotypes are described in the Appendix B. Haploids of phenotypic groups A,B,C and D were derived by haploidisation of DU260 on meth 2% - SM-agar. Haploids of phenotypic groups E,F,G and H were derived by haploidisation of DU260 in axenic medium. Representative strains from each group are A: HU167; B: HU171; C: HU168; D: HU178; E: HU193; F: HU197; G: HU202; H: HU199.

† Haploids of phenotypic groups C and D are called 'impotent'. Only 7 out of 72 attempts to make macrocysts between representative strains HU101, HU178 or HU104 and tester strains of mata mating-type were successful; no macrocysts were formed in 72 crosses to matA mating-type tester strains.

TABLE 7

Mating-type haploid segregants from diploid DU260

	Phenotypic group of DU260 haploid segregants*								Parental Strain		Tester Strain	
	A	B	C	D	E	F	G	H	HU1 (matA1)	NP158 (mata2)	TS12 (matA1)	WS582 (mata351)
No. of independently derived isolates tested	35	5	35	12	4	1	6	2				
Tester strain TS12 (matA1)	-	-	-	-	-	-	-	-	-	+	-	+
Tester strain WS582 (mata351)	+	+	(+)†	(+)†	+	+	+	+	+	-	+	-



and so, by haploidising the diploid on meth 2% - SM-agar, the HU1 linkage group II (acrA) was selected (phenotypic groups A,B,C,D: Table 7). The mata2 allele was conceivably on the NP158 linkage group II. Therefore it was necessary to select haploids with this linkage group. Since strain NP158 is an axenic grower, (Williams, 1976), haploid segregants were selected by haploidising DU260 in axenic medium. When this approach was taken the diploid DU260 haploidised, but the haploid segregants grew poorly if at all in axenic medium. Nevertheless all haploids selected in this way were yellow (whiA+), methanol sensitive (acrA+) and had thin elliptical spores (sprB) thus indicating that the NP158 linkage group II had been selected. Mating-type tests showed that these haploids were competent matA1 strains and so DU260 still appeared to possess only one functional mating-type. This suggested that there was a change at the mating-type locus which was involved with vegetative incompatibility. However if, during haploidisation of DU260, there was non random segregation of some of the chromosomes, the mata2 allele could still be present in the diploid but undetected, since only three or possibly four of the seven linkage groups in DU260 are marked. In a search for this possibly 'unexposed' allele, DU260 was haploidised more extensively. The results (shown collectively with those of the initial experiment in Table 7) are entirely consistent with the first findings. Of 100 haploid segregants of independent origin (i.e. derived from independent haploidisation events) tested for mating-type, no mata2 haploids were detected.

'Impotent' haploid segregants of phenotypic groups C and D were effectively matA1 strains of very low mating competence (Table 7),

since macrocysts were sometimes formed when representative strains HU101, HU178 and HU104 were repeatedly paired with a mata tester strain. Alternatively, impotence could have been a reflection of genetic background clashes between the tester strain, WS582 and strains of phenotypic groups C and D. To test this, representative haploids of phenotypic groups A,B,C and D were crossed, using the quantitative technique (2.2.C(i)) to three different tester strains of each mating-type (see Table 8). Tester strains WS582 and WS583 are wild isolates and are totally unrelated to the haploid segregants; strains NP158 and HU1 are the parental strains of DU260 from which the haploid segregants were derived; strains HU89 and HU167 are themselves haploids, recombinant for HU1 and NP158 chromosomes. Of all the tester strains the last two are considered to be the most closely related to the haploid segregants.

A genetic background effect on macrocyst production was shown to occur in phenotypic groups A and to some extent B in which better mating occurred with HU89, the partially isogenic strain (Table 8). However, 'impotent' strains did not become sexually competent even when paired with strain HU89.

(ii) Parasexual diploid formation between haploid segregants of DU260 and tester strains of each mating-type

Haploid segregants of DU260 were shown to express only one mating-type (matA1). If vegetative incompatibility is associated with the mating-type locus as predicted from the study on independent wild isolates (2.3.B), one would expect these haploid segregants, of matA1 mating-type, to form stable parasexual diploids with a matA1 tester strain and not with a mata2 tester strain. Representative haploids of phenotypic groups A,B,C,D,E,F,G and H (see Appendix B) were therefore tested for their ability to make parasexual diploids when crossed with tester strains of each mating-type, HU180 (matA1) and HM3 (mata2).

TABLE 8

Mating competence of haploid segregants of DU260 when paired with related or unrelated tester strains

		Number of macrocysts formed						
		Phenotypic group of DU260 haploid segregants						
		A		B	C		D	
Haploid segregants		HU97	HU167	HU107	HU104	HU168	HU101	HU178
Tester Strain	Relationship of tester strain to DU260 segregants							
matA								
WS582	unrelated	-(10)	1(6)	2(5)	-	-	-	-
NP158	parent of DU260	26	13	3	-	-	-	-
HU89	partially 'isogenic'	42	36	8	-	-	-	-
matA1								
WS583	unrelated	-	-	-	-	-	-	-
HU1	parent of DU260	-	-	-	-	-	-	-
HU167	partially 'isogenic'	-	-	-	-	-	-	-

Results of a single experiment, using the "quantitative-technique" of macrocyst formation (1.2.C). Amoebae were pregrown in the dark on LP agar. The number of macrocysts were scored after 7 days (poorly developed macrocysts are shown in parenthesis).

When crossed parasexually with tester strain HU180 (matA1) the haploid segregants of DU260 fell into two classes (Table 9). Class (1) includes all haploids of phenotypic groups C and D and haploid HU193 of phenotypic group E. In these crosses parasexual diploids were detected within 4 days after plating and at the expected frequency of approximately 10^{-5}. Class (2) includes haploid segregants of phenotypic groups A,B,F,G,H and strain HU195 from phenotypic group E. In these crosses parasexual diploids were detected within 14 days after plating, at a frequency of about 10^{-6}. When diploids formed between strains HU167, HU169, HU173 or HU171 (i.e. of phenotypic groups A & B) and HU180, were purified and tested for growth at 26.8±0.3°C they were found to be slow growers.

This suggests that haploid segregants of class (2) when crossed parasexually with strain HU180, form partially ts diploids. Another possible explanation for poor parasexual diploid formation is that class (2) haploid segregants, when crossed with HU180, may exhibit another vegetative incompatibility locus which is unlinked to the mating-type locus. In view of the apparent single locus mating-type associated control of vegetative incompatibility shown by the wild isolates (Table 5), this alternative seemed unlikely. However, to test it, an experiment was performed in which the haploid segregants were paired with another strain, HU77, of NC4 origin, but of different lineage to HU180 (Appendix B). In addition, to test whether or not the failure to form parasexual diploids was an inherited trait, the matA1 parental strain of DU260 (HU1) was crossed with both HU180 and HU77. If a second vegetative incompatibility locus was present in the haploid segregants, parasexual diploid formation with HU180 would be inhibited in attempted crosses with all NC4 derivatives. The results (Table 10) show that the failure to form parasexual diploids is inherited from the parental strain

TABLE 9 (legend)

Haploid segregants of DU260 were crossed (multi-well pick technique: 2.2.D(i)b) with tester strains HU180 (matA1) and HM3 (mata2) and the presence or absence of parasexual diploids were scored.

+: parasexual diploids detected within 7 days;

(-): no parasexual diploids detected within 7 days, but slow growing diploids detected at low frequency ($\sim 10^{-6}$) within 14 days;

-: no parasexual diploids detected within 14 days. Crosses between haploids of phenotypic groups A,B,C or D and the two tester strains were repeated five times. Crosses between haploids of phenotypic groups E,F,G or H and the two tester strains were repeated four times. The results of these experiments were the same each time.

* Haploids of phenotypic groups A,B,E,F,G and H express a matA1 mating-type. Haploids of groups C and D are essentially impotent (Table 7). All of the haploids are derived from independent haploidisation events.

† In the cross HU193 x HU180 parasexual diploids were formed within 7 days in each of the four experiments, but in each case the diploids were fewer and slower growing than diploids formed in the crosses of phenotype C strains x HU180 and phenotype D strains x HU180.

‡ Strain HU197 made one slow growing diploid within 7 days in one cross with HU180. In the other 3 experiments no diploids were detected.

** Inconclusive evidence for the formation of one diploid in the cross HU177 x HM3 was obtained in one experiment (see text).

TABLE 9

Parasexual diploid formation between haploid segregants of DU260 and tester strains HU180 (matA1) and HM3 (mata2)

Phenotypic group	Strain tested*	Parasexual diploid formation: Tester strains HU180 (matA1)	Parasexual diploid formation: Tester strains HM3 (mata2)
A	HU167	(-)	-
	HU169	(-)	-
	HU173	(-)	-
B	HU171	(-)	-
C	HU168	+	-
	HU170	+	-
	HU172	+	-
	HU177	+	-**
D	HU174	+	-
	HU175	+	-
	HU176	+	-
	HU178	+	-
E	HU193	+†	-
	HU195	(-)	-
F	HU197	(-)‡	-
G	HU196	(-)	-
	HU202	(-)	-
H	HU192	(-)	-
	HU199	(-)	-

TABLE 10

Parasexual diploid formation between haploid segregants of diploid DU260 and tester strains of each mating-type

		Parasexual diploid formation		
		Tester strains		
Phenotypic group	Strain tested	HU77 (*matA1*)	HU180 (*matA1*)	HM3 (*mata2*)
A	HU167	+	(-)	-
	HU97	+	(-)	-
B	HU107	+	(-)	-
C	HU168	+	+	-
	HU104	+	+	-
D	HU101	+	+	-
	HU178	+	+	-
E	HU193	+	+	-
	HU195	+	(-)	-
F	HU197	+	(-)	-
G	HU196	+	(-)	-
	HU202	+	(-)	-
H	HU192	+	(-)	-
	HU199	+	(-)	-
matA1 parent of DU260	HU1	+	(-)	-

Haploid segregants of diploid DU260, and HU1 the *matA1* parent, were crossed (multi-well pick technique 2.2.D(i)b) with tester strains HU77 (*matA1*), HU180 (*matA1*) and HM3 (*mata2*) and the presence or absence of parasexual diploids was scored; +: parasexual diploids detected within 7 days; (-): no parasexual diploids detected within 7 days, but slow growing diploids detected at low frequency ($\sim 10^{-6}$) within 14 days; -: no parasexual diploids detected within 14 days.

HU1, since crosses between HU1 and HU180 produced partially ts diploids. However, since HU77 forms parasexual diploids readily with all DU260 haploid segregants and also with the parental strain HU1, evidence for another vegetative incompatibility locus in D. discoideum is not sustained. A similar problem to that of HU1 x HU180 crosses has been observed previously with other mutant strains of NC4 (Williams & Newell, 1976).

As would be expected from the postulated involvement of mating-type with vegetative incompatibility, the crosses between all haploid segregants and the mata2 tester strain, HM3, were unsuccessful (Tables 9 & 10). However, in one experiment, inconclusive evidence for parasexual diploid formation between strains HU177 and HM3 was obtained (see Table 9). The colony had round spores (sprA) of diploid size, (sprA inherited from strain HU177, see Appendix B). Since the diploid was lost during attempts to purify it, a definitive investigation of its genotype by checking for other markers could not be made. Diploids are normally characterised by checking spore size and shape, fruiting body colour, morphological characteristics, drug markers and if there is still doubt chromosome number (2.2.D(ii)a). The diploid could have been an isogenic diploid of HU177 or a hybrid between HM3 and HU177 (sprA is a dominant spore shape marker). During the experiments described in Tables 9 and 10 approximately 4×10^7 amoebae were plated in crosses 9 & 10 between strains of opposite mating-type (i.e. between HM3 and haploid segregants of DU260).

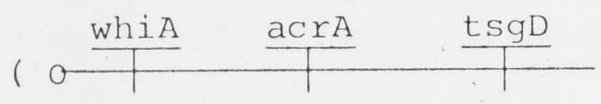
Therefore, on the basis of the estimated frequency of illegitimate diploid formation (10^{-8} to 2×10^{-8} see 2.3.C) one would not have expected to detect an illegitimate diploid during these experiments. However, when these results are combined with the results of other such crosses (Table 15) the detection of an illegitimate diploid

was statistically likely.

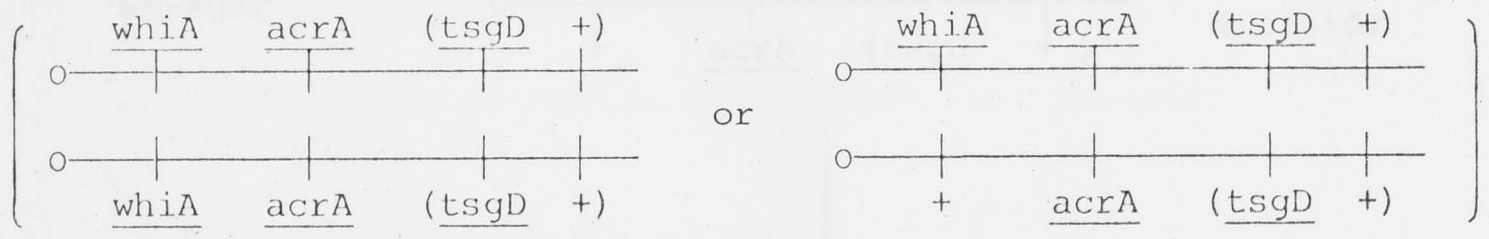
In summary therefore it appears that diploid DU260 expresses only one mating-type allele and only one vegetative incompatibility allele, both of which are of the matA1 parental type.

(iii) Analysis of crossing-over in linkage group II of DU260

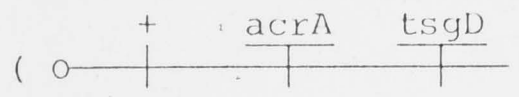
When diploid DU260 was initially haploidised on meth 2% - SM-agar (2.3.D) the frequency of haploid segregants detected which resulted from cross-over diploids, (i.e. yellow methanol resistant, ts haploids: Fig. 7) appeared to be higher than normally expected. Normally one expects white, methanol resistant ts, haploids,



ts, diploids



but very rarely, yellow, methanol resistant, ts haploids



. This could only be analysed accurately by scoring the ploidy and spore colour of every colony that appeared during haploidisation of DU260 on methanol 2% - SM-agar. Such an analysis was made in conjunction with isolating a large number of haploid segregants of independent origin for mating-type analysis (2.3.D(i)). Approximately 2×10^4 amoebae of DU260 were plated on to each of eighty meth 2% - SM-agar plates together with K. aerogenes. The amoebae for each plate were taken from a different clone of DU260 on SM-agar to ensure that haploid segregants on each plate were of independent origin. With 2×10^4 amoebae per plate, only between two and five haploids were expected to appear on each plate, thus allowing colonies late to appear (e.g. slow growers) to be detected. The fast growing colonies were

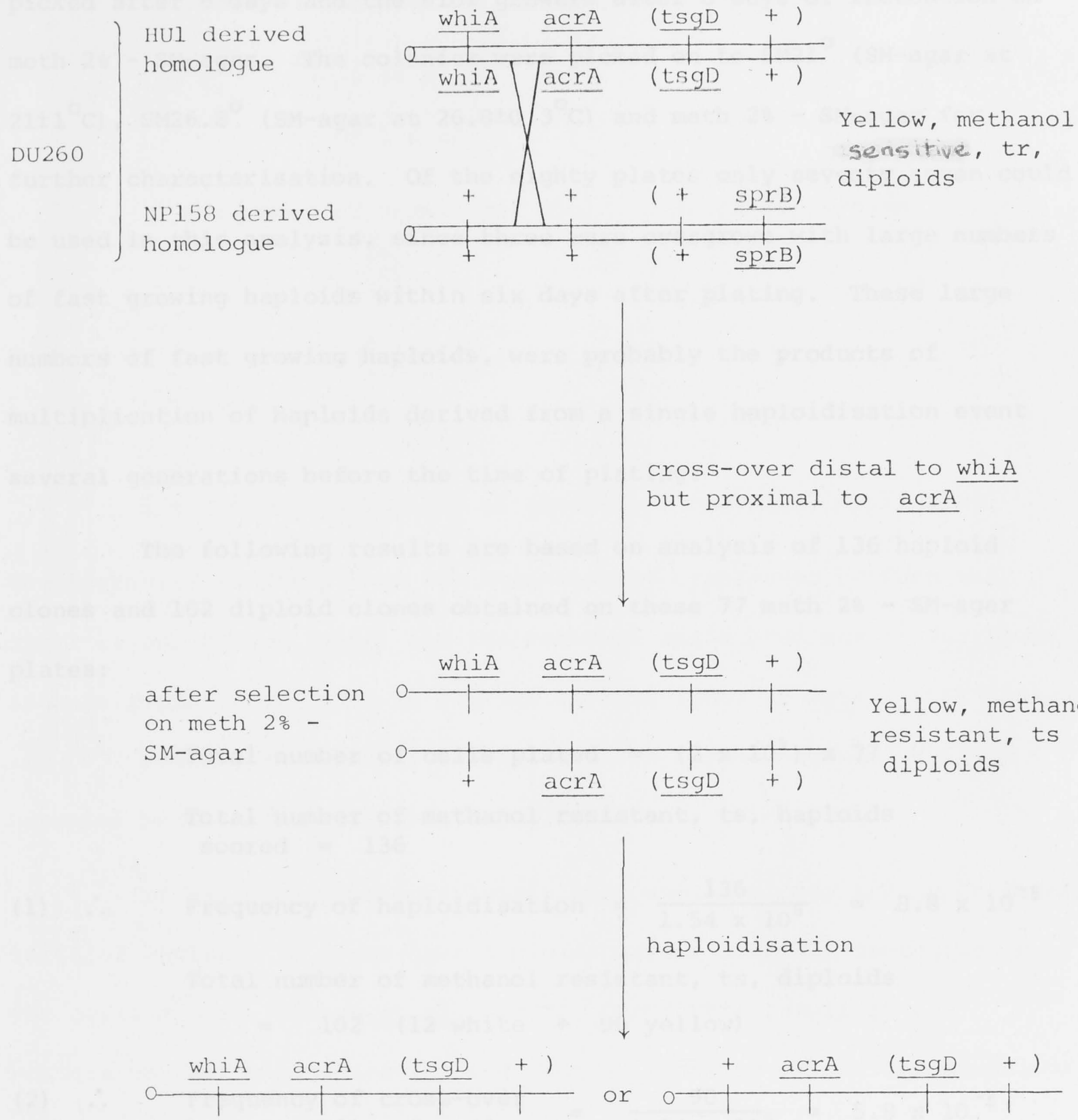


Fig. 7 A graphical demonstration of haploidisation on meth 2% - SM-agar after a cross-over distal to whiA and proximal to acrA1 on linkage-group II of diploid DU260.

picked after 6 days and the slow growers after 8 days of incubation on meth 2% - SM-agar. The colonies were picked on to SM21^{o} (SM-agar at 21±1^{o}C), SM26.8^{o} (SM-agar at 26.8±0.3^{o}C) and meth 2% - SM-agar for further characterisation. Of the eighty plates only seventy seven could be used in this analysis, since three were overgrown with large numbers of fast growing haploids within six days after plating. These large numbers of fast growing haploids, were probably the products of multiplication of haploids derived from a single haploidisation event several generations before the time of plating.

The following results are based on analysis of 136 haploid clones and 102 diploid clones obtained on these 77 meth 2% - SM-agar plates:

Total number of cells plated = $(2 \times 10^{4}) \times 77$

Total number of methanol resistant, ts, haploids scored = 136

(1) $\therefore$ Frequency of haploidisation = $\frac{136}{1.54 \times 10^{6}} = 8.8 \times 10^{-5}$

Total number of methanol resistant, ts, diploids

= 102 (12 white + 90 yellow)

(2) $\therefore$ Frequency of cross-over between <u>whiA</u> and <u>acrA</u> = $\frac{90}{1.54 \times 10^{6}} = 5.8 \times 10^{-5}$

(3) $\therefore$ Expected frequency of cross-over + haploidisation (assuming these events to be unrelated)

= (1) x (2)

= $(8.8 \times 10^{-5}) \times (5.8 \times 10^{-5})$

= 5.1×10^{-9}

Total number of yellow, methanol resistant, ts haploids

= 7

$\therefore$ Total number of haploids derived from cross-over diploids

= 14 (i.e. yellow + equal number of white) (Fig. 7)

(This assumes that only one haploid arises from the haploidisation of a diploid; see Williams & Barrand, 1978.)

(4) $\therefore$ Observed frequency of cross-over + haploidisation

$$= \frac{14}{1.54 \times 10^{6}}$$

$$= 9.1 \times 10^{-6}$$

Frequency of cross-over diploids which haploidise

$$= \frac{(4)}{(2)}$$

$$= \frac{9.1 \times 10^{-6}}{5.8 \times 10^{-5}}$$

$$= 1.6 \times 10^{-1}$$

i.e. approximately 16% of cross-over diploids, haploidise.

These results indicate that the frequency of cross-over between the spore colour marker (whiA) and the methanol resistance marker (acrA) on linkage group II of DU260 is similar to that found in matA1/matA1 diploids and DP72 which, like DU260, is another diploid formed between strains of opposite mating type (Williams unpublished).

It has been shown previously that when diploids are haploidised, the ratio of haploids to cross-over diploids varies markedly depending on the strains used (Mosses et al., 1975). From this result it was postulated that haploidisation and mitotic crossing-over are independent events. However, the results detailed above show that these two events are not entirely unrelated as previously thought. Approximately 16% of cross-over diploids haploidise, compared to approximately 0.01% (see (1) above) in normal diploids. Such a result has now been observed in a diploid formed between strains of the same mating-type (Williams unpublished). Therefore this phenomenon is not related only to illegitimate diploids, and is not considered further.

2.3.E Analysis of DP72 by characterisation of haploid segregants

Diploid DP72 was not analysed as extensively as DU260. Haploid segregants were obtained by haploidisation of DP72 on meth 2% - SM-agar and on cobaltous chloride 350 µg/ml - SM-agar. The haploids were characterised into phenotypic groups (Table 11 and Appendix B) and were tested for mating-type using the 'toothpick technique'.

(i) Mating-type analysis of haploid segregants

All segregants were sexually competent, and all expressed the mata mating-type (Table 11). Hence this preliminary investigation suggests that illegitimate diploid, DP72, has only one mating-type allele.

(ii) Parasexual diploid formation between haploid segregants of DP72 and tester strains of each mating-type

Representative haploid segregants of each phenotypic group (A, B and C) of DP72 were tested for their ability to make parasexual diploids when crossed with a mata2 tester strain (HM3 or HU156) and a matA1 tester strain, (HU128, HU180 or HU154) (Table 12). Parasexual diploids were detected when haploid segregants were crossed with the mata2 tester strain but not when crossed with the matA1 tester strain. Therefore it appears that DP72 has only one vegetative incompatibility allele and that it is of the mata2 parental type.

2.3.F Analysis of DU454 by characterisation of haploid segregants

The mating-type analysis of haploid segregants of illegitimate diploids DU260 and DP72 shows that only one mating-type is expressed (DU260 haploid segregants = matA1; DP72 haploid segregants = mata2). One could hypothesise that illegitimate diploids can be formed between haploids of opposite mating-type only when one of the mating-type alleles is excluded (either functionally or physically). A way of

TABLE 11

Mating-type of haploid segregants from DP72

	Macrocyst formation						
	Phenotypic group of DP72 haploid segregants*					Parental strains	
	A		B	C			
Haploid segregants	X37	X49	X39	X42	X44	X23 (*matA1*)	NP84 (*mata2*)
Tester strain							
TS12 (*matA1*)	+	+	+	+	+	-	+
WS582 (*mata351*)	-	-	-	-	-	+	N.D.

Representative independent haploid segregants of diploid DP72 were paired with tester strains of each mating-type and after 7 days were scored for macrocysts ('toothpick technique' 2.2.C(ii)).

+: macrocysts formed;

-: no macrocysts formed;

N.D.: not determined.

* The haploid segregants are classified into groups according to their phenotype (Appendix B).

TABLE 12

Parasexual diploid formation between haploid segregants of DP72 and tester strains of either mating-type

	Parasexual diploid formation				
	Phenotypic group of DP72 haploid segregants			Parental strains	
	A	B	C		
Haploid segregants	X37	X39	X42	X23 (matA1)	NP84 (mata2)
Mating type of tester strain					
matA1	-	-	-	+	-
mata2	+	+	+	-	+

Representative independent haploid segregants of diploid DP72 were paired with tester strains of matA1 (HU128, HU180 or HU154) and mata2 (HM3 or HU156). Each cross was performed in at least two experiments using the 'multi-well pick technique' (2.2.D(i)b) in combination with the 'complementing ts technique' (2.2.D(ii)a).

+: Parasexual diploids detected in at least one experiment;

-: No parasexual diploids detected in any experiment.

definitively testing this hypothesis would be to search for the mating-type locus on each of the seven linkage groups. To do this one would need to construct an illegitimate diploid in which all of the linkage groups are recognisable i.e., a diploid with a marker on each of the seven linkage groups. To this end, the diploid DU454 was constructed recently (2.3.C). Strain DU454 has six of the seven linkage groups marked (linkage group V is still uncharacterised genetically in D. discoideum) and is heterozygous for all parental markers (Appendix B). This was determined during a preliminary investigation of seventeen independent haploid segregants of DU454. The diploid was haploidised on SM-agar containing either 2% methanol, 350µg/ml cobaltous chloride, 500 µg/ml cycloheximide or 20 µg/ml ben late.

(i) Mating-type analysis of haploid segregants

The seventeen haploids are sexually competent, but all express the same mating-type; matA1 (Table 13). However, an analysis of the genotype of these haploids showed that some of the chromosomes of DU454 did not segregate randomly during haploidisation. All of the haploids are cycloheximide resistant, α- mannosidase negative and all but two have white spore colour even though the diploid is clearly sensitive to cycloheximide (∴ cycA1/+), α- mannosidase positive (∴ manA1/+) and has yellow spores (∴ whiA1/+). No attempt has yet been made to analyse DU454 more thoroughly, as was done for DU260. These preliminary results are consistent with the hypothesis that parasexual diploids can be made between strains of opposite mating-type only when just one parental mating-type allele is functional. However, since all classes of segregants have not been analysed it is still possible that DU454 possesses the mata2 mating-type allele.

TABLE 13

Mating-type of haploid segregants from DU454

Independent haploid segregants*	Source of linkage groups†						Macrocyst formation: Mating-type of tester strain	
	I	II	III	IV	VI	VII	matA1	mata2
HU299	X	X	N.D.	X	X	X	-	+
HU301	X	X	N.D.	X	X	X	-	+
HU315	X	X	N.D.	X	X	X	-	+
HU320	X	X	N.D.	X	X	X	-	+
HU300	X	X	N.D.	Z	X	X	-	+
HU305	X	X	N.D.	Z	X	X	-	+
HU314	X	X	N.D.	Z	X	X	-	+
HU321	X	X	N.D.	Z	X	X	-	+
HU308	X	X	N.D.	X	X	Z	-	+
HU312	X	X	N.D.	X	X	Z	-	+
HU316	X	X	N.D.	X	X	Z	-	+
HU318	X	X	N.D.	X	X	Z	-	+
HU311	X	X	N.D.	Z	X	Z	-	+
HU313	X	X	N.D.	Z	X	Z	-	+
HU319	X	X	N.D.	Z	X	Z	-	+
HU317	X	(Z+X)	N.D.	X	X	Z	-	+
HU329	X	Z	N.D.	X	X	X	-	+
Parental haploids								
HU227 (matA1)	X	X	X	X	X	X	-	+
HU89 (mata2)	Z	Z	Z	Z	Z	Z	+	-

(ii) Parasexual diploid formation between haploid segregants of DU454 and tester strains of each mating-type

Sixteen of the seventeen haploid segregants of DU454 were tested in one experiment for their ability to make parasexual diploids when crossed with tester strains of each mating-type. Table 14 shows that six of the sixteen haploids made parasexual diploids when crossed with tester strains of matA1 mating-type but not when crossed with the mata2 tester strain, and the remaining ten haploid segregants did not make parasexual diploids when crossed with tester strains of either mating-type.

In view of the preliminary nature of this experiment the failure of the ten haploids to produce parasexual diploids is not considered to be significant. Repeat experiments should be performed with alternative tester strains to avoid superficial incompatibilities of the kind encountered in similar crosses between HU180 and haploid segregants of DU260. These results though limited, are consistent with the hypothesis that parasexual diploids can be formed between strains only of the same mating-type and further that the mating-type locus codes for vegetative incompatibility.

2.3.G Further attempts to find both mating-type alleles in illegitimate diploids D. discoideum

Using another approach in the search for both mating-type alleles in illegitimate diploids, strains DU260, DP72 and DU454 were tested for their ability to 'self' (using the 'toothpick technique': 2.2.C(ii)). No macrocysts were formed by any of these diploids when 'selfed'. Although this supports the hypothesis that illegitimate diploids possess only one mating-type allele it does not prove it. Bisexual wild isolates do not self, but may possess both mating-type

TABLE 14

Parasexual diploid formation between haploid segregants of DU454 and tester strains of either mating-type

Independent haploid segregants	Source of linkage groups*						Parasexual diploid formation: Mating-type of tester strain	
	I	II	III	IV	VI	VII	matA1	mata2
HU299	X	X	N.D.	X	X	X	+	-
HU301	X	X	N.D.	X	X	X	-	-
HU315	X	X	N.D.	X	X	X	+	-
HU320	X	X	N.D.	X	X	X	-	-
HU300	X	X	N.D.	Z	X	X	+	-
HU305	X	X	N.D.	Z	X	X	+	-
HU314	X	X	N.D.	Z	X	X	+	-
HU321	X	X	N.D.	Z	X	X	-	-
HU308	X	X	N.D.	X	X	Z	-	-
HU312	X	X	N.D.	X	X	Z	-	-
HU316	X	X	N.D.	X	X	Z	-	-
†HU318	X	X	N.D.	X	X	Z	N.D.	N.D.
HU311	X	X	N.D.	Z	X	Z	-	-
HU313	X	X	N.D.	Z	X	Z	-	-
HU319	X	X	N.D.	Z	X	Z	-	-
HU317	X	(X+Z)	N.D.	X	X	Z	-	-
HU329	X	Z	N.D.	X	X	X	+	-
Parental haploids								
HU227 (matA1)	X	X	X	X	X	X	+	-
HU89 (mata2)	Z	Z	Z	Z	Z	Z	-	+

alleles (see Discussion 2.4.C).

2.3.H Genetic background problems of illegitimate diploids

Although the two illegitimate diploids, DU260 and DP72 show normal growth, the haploid segregants of these diploids display defective growth morphologies as a result of deleterious combinations of the parental chromosomes. The haploid segregants of DU454 display no such growth abnormalities, possibly because the parental haploids of DU454 are partially 'isogenic'. The morphology of all these strains is described in Appendix B.

2.3.I Relationship of parasexual diploid formation to the mating-type locus in D. discoideum

Independent haploid wild isolates have been tested for their ability to make parasexual diploids with tester strains of each mating-type (see 2.3.B), as have independent haploid segregants of illegitimate diploids DU260, DP72 and DU454 (see 2.3.D(ii), 2.3.E(ii) and 2.3.F(ii) respectively). Strain HU89, another independently derived haploid of mixed NC4-V12 genotype (see 2.3.C) has also been tested for its ability to make parasexual diploids with tester strains of each mating-type. In addition, control experiments were performed in which strains of the same mating-type were crossed parasexually. The results, summarised in Table 15, show that parasexual diploids are formed $\sim 10^3$ times more frequently in crosses between strains of the same mating-type than in crosses between strains of opposite mating-type. Therefore there is a vegetative incompatibility between strains of opposite mating-type. This incompatibility is expressed regardless of whether the cross is performed between strains of different genotype (e.g. tester strains x wild isolates: see 2.3.B) or between strains of similar genotype (e.g. HU89 x HU227 see 2.3.C). Throughout the course

TABLE 15

Quantitative date on parasexual diploid formation between strains of like and unlike mating-type

Mating Types of strains crossed	Origin of strains crossed*	Total Number of cells tested (approximate)†	Number of diploids isolated	Frequency of parasexual diploid formation after 7 days. (approximate)***	
matA/matA	NC4/NC4	10^8	464‡	4.6×10^{-6}	(1)
	NC4-V12/NC4	1.5×10^8	384	2.5×10^{-6}	(2)
	Other Isolates/NC4	2×10^7	40	2×10^{-6}	(3)
matA/mata	NC4/V12	2.5×10^8	3††	1.2×10^{-8}	
	NC4-V12/V12	1.1×10^8	1**	9.1×10^{-9}	
	Other Isolates/V12	2×10^7	0	$<5 \times 10^{-8}$	
mata/matA	V12/NC4	——— as above ———			
	NC4-V12/NC4	1.7×10^8	1‡‡	6×10^{-9}	(4)
	Other Isolates/NC4	6×10^6	0	$<10^{-7}$	
mata/mata	V12/V12	10^7	5	5×10^{-7}	(5)
	NC4-V12/V12	8×10^7	186	2.3×10^{-6}	(6)
	Other Isolates/V12	6×10^6	4	6×10^{-7}	(7)

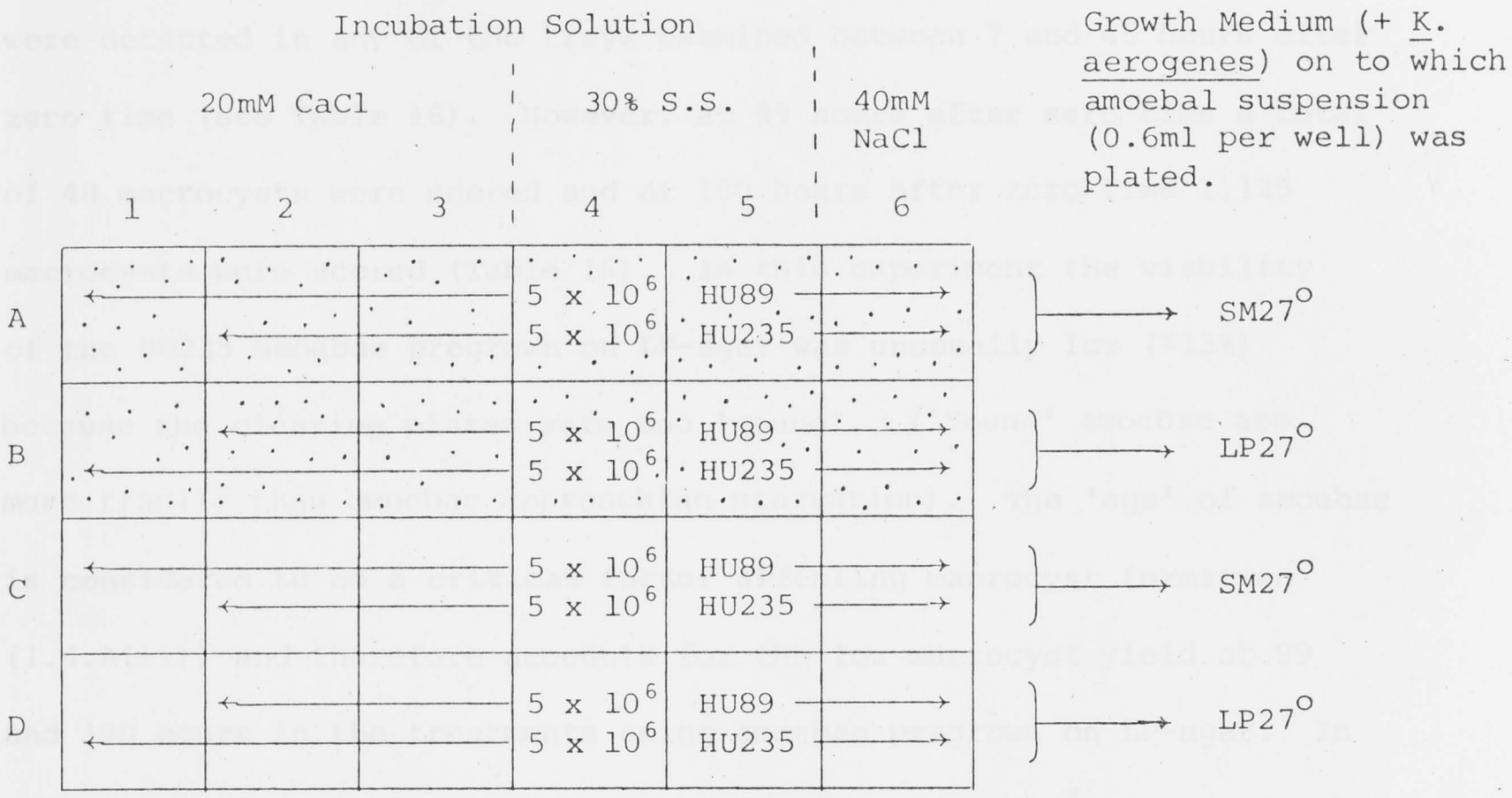
of this work, no more than one vegetative incompatibility locus has been detected. Furthermore, all available evidence suggests that this locus is either linked to or coincident with the mating-type locus.

2.3.J Can vegetative incompatibility be overcome by transferring cytophagic cells to conditions selective for parasexual diploids?

Cytophagic cells are binucleate cells formed by the fusion of two starving amoebae of opposite mating-type during the early stages of sexual reproduction in D. discoideum (Fig. 3). Two well marked sexually competent strains of opposite mating-type, HU89 (mata2) and HU235 (matA1), were used in a quantitative experiment to test whether or not cytophagic cells could form parasexual diploids and divide mitotically when provided with a food supply. Amoebae of strains HU89 and HU235 were mixed and incubated using a modification of the 'quantitative technique' for macrocyst formation (1.2.C). Modifications of the technique included 1) variation of incubation solutions (30% S.S. and 40mM NaCl were used in addition to the usual 20mM $CaCl_2$); 2) interruption of starvation by transfer of the suspension containing developing macrocysts to growth medium at 26.8±0.3°C.

The procedure is most easily explained in the form of a figure (see Fig. 8). Eight replicate trays, such as the one shown in Fig. 8, were prepared and wrapped in foil for dark incubation (see 'quantitative technique': 1.2.C). At each time interval (7,11,19,24,34,45,99 and 190 hours after wrapping the trays in foil) one of the eight trays was unwrapped, examined for macrocysts and 0.6 ml of the suspension from each well was transferred to growth medium (LP or SM agar with K. aerogenes) and then placed at the restrictive temperature, 26.8±0.3°C. The entire contents of each well were removed and whirlimixed before taking a 0.6ml sample to ensure that the clumps of cells were disrupted.

Fig. 8 Experimental procedure for transferring cytophagic cells from conditions favouring macrocyst production to conditions selective for parasexual diploids



Graphical layout of tissue-culture dish Falcon FB-16-24TC with 24 wells. Each well contained 1ml of the appropriate incubation solution.

Amoebae of each strain were pregrown on SM-agar in the dark at 21±1°C before preparation for dark incubation (see 'quantitative technique': 1.2.C).

Amoebae of each strain were pregrown on LP-agar in the dark at 21±1°C before preparation for dark incubation (see 'quantitative technique': 1.2.C).

Wells A1, B1, C1 and D1 were used to check the viability of each strain.

Two growth plates were used per well so that only 0.3ml of amoebal suspension was plated on to one growth plate. (This was done to avoid problems of 'leaky' growth at 26.8±0.3^{o}C). The growth plates were examined for up to 14 days after plating. No parasexual diploids were detected on any of the growth plates (Table 16). No macrocysts were detected in any of the trays examined between 7 and 45 hours after zero time (see Table 16). However, at 99 hours after zero time a total of 48 macrocysts were scored and at 190 hours after zero time 1,125 macrocysts were scored (Table 16). In this experiment the viability of the HU235 amoebae pregrown on LP-agar was unusually low (≃13%) because the clearing plates were too 'young'. ('Young' amoebae are more fragile than amoebae approaching starvation). The 'age' of amoebae is considered to be a critical factor affecting macrocyst formation (1.4.A(ii)) and therefore accounts for the low macrocyst yield at 99 and 190 hours in the treatments using amoebae pregrown on LP-agar. In this experiment there were 142 macrocysts formed/4 x 10^{7} amoebae in the 20mM $CaCl_2$ treatment at 190 hours i.e. 35 macrocysts/10^{7} amoebae (Table 16). In chapter 1 the average macrocyst production for LP grown amoebae under the same conditions was 174 macrocysts/10^{7} amoebae (Fig. 5), i.e. 5 x the number of macrocysts reported in Table 16 . The amoebae pregrown on SM-agar were 'well cleared' and this resulted in good macrocyst production, comparable to that found for macrocyst experiments in chapter 1 (Fig. 5).

Using the results of macrocyst production (Table 16) one can make predictions regarding expected parasexual diploid formation. For instance at 190 hours after zero time, wells A2, A3, B2 and B3 (Fig. 8) collectively contained 730 macrocysts. At 99 hours the same wells of a replicate plate collectively contained 34 macrocysts. Therefore between 0 and 99 hours, approximately 700 cytophagic cells at various

TABLE 16

Transfer of presumptive sexual diploids to growth conditions selective for parasexual diploids

Time after zero time (hours)†	Prior growth medium at 21°**	Number of macrocysts formed*			Number of parasexual diploids detected÷					
		Incubation solutions								
		20mM $CaCl_2$	30% S.S.	40mM NaCl	20mM $CaCl_2$		30% S.S.		40mM NaCl	
					Growth medium for parasexual diploid isolation at 27°					
					SM	LP	SM	LP	SM	LP
7-45	SM	(-)	(-)	(-)	-	-	-	-	-	-
	LP	(-)	(-)	(-)	-	-	-	-	-	-
99	SM	34	10	(-)	-	-	-	-	-	-
	LP	(-)	4	(-)	-	-	-	-	-	-
190	SM	730	240	8	-	-	N.D.	N.D.	N.D.	N.D.
	LP	142	5	(-)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

stages of development were present in these wells. 60% of each well was plated on to 2 plates of either SM or LP agar (Fig. 8) together with K. aerogenes and placed at the restrictive temperature 26.8±0.3°C. If cytophagic cells were able to form parasexual diploids and divide mitotically in the presence of a food supply, one would have expected to detect in the order of 400 parasexual diploids on these 'early' plates (i.e. between 0 and 99 hour samples). No parasexual diploids were detected, however, and so it is probable that cytophagic cells can not be induced to form parasexual diploids by switching the conditions of development in this way.

2.4. DISCUSSION

2.4.A General approach

The ultimate aim of this research is to establish strains of D. discoideum that are used in parasexual genetic analysis that can readily undergo meiosis, so that the advantages of both the sexual and parasexual cycles can be exploited. Delayed macrocyst germination is at present the major obstacle to sexual genetic analysis in D. discoideum (Wallace, 1977). The problem may be in part alleviated by removing genetic background differences. This could be achieved by transferring the mating-type locus of one parental strain into the background of the other. Before such manipulation of the mating-type locus can begin, it is necessary to map its position in the genome. One approach to mapping the mating-type locus would be to construct parasexual diploids between strains of opposite mating-type and to determine the linkage of mat using haploidisation (Katz & Sussman, 1972; Williams et al., 1974a). However, my results show that such a simple approach is not possible in D. discoideum. Parasexual diploids possessing both mating-types have not been detected because of a system of vegetative incompatibility. Therefore the task of mapping the mating-type locus has become more complicated. In fact, the emphasis of this thesis was shifted to an examination of vegetative incompatibility rather than the germination of macrocysts.

2.4.B Genetic background differences in D. discoideum

The problem of interbreeding strains of different genetic background has been discussed in relation to macrocyst germination (General Introduction). One might expect hybrids to express problems of genetic background in other cell functions as shown in systems such as N. crassa (Kafer, 1977) or Phycomyces (Eslava et al., 1975). Two

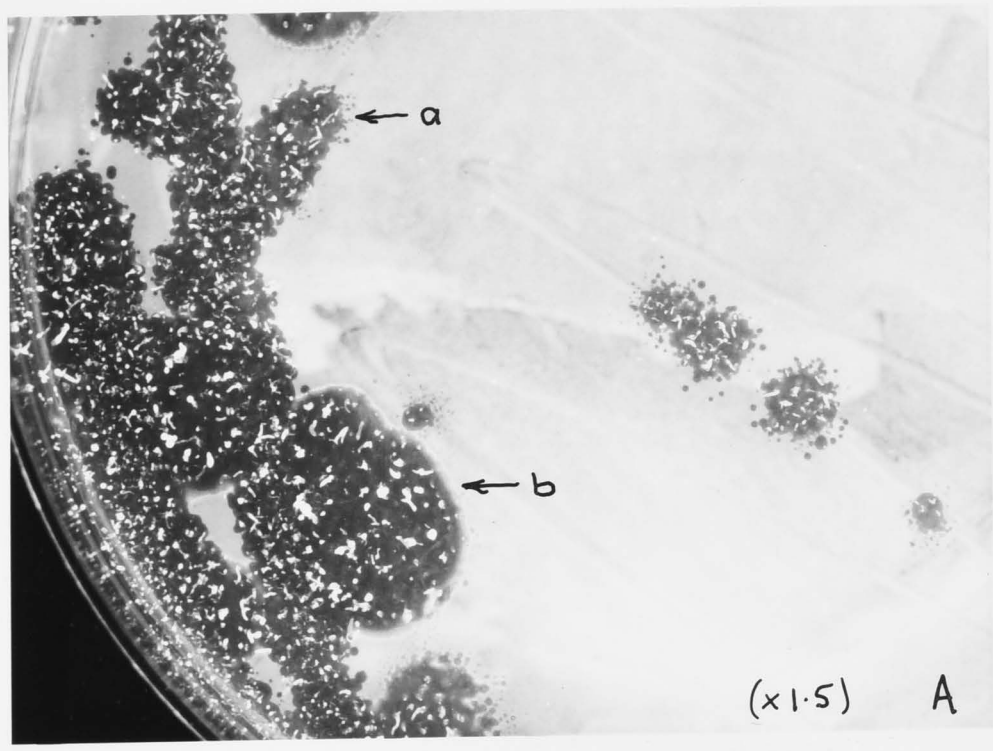
diploids of D. discoideum, DP72 and DU260 formed between haploids of different geographical origin, have been examined in some detail and haploid segregants from them do indeed express problems which are possibly related to a deleterious combination of parental chromosomes.

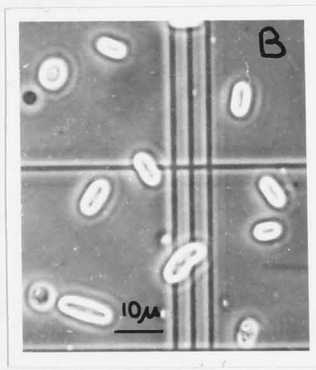
(i) Effects of genetic background differences on growth and differentiation in haploid segregants of DP72 and DU260

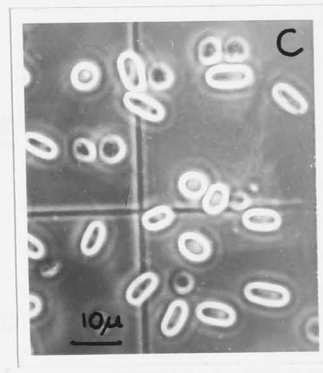
Some haploid segregants of DP72 (e.g. strains X49 and X37: see Appendix B) are very slow growers, perhaps because of their tendency to differentiate in the presence of a food supply (bacteria). Normal strains will not aggregate while there is food available, but X49 and X37 aggregate in the middle of a lawn of bacteria.

These may be interesting strains for use in the study of the switch from vegetative growth to aggregation, since they seem to be permanently programmed into the latter stage of development (Rickenberg et al., 1975; Marin, 1977).

Haploid segregants of phenotypic groups D and G of DU260 (e.g. strains HU178 and HU202: see Appendix B) grow exceedingly slowly, although unlike X49 and X37 they differentiate normally. Strains HU178 and HU202 were maintained on streak clone plates (1.2.B(ii)) and were found to sector at high frequency fast growing colonies, of characteristic morphology (see photo: Fig. 9). Although this unusual phenomenon has not been extensively analysed, the following observations offer a tentative explanation. The spores of the slow growing colonies were very variable in size, whereas the spores of the fast growing sectors were of constant haploid size (e.g. see photo: Fig. 9). Since spore size is correlated to ploidy (Sussman & Sussman, 1962) it was thought that the slow growing colonies may have represented a stable diploid-haploid population and that the fast growing colonies may have







(A) Colony morphology of haploid segregant, HU202:

(a) slow grower; (b) fast growing sector

(B) Spores of slow grower

(C) Spores of fast grower

Fig. 9 Slow growing haploid segregants of DU260 sector fast growing colonies at high frequency

represented a fast growing purely haploid sector. Some years ago Sussman published several papers on such a phenomenon but no firm conclusions were drawn (Ennis & Sussman, 1958 ; Sussman & Ennis, 1959; Sussman & Sussman, 1962). Improved cytological techniques (Brody & Williams, 1974; Robson & Williams, 1977) make a more definitive study of this phenomenon possible. A cursory cytological examination of the slow growing HU178 does indeed support the prediction that HU178 is a stable strain of mixed ploidy (haploid-diploid).

All the above effects are unrelated to the mating-type and to vegetative incompatibility (2.4.E). All the factors segregate independently of mating-type and, in particular, the mating competence is the same for both slow growers (e.g. HU178 & HU101) and their fast growing sectors.

(ii) Effects of genetic background differences on mating competence in haploid segregants of DU260

Haploid segregants of phenotypic groups C and D derived from DU260 (e.g. strains HU104 and HU101, see Appendix B) are impotent (Table 7). An analysis of the segregation pattern of impotence suggests possible linkage of the impotence factor to the HU1 derived linkage group II (no methanol sensitive haploids are impotent). However, a second unspecified NP158 derived linkage group must also be involved (only 50% of the methanol resistant haploids are impotent: Table 7). It is proposed that impotence results from a clashing combination of parental chromosomes and thus is another expression of the genetic background differences between parental haploids HU1 and NP158.

(iii) Progress in overcoming genetic background differences

In a recent experiment, a third parasexual diploid, DU454, was formed between strains of opposite mating-type. This diploid was,

however, formed between two partially 'isogenic' strains, HU89 x HU227. Strain HU89 is a haploid possessing both NC4 and V12 chromosomes. It has a very NC4-like appearance but a V12 mating-type; HU227 is derived totally from NC4. No haploid segregants from DU454 have shown such gross problems as those described for certain haploid segregants of DP72 and DU260. Therefore, even though 'isogenic' strains of opposite mating-type have not yet been obtained, strains recently constructed may have largely overcome the major genetic background problems.

2.4.C Regulation of mating in D. discoideum - the number of alleles at the mating-type locus

During experiments in which the 'toothpick technique' was tested for reliability (2.3.A) the mating-competence of a series of American wild isolates of D. discoideum was re-examined (Erdos et al., 1973b). The results of these experiments suggest that mating in D. discoideum is controlled by two alleles at a single locus and not three alleles as suggested by others (Table 3; Erdos et al., 1973b; Wallace, 1977). These findings, although not directly related to vegetative incompatibility, are interesting in themselves and so will be discussed here.

There is a general agreement that mating in D. discoideum is controlled by alleles at a single locus since no contradictory evidence has been presented. Furthermore, most of the evidence supports a two allele mating-type system of control (Erdos et al., 1973b). However, the possible existence of more than two alleles at this locus has been postulated, in order to explain the unusual mating behaviour of strains WS112b and WS216-2 (Erdos et al., 1973b; Wallace, 1977). These two strains produce macrocysts successfully when crossed with strains of either mating-type matA or mata but they do not 'self' (Erdos et al., 1973b).

My additional evidence discussed below, is consistent with strains WS112b and WS216-2 being pseudo-homothallic rather than representing strains with a third mating-type allele. True homothallic strains, AC4 and Za-3a, 'self' and therefore may possess both matA and mata mating-type alleles. More complex explanations for homothallism are possible but will not be considered here (see 2.4.G(iii)). Unlike AC4 which produces large numbers of macrocysts when 'selfed' or when mixed with strains of either mating-type, Za-3a is a weak 'selfer' and expresses the matA mating-type more strongly than the mata mating-type (Table 4). The strains WS112b and WS216-2 do not self and both express the mata mating-type more strongly than the matA mating-type. I have shown (Table 2) that the balance of mating-type alleles present in a cross (taken as the cell number ratio of the matA strain: mata strain) significantly affects macrocyst production. It is essential to have a high number of amoebae of mating-type matA participating in the cross to ensure good macrocyst production. It is proposed that strains WS112b and WS216-2 are in fact pseudo-homothallic, but unlike Za-3a, which has a fully competent matA reaction, WS112b and WS216-2 have a matA reaction of low competence and are therefore unable to make macrocysts when 'selfed'. The mating reaction of strains WS112b and WS216-2 can hence be explained without referring to a third allele. Wallace (1977) although claiming that these strains do have a third allele, admits the alternative that they may carry both mating-type alleles mata and matA.

In this study the asexual wild isolates of D. discoideum (Erdos et al., 1973b) that could be analysed successfully made parasexual diploids when crossed with the matA tester strain, but not when crossed with mata tester strains (see Table 5). On the basis of the vegetative incompatibility reaction of these crosses (see 2.4.D) one could predict

that asexual strains conform to the one locus - 2 allele system of mating as proposed for the heterothallic strains above, and that for some reason macrocyst production is inhibited in these strains. In support of this prediction, the 'asexual strain' WS380b in one experiment, produced macrocysts when crossed with the mata tester strain WS582 (i.e. WS380b appears to be a heterothallic matA strain of very poor mating competence).

2.4.D Characterisation of vegetative incompatibility in D. discoideum by an investigation of independent wild-isolates

Strains derived from NC4 and V12 form parasexual diploids with great difficulty. It was considered that a system of vegetative incompatibility could be operating either at the mating-type locus as in Neurospora (Beadle & Coonradt, 1944; Gross, 1952; Newmeyer, 1970), or through some other incompatibility specific to the NC4-V12 cross. To investigate these alternatives, the independent wild isolates of various mating classes, as reported by Erdos et al., (1973b) were tested for their ability to make parasexual diploids when crossed with tester strains of either mating-type (see Results 2.3.B). Regulation of somatic cell fusion has not previously been examined in cellular slime moulds, but the comprehensive studies on vegetative incompatibility in the primitive Ascomycetes, N. crassa and Aspergillus sp. (Garnjobst & Wilson, 1956; Jinks & Grindle, 1963; Kwon & Raper, 1967; Mylyk, 1975), have provided a most useful conceptual framework since in many respects the regulation of vegetative incompatibility in the two groups of organisms is analogous. The requirement for identity at the het loci for heterokaryon formation in the primitive Ascomycetes is analagous to the requirement for identity at the mating-type locus for parasexual diploid formation in D. discoideum. However the system of vegetative

incompatibility is controlled at only one locus in D. discoideum compared to genes at a number of different loci, one of which is the mating-type locus, in the primitive Ascomycetes. This difference has a significant effect that is best illustrated by comparing the frequency of vegetative compatibility between wild isolates. All of the eleven heterothallic wild isolates of D. discoideum tested were vegetatively compatible with strains of the same mating-type. Mylyk (1976) performed 64 crosses between different wild isolates of N. crassa of the same mating-type and found only two strains with identity at 5 het loci examined.

The invariable relationship between mating-type and vegetative incompatibility in wild isolates of D. discoideum is considered to be strong evidence for the two functions being controlled at the same locus. The mating-type locus has been shown conclusively to have such a dual function in N. crassa (Griffiths & Delange, 1978).

A further affinity of vegetative incompatibility with the mating-type locus in D. discoideum is demonstrated by strains thought to possess both mating-type alleles (i.e. homothallic and bisexual strains: 2.4.C). In all cases the strains appeared to be vegetatively incompatible with strains of either mating-type (2.3.B).

2.4.E Further evidence concerning the relationship of vegetative incompatibility with the mating-type locus in D. discoideum using genetically characterised strains

(i) Haploid segregants of illegitimate diploids express only one mating-type allele

The studies described in 2.3.B using independent wild isolates were followed by a detailed investigation of the vegetative incompatibility using genetically characterised strains (2.3.D). It

seemed significant that illegitimate diploids (i.e. parasexual diploids formed between strains of opposite mating-type: 2.3.C) gave rise to haploid segregants of only one mating-type when haploidised. Of the three illegitimate diploids so far formed, two segregate haploids of matA1 (i.e. diploids DU260 and DU454) and one segregateshaploids of mata2 (i.e. diploid DP72) (see Tables 7,11 & 13). This result suggests that either the mating-type locus has become homozygous or that one of the parental mating-type alleles has been mutated during illegitimate diploid formation. This is supported by the finding that the three diploids do not 'self' when tested for mating-competence (2.3.G). Hence it appears that parasexual diploids cannot be formed between strains of opposite mating-type while both mating-type alleles are being expressed.

(ii) Haploid segregants of illegitimate diploids express only one vegetative incompatibility allele

As determined in the study on independent wild isolates (2.3.B), vegetative incompatibility and mating-type are invariably linked. In addition haploid segregants of illegitimate diploids DP72, DU260 and DU454 are vegetatively incompatible with tester strains of opposite mating-type, but are all vegetatively compatible with tester strains of the same mating-type (see Tables 10, 12 & 14). Therefore it follows that haploid segregants of illegitimate diploids express only one vegetative incompatibility allele as well as only one mating-type allele.

(iii) Significance of forming illegitimate diploids that have identity at both the vegetative incompatibility locus and the mating-type locus

It is significant that in all three of the illegitimate diploids formed, the vegetative incompatibility locus and the mating-type locus

express only one parental allele and that for both loci the allele is inherited fromthe same parent. Since the formation of illegitimate diploids selects for overcoming vegetative incompatibility, one would expect identity only at the vegetative incompatibility locus. The chance of losing (effectively) the mating-type locus as well as the vegetative incompatibility locus in all three diploids is equal to the (mutation frequency)^3 = $(10^{-6})^3$ = 10^{-18} i.e. there is a very low chance of effectively changing two loci simultaneously by mutation. However, a possible alternative, which fits the available data and allows the two functions to be controlled by separate, but linked, genes is that homozygosis at the vegetative incompatibility locus results from a cross-over event. The vegetative incompatibility and mating-type could become simultaneously homozygous if the mating-type locus is distal to the vegetative incompatibility locus and there is a cross-over proximal to the vegetative incompatibility locus.

(iv) Evidence that a cross-over event in DU260 provisionally maps the mating-type locus

Illegitimate diploids DP72 and DU454 are of no use for mapping the mating-type locus of D. discoideum. Illegitimate diploid DU260, however, does provide a possible lead to locating the mating-type locus.

Vegetative incompatibility in D. discoideum could be overcome as a result of mitotic recombination leading to homozygosis at the mating-type locus. Such an event would render any markers distal to the mating-type locus homozygous. Therefore homozygosis for any chromosomal marker in an illegitimate diploid could indicate the position of the mating-type locus. The spore shape marker, sprA, located on linkage group I in D. discoideum, is homozygous in DU260 (see Fig. 11 Appendix B), thus using this argument the mating-type locus is

located on linkage group I. Wallace, (1977) using limited meiotic analysis suggested that the mating-type locus was not on linkage group I because it segregated independently of cycA. However, it is common in lower eukaryotes (e.g. A. nidulans and S. cerevisiae) for each chromosome to comprise several meiotic linkage groups (Kafer, 1977; Sherman & Lawrence, 1974) and so these results are not necessarily contradictory.

2.4.F A search for the physiological cause of vegetative incompatibility

By discovering the physiological basis of vegetative incompatibility one could perhaps find a way of overcoming it. There are three stages of parasexual diploid formation at which vegetative incompatibility could act. These are: plasmogamy, karyogamy and cell division of the diploid.

(i) Plasmogamy

In the Myxomycetes and N. crassa, which are lower eukaryotes in which vegetative incompatibility has been extensively studied, it appears that recognition of the incompatibility between two 'unlike' colonies occurs after plasmogamy (Carlile, 1973; Clark & Collins, 1973; Garnjobst & Wilson, 1956). Carlile (1973) suggests that the 'f' locus in the acellular slime mould Physarum polycephalum does not inhibit initiation of fusion but prevents the maintenance of stable protoplasmic bridges between plasmodia. In the acellular slime mould Didymium iridis the failure of stable plasmodial fusion is also found to be due to a reaction after plasmogamy (Clark & Collins, 1973). The fungus N. crassa, which has a similar genetic regulation of vegetative incompatibility to D. discoideum (see 2.4.D), also imposes a block to stable somatic fusion after plasmogamy (Garnjobst & Wilson, 1956). The plasma membrane itself has been implicated in this incompatibility reaction in N. crassa

(Williams & Wilson, 1966) and this is supported by the fact that the mating-type locus has been linked to the control of structure in the plasma membrane (Kappy & Metzenberg, 1967).

Whereas strains of opposite mating-type are incompatible during somatic fusion of N. crassa, the same strains fuse compatibly during sexual reproduction, supposedly because sexual interaction is mediated by specialised structures (Perkins & Barry, 1977). In D. discoideum both mating and somatic fusion require a union between morphologically indistinguishable amoebae, and, since haploids of opposite mating-type fuse successfully during macrocyst formation, one must assume that the same haploids fuse successfully during parasexual diploid formation. An experiment was performed in which amoebae of opposite mating-type were incubated under macrocyst forming conditions and then transferred to growth conditions selective for mitotically dividing parasexual diploids (2.3.J). No parasexual diploids were formed and hence it may be concluded that vegetative incompatibility is imposed some time after plasmogamy.

(ii) Karyogamy

The fate of opposite mating-type nuclei during parasexual diploid formation can be examined genetically. Homozygosis at the mating-type locus via mitotic recombination is one proposed mechanism for overcoming vegetative incompatibility during illegitimate diploid formation in D. discoideum (see 2.4.E (iii)). Mitotic recombination is only possible after nuclear fusion, and so any evidence for this proposed mechanism would indicate successful nuclear fusion and hence that vegetative incompatibility imposes a block after karyogamy. Circumstantial evidence for homozygosis at the mating-type locus via mitotic recombination in illegitimate diploid DU260, is discussed in section 2.4.E(iv).

(iii) Cell division of the diploid

(a) Cell cycle block

It is conceivable that diploids formed between haploids of opposite mating-type are in effect zygotes and can only divide meiotically. In the yeast, Saccharomyces cerevisiae haploids of opposite mating-type fuse to form a zygote which can then divide meiotically or mitotically depending upon the medium on which it is placed (Shilo et al., 1978; Gunge & Nakatomi, 1972). In view of this, a preliminary experiment, in which attempts were made to redirect zygotes of D. discoideum into a growth phase, was performed (see Results 2.3.J). No stable parasexual diploids were detected. However, yeast zygotes are quite simple structures when compared to the sexual structure of D. discoideum (i.e. the macrocyst) and are probably much more easily switched to a cycle of mitotic division. In cellular slime moulds the binucleate cytophagic cell (prezygote) is surrounded by peripheral cells very early in its development (Erdos, Nickerson & Raper, 1972; Filosa & Dengler, 1972) and even begins to phagocytose its neighbours before becoming a diploid (Wallace, 1977). It is therefore possible that, at the time of diploid formation, zygotes of D. discoideum are irreversibly locked into the sexual cycle and so further attempts to find appropriate conditions for switching zygotes into the vegetative mode were not pursued.

(b) Unstable diploids

It could be suggested that parasexual diploids, when formed between strains of opposite mating-type are so unstable that when placed in a growth environment they haploidise either mitotically or meiotically. However, some of the haploid segregants so formed would have a combination of parental chromosomes lacking in ts mutations, such that they could grow at the restrictive temperature. Therefore, had this occurred, tr

haploid recombinant segregants would undoubtedly have been isolated during the numerous attempts at illegitimate diploid formation (in all such experiments any tr colonies were checked for phenotypic characteristics such as spore size, shape and colour, diffusible brown pigment, colony morphology and, in some cases drug markers; all of these colonies were tr revertants of the parental haploids and were found at the expected reversion frequency for the ts mutations used).

Collectively, the evidence concerning the physiological cause of vegetative incompatibility between strains of opposite mating-type most favours a cell cycle block in which the diploid is locked into the sexual cycle and hence can divide only by meiosis.

2.4.G Attempts to overcome vegetative incompatibility and thus provide a system in which sexual and parasexual genetics can be used in crosses between the same strains

Two possible ways of overcoming vegetative incompatibility have been considered while discussing the search for the physiological cause of vegetative incompatibility. They are:

(i) Identity at the mating-type locus via a cross-over event

By becoming homozygous at the mating-type locus (and hence vegetative incompatibility locus) via a process of crossing-over the two strains become vegetatively compatible (see 2.4.F(ii)). The mechanism is of no immediate value, since the haploid segregants of diploids so formed would be all of the same mating-type and would still be vegetatively incompatible with strains of opposite mating-type.

(ii) Transfer of the zygote to a growth environment

Zygotes of S. cerevisiae can be redirected from meiosis to mitosis by transfer to a growth environment (Shilo et al., 1978; Gunge & Nakatomi, 1972). However, for reasons discussed in section 2.4.F(iii)a, it is considered unproductive to pursue this approach in D. discoideum.

Several other possible mechanisms for overcoming vegetative incompatibility in D. discoideum are:

(iii) Selection of a "mutator-type" mutation to control inter-conversion of mating-type alleles

One could select a "mutator-type" mutation of the kind shown to cause 'homothallism' in yeast, i.e. there are genes in S. cerevisiae, unlinked to the mating-type locus, which control the interconversion of **a** to α or α to **a** (mating-type alleles). They are detected by sporulation in a 'single' mating-type population (Hopper & Hall, 1975; Harashima et al., 1974; Blamire & Melnick, 1975). There is no evidence for such genes in D. discoideum, since in the numerous intra-mating-type tests performed, no macrocysts have been detected. Therefore, as in Neurospora (Griffiths & Delange, 1978), the mating-type alleles in D. discoideum appear to be very stable.

(iv) Deletion of one mating-type allele

Deletion of one mating-type allele is shown to remove vegetative incompatibility in bisexual heterokaryons of Neurospora (Delange & Griffiths, 1975). 'Escaped' homokaryons were not isolated in the study of Delange & Griffiths (1975) and the deletion was thought to be lethal.

Even if it were possible to obtain viable cells with a deleted mating-type locus in D. discoideum, these strains would be of little value for sexual genetics since the capacity to mate would be lost.

(v) Isolation of modifier genes to suppress vegetative incompatibility

One could isolate modifier genes which suppress vegetative incompatibility at the mating-type locus, such as 'tol' or 'tol'-like genes in Neurospora (Newmeyer, 1970; Metzenberg & Ahlgren, 1973) and CSP or ste mutations in yeast (Hopper & Hall, 1975; Mackay & Manney, 1974). The recessive mutation 'tol', which is unlinked to mating-type, suppresses vegetative incompatibility at the mating type locus without inhibiting the mating reaction in N. crassa (Newmeyer, 1970). Isolation of such a mutant in D. discoideum would be invaluable since any strain into which it was transplanted could undergo both the sexual and parasexual cycle with a strain of opposite mating-type. In N. crassa 'tol' was isolated in a haploid strain carrying a duplication which resulted in heterozygosity for mating-type (Newmeyer, 1970).

(vi) Selection of mutations at the mating-type locus itself

Mutations at the mating-type locus have been reported in many fungi. e.g. S. cerevisiae (MacKay & Manney, 1974; Kassir & Simchem, 1976), Schizosaccharomyces pombe (Meade & Gutz, 1976), Schizophyllum commune (Raper & Raper, 1973) and N. crassa (Griffiths & Delange, 1978).

The mutational approach is potentially of great value for isolating strains of D. discoideum that can be used for both sexual and parasexual genetic analysis. A mutational approach would require selection of either a mating mutant from a vegetative compatible strain (i.e. say a matA strain that functionally expresses mata in its mating

reaction), or a mutant rendered vegetatively compatible from a vegetatively incompatible strain (i.e. say a mata strain that functionally expresses a matA vegetative incompatibility reaction when crossed parasexually with another matA strain).

The first approach (i.e. a change to the mating reaction) has a theoretical problem. The mating-type alleles in D. discoideum are very stable (2.4.G(iii)) and therefore it is possible that the mating-type genes cannot be mutated in such a specific way even by induced mutation.

The second approach has been attempted successfully in N. crassa in which the mating-type alleles are also very stable (Griffiths & Delange, 1978). Normally (A + a) heterokaryons grow very poorly. However Griffiths and Delange (1978) selected vigorous (A + a) heterokaryons after mutagenising the 'a' parent. On analysis of these heterokaryons they demonstrated the loss of the 'a' vegetative incompatibility function. Griffiths & Delange (1978) argue that the (A + a) combination actively inhibits heterokaryon formation and that their approach therefore selects for loss of an active (inhibitory) function by mutation to an inactive state. Most of the mutants obtained by Griffiths & Delange (1978) were effectively asexual due to simultaneous loss of mating competence. However, in two out of 30 mutants the vegetative incompatibility was lost while the mating functions were retained.

An analogous mutational approach could be used in D. discoideum. It is hoped that, by mutating a mata2 strain, a mutant could be selected which is vegetatively compatible with a matA1 strain (i.e. can successfully make parasexual diploids with a matA1 tester strain) and yet retains its mata2 mating competence, thus producing a mutant that could be used both sexually and parasexually in crosses with the same strain.

In D. discoideum, the strain HU89 would be a suitable 'treatment' strain, since it is of mata2 mating-type and is already partially NC4 in its chromosomal make-up (see Results 2.3.C). The mating-type locus could be readily mapped using parasexual genetics even in strains which had lost their mating capacity. The remaining V12 chromosomes could then be easily exchanged with NC4 chromosomes in steps towards 'isogenicity'.

2.4.H Speculations on evolutionary advancement of incompatibility systems

Throughout a wide range of organisms it has been shown that cells can recognise the difference between 'self' and 'not self'. The highly evolved immune system of vertebrates, the relatively simple allogenic reaction in compound ascidians and self incompatibility in flowering plants serve as examples (Burnet, 1971; Klein, 1975; Oka & Watanabe, 1957; Oka, 1970).

Burnet (1971), in a review on evolution of immunity, states that "recognition must always be a basic phenomenon of immunity". In the compound ascidian Botryllus, fusion of colonies is controlled by a "single locus multiple allele" system (Oka, 1970). Oka (1957, 1970) has shown that colonies of Botryllus fuse successfully when they have at least one allele in common at that locus, but that fertilisation appears to be possible only between colonies which do not fuse. Burnet (1971) uses this information as an example of 'self' 'not self' recognition and speculates that the destructive response to vegetative interaction between two unlike colonies may represent the phenotypic expression of ancestral genes to the immune system. Oka (1970) and Burnet (1971) direct attention to the similarity of self-sterility in Botryllus and self-incompatibility in the flowering plants. In both of these systems there is a positive recognition system which triggers a

destructive reaction. Indeed, the plant kingdom offers many examples of recognition systems, but the literature will not be reviewed here, as it is beyond the scope of this thesis.

Another worker, Klein (1975), examines the immune response in the vertebrates and states that it is a polygenically controlled trait of which the major histocompatibility complex (MHC) is a part. Klein speculates that the MHC, which is a complex group of genes controlling allograft reactions and immune response in vertebrates, has evolved from an already complex gene by intrachromosomal duplication. Klein also suggests that the complex ancestral gene could have evolved from the invertebrate incompatibility systems, such as the mating system of the fungus Schizophyllum commune. Both the MHC and the mating system of S. commune provide a means of cell type recognition via a cell surface reaction and both systems are controlled by two closely linked highly polymorphic regions of the genome (Klein, 1975). The MHC, in controlling allograft reactions and immune response, triggers a destructive interaction when the organism recognises 'not self' interference. This is fundamentally different from the mating system of S. commune which requires a 'not self' recognition for a successful non-destructive interaction (i.e. interacting haploids of S. commune must have different sex factors for a successful mating to occur). While the mating system of S. commune was chosen as an example of a possible ancestral incompatibility system partly becasue of its complexity, there may be more value in looking at the vegetative incompatibility system of fungi in which 'unlike' alleles induce an active response (e.g. in N. crassa; Griffiths & Delange, 1978). N. crassa is a primitive Ascomycete and, as described previously in this chapter, has a one locus - 2 allele mating-type system, the alleles of which control vegetative fusion as well as sexual reproduction (see 2.4.D). In N. crassa a 'not self' recognition during

vegetative interaction between hyphae of opposite mating-type triggers a destructive reaction and is therefore analogous, although at a grossly simplified level, to the immune response in vertebrates. It is apparent that within the fungi alone the complexity of the mating system has greatly increased during evolution (e.g. the mating system of N. crassa vs. S. commune) and it is perhaps feasible that a complex incompatibility system such as that of the immune response in vertebrates could have evolved from the simple mating-type locus, via vegetative incompatibility of the lower eukaryotes.

A major drawback in comparing recognition systems of the fungi to those of animals is that, in contrast to animal cells, fungal cells are encased in a cell wall and the cell wall has been shown to have some control on fusion in several species of fungi (Dales & Croft, 1977; Sipiczki & Ferenczy, 1977a; 1977b; Gunge & Tamaru, 1978).

It is possible that the large numbers of het loci in the fungi (other than the mating-type locus) are in some way associated with the cell wall (Dales & Croft, 1977). D. discoideum, which has no cell wall, is more simple since it has only one vegetative incompatibility gene which is located at the mating-type locus. The complexity of the mating-type locus and the associated vegetative incompatibility in D. discoideum seems similar to that of N. crassa, but D. discoideum has the advantage that vegetative incompatibility at the mating-type locus can be studied without the interference of other vegetative incompatibility loci. Since D. discoideum is a naked amoeba it has the additional advantage of being more amenable to biochemical studies than the fungi. Hence D. discoideum is becoming an attractive system for studies on simple 'self' - 'not self' recognition.

APPENDIX A ISOLATION OF AUSTRALIAN CELLULAR SLIME MOULDS

1. INTRODUCTION

Cellular slime moulds have been isolated from forest soils and decaying forest litter in both temperate and tropical regions throughout the world, with the greatest diversity of species occurring in the tropical and subtropical zones (Cavender, 1973; Olive, 1975; Cavender, 1976a and b). The Dictyostelium mucoroides complex is the most widespread species. It has been isolated on all of the five continents surveyed (Cavender, 1973). It is the dominant species in Europe and East Africa (Cavender, 1969a and b) and shares dominance with other species such as Polysphondylium pallidum, Polysphondylium violaceum, Dictyostelium purpureum and sometimes Dictyostelium polycephalum and Dictyostelium minutum, in S.E. Asia, America and Canada (Cavender and Raper, 1965; Cavender & Raper, 1968; Cavender, 1972; Cavender, 1976b). D. discoideum is a rare species of cellular slime mould, having been isolated only on the American continent (Raper, 1935; Cavender & Raper, 1965; Cavender & Raper, 1968).

Australian cellular slime moulds have not been described previously. During attempts to isolate Australian D. discoideum (for reasons outlined in the General Introduction) a survey has been made of the dictyostelid microflora in soils of three areas of virgin Australian bush. The surveys were: (1) A seasonal survey of the Brindabella Range, A.C.T. (2) A comprehensive study of a transect in New England National Park, N.S.W. (3) An examination of some soil samples from Lizard Island, Queensland.

2. MATERIALS AND METHODS

2.A Materials

All media and chemicals are described in Appendix B.

2.B Method of isolating and storing cellular slime moulds

We used an adaptation of the method described by Cavender & Raper (1965a) for isolating cellular slime moulds from the wild. Soil samples were collected, with a spatula, from just below the leaf litter. Two samples were taken at each site; a 1 ml sample and a "spare" sample of about 5 ml. The samples were collected in sterile graduated 10 ml plastic tubes and lids were lightly secured. On returning to the laboratory, the soil samples were stored overnight at 4^{o}C. (The 'spare' samples were stored at 4^{o}C until required.) On the following day the 1 ml samples were diluted to a final volume of 2.0ml with S.S. They were whirlimixed vigorously until the soil was completely suspended and then 0.1 ml of each suspension was plated on to Hay agar together with 0.1 ml of a thick suspension of K. aerogenes. An Oxford sampler (100µl) was found to be the most effective implement for transferring the thick soil suspension. The plates were incubated at 21 ± 1^{o}C. Colonies of cellular slime moulds began to appear after 4 days. The larger dictyostelid slime moulds could be identified with the naked eye, but in order to identify smaller cellular slime moulds (e.g. D. minutum), the Hay agar plates were examined using a Leitz binocular microscope at 10x magnification. Olive (1975) was used as a guide to the classification of cellular slime moulds. Spores were taken from the mature fruiting bodies, using a sterile wooden toothpick and were deposited into a drop of sterile S.S. on an SM/5 agar plate, prespread with K. aerogenes. The spores were streaked across the plate using a sterile platinum loop. In this way the isolates were purified by clonal passage. Spores of the purified colony were dried onto silica gel for storage (1.2.B).

3. RESULTS

3.A A seasonal survey of the Brindabella Range, A.C.T.

Soil samples were taken from the same six sites at various times throughout the year. Each site differed in altitude, vegetational cover, soil type and aspect (Table 17). The seasonal distribution of dictyostelid slime moulds is shown in relation to these variables (Table 17). The sheltered more temperate sites (sites 5 and 8) support the highest annual slime mould population of the six sites tested. These sites have their highest slime mould population in late spring. The more exposed sites have their highest slime mould population in the autumn. However, the numbers of colonies scored per gram of soil are too low to make reliable correlations with any of the environmental variables. The D. mucoroides complex (probably including D. giganteum) was the most abundant and most frequently isolated species. It was isolated at all of the six sites. P. pallidum and D. minutum have been isolated at two of the sites although infrequently and P. violaceum was only isolated once at one site. All of these species were isolated on Hay agar and were purified by clonal passage on SM/5 agar for storage on silica gel (2.B above). Two other possible species of cellular slime mould were isolated in addition to the four above. One was similar in appearance to Dictyostelium lacteum. It was abundant at all sites and had very tiny fruiting bodies. However, since the spore heads were firmly attached to the stalk, it was difficult to collect spores on the end of a sterile toothpick without either touching the plate or without collecting the stalk as well. Repeated attempts to purify this isolate were hindered by fungal contamination. Although amoebae were present in the vicinity of the fruiting body the isolate could not, with certainty, be classified as a cellular slime mould. Therefore these data have been excluded from Table 17. The other isolate

TABLE 17

The distribution of dictyostelid slime moulds in the Brindabella Range, A.C.T.

Altitude (metres)	Site	Aspect	Soil	Understorey	Cover	Av. number of cellular slime moulds/gm. soil*				Species†			
						early spring	late spring	autumn	winter	early spring	late spring	autumn	winter
750	8	sheltered gully N.	washed out gravelly, twiggy	none	casuarinas	20	140	N.D.	20	a,d	a,c	-	a
900	5	very sheltered N.W.	moist and mossy	wattles, small gums, tussock grass, fallen trees	tall gums (~ 100ft.)	60	180	20	10	a,c	a	a	a
1200	2	steep well protected S.	rocky moist lots charcoal	relatively open, wattles tussock grass, straggly bushes	tall straight gums	0	0	20	80	-	-	a	a
1200	1	exposed, steep N.E.	moist, small stones roots of grass	open, sparse bracken, tussock grass	snow gums	10	0	20	0	a	-	b	-
1450	3	exposed, flat windy S.E.	moist, humus, small rocks.	short bushy shrubs, lots dry dead timber	straggly snowgums	20	20	40	10	b	a	a	a
1450	4	moderately protected S.W.	moist, lots small stones	straggly shrubs lots tussock grass	relatively dense snow-gums	0	0	40	0	-	a	a	-

The soil samples were treated as described in the MATERIALS AND METHODS (2.B).

* The average number of cellular slime moulds /gm. soil = the average number of colonies/0.1 ml soil suspension x 20.

† a = *D. mucoroides* complex; b = *P. pallidum*; c = *D. minutum*; d = *P. violaceum*.

had similar compound fruiting structures to D. polycephalum but since some isolates of D. mucoroides occasionally fruit with a similar clumping pattern, the isolate in question was not classified as D. polycephalum in Table 17. D. discoideum was not detected at any of the sites sampled.

3.B A comprehensive survey of a transect in the New England National Park, N.S.W.

The Brindabella Range is largely subalpine and varies from harsh exposed country with sparse straggly snow gums to more sheltered temperate sclerophyll forest. Eucalypt is the major hardwood throughout. Since D. discoideum was not detected in the Brindabellas, we decided to survey a more temperate area. New England National Park was chosen because it has a large range in altitude, with a correspondingly large range in vegetation from subtropical to subalpine. Furthermore, the forests were largely virgin bush and included stands of Antartic beech which offered a different leaf litter and had a different type of understorey to that of the eucalypt. A transect was taken in which the vegetation ranged from subtropical to temperate rain forest. Soil samples were collected from the forest floor on either side of a fire trail (but well away from the trail) at intervals of about 200 metres. The results of the survey have been tabulated to show the distribution of dictyostelid slime moulds in relation to altitude (Table 18). The vegetation varied with altitude. The highest soil samples were taken from the floor of beech forest (1100-1300 metres). Even though the soils were very moist and the leaf litter thick, the cellular slime mould population was surprisingly small. From about 700-1100 metres the vegetation could be classified as dry sclerophyll forest. Although the understorey varied the major hardwood was always eucalypt. Most of the cellular slime moulds isolated during this survey were isolated in this

TABLE 18

The distribution of dictyostelid slime moulds of New England National Park, N.S.W. †

Range in altitude (metres)	Type of vegetation	Major hardwood	Number of sites samples	Number of species detected	Ave. number of cellular slime moulds/ gm. soil*
500 - 700	subtropical	eucalypt	10	3	8
700 - 900	dry sclerophyll	eucalypt	13	4	27
900 - 1100			9	4	32
1100 - 1300	temperate rainforest	Antarctic beech	18	1	7

The soil samples were treated as described in the MATERIALS AND METHODS : 2.B. Three 1 ml samples were taken from each site and these were examined on returning to the laboratory (3 to 5 days after collection). The total number of colonies detected were: 71 D. mucoroides, 25 P. pallidum, 12 P. violaceum, 2 D. purpureum and 1 D. minutum. An examination of the 'spare' soil samples, 9 days later, gave similar results to those tabulated above and the numbers of colonies detected in this second experiment were: 55 D. mucoroides, 19 P. pallidum, 2 P. violaceum, 1 D. purpureum and 1 D. minutum.

* The average number of cellular slime moulds/gm soil = number of colonies per site per hay agar plate x 20.

† This survey was conducted in early spring.

section. The transect ended at the base of the valley. The creek bed at the base of the valley was surrounded by subtropical rainforest (~ 500-700 metres). The major hardwood was still eucalypt, but the understorey was thick and included more ferns, bracken and mosses than did the higher altitudes. These samples were taken mainly from the banks of the small creek, but the soil was quite dry.

Overall the density of cellular slime moulds appeared to be unrelated to soil moisture. The moist soil of the beech forest and the dry soil of the creek bed both contained a low density of slime moulds. There were also moist as well as dry samples containing a high density of slime moulds. Some samples yielded particularly high numbers of dictyostelid slime moulds but the corresponding sample sites had no obvious environmental variables in common.

D. mucoroides was the most common species of cellular slime mould found in New England National Park. It was found at 50% of the sites sampled. P. pallidum was also quite common. It was found at 20% of the sites samples. The species P. violaceum, D. purpureum and D. minutum were less common. They were found at 10%, 4% and 2% of the sites respectively.

3.C An examination of cellular slime moulds from several sites on Lizard Island, Queensland

Dr Adrian Gibbs collected soil from several sites of different vegetation on Lizard Island, an island situated off the north east coast of Queensland. This provides quite a different habitat to those already studied in Australia. The survey was based on very few samples. However the distribution of cellular slime moulds (Table 19 legend) was similar to that found elsewhere in Australia.

TABLE 19

The distribution of dictyostelid slime moulds on Lizard Island, QLD

Type of vegetation	Number of sites sampled	Av. number of cellular slime moulds/gm.soil	Species isolated*
rainforest	4	35	a,e
dry sclerophyll scrub	3	7	c
pandoras	3	-	
wet mangrove	1	-	
under the mangrove	6	10	b

The soil samples which were collected by Dr. Adrian Gibbs, were treated as described in the MATERIALS AND METHODS (2.B).

* In this survey the total number of colonies detected was:

6 *D. mucoroides* complex (a); 3 *P. pallidum* (b);

1 *D. minutum* (c); 1 *D. purpureum* (e).

3.D Strains with interesting features

Several of the isolates had features of particular interest. These features were unrelated to the work described in this thesis and so will not be discussed in detail here. The strains are briefly described in Table 20.

4. DISCUSSION

The surveys described in this Appendix were primarily initiated in a search for D. discoideum. Therefore the emphasis has been on examining the common dictyostelid slime moulds of a large number of samples rather than on examining the complete microflora of any particular area. The data collected have shown that all of the major dictyostelid species exist in Australia, although at lower density than elsewhere in the world. Much of the arable land in Australia has been used for agriculture and therefore the remaining virgin bushland is apt to be marginal. This is perhaps why the three areas of virgin bushland surveyed support a low density of cellular slime moulds.

The aim of isolating D. discoideum was to obtain two strains of opposite mating-type from the same soil sample. It was supposed that such strains would be virtually isogenic and that macrocysts formed between them could therefore germinate more readily (see General Introduction). Wallace (1977) has in fact used this approach with American wild isolates of D. discoideum. However, he showed that macrocysts so formed did not germinate more readily.

Since D. discoideum was not isolated at any of the sites surveyed and in view of the recent findings of Wallace (1977), the search for Australian D. discoideum was discontinued.

TABLE 20

Strains with interesting features

Strain name	Species	Isolation area	Date of isolation	Feature of particular interest
ANU11 ANU12 ANU13	D. mucoroides	Brindabellas	May, 1976	Large aggregation territories on H_2O agar but normal small territories on LPS agar
ANU4	D. mucoroides	Brindabellas	April 1976	Temperature sensitive for growth (ts) restrictive temperature = $26.8 \pm 0.3^{\circ}C$
ANU60	P. violaceum	N.E.N.P.*	October,1977	Irregular branching; doesn't always branch; has a curly stalk and possibly a basal disc(?).
ANU84 ANU85 ANU86	P. pallidum	N.E.N.P.	October,1977	Haploids with round spores instead of the usual wild type elliptical shape.
ANU110 ANU120	D. mucoroides	N.E.N.P.	October,1977	Stalks branch more than is usual for D. mucoroides
ANU95	D. mucoroides	N.E.N.P.	October,1977	Very pale yellow spore heads, curly stalk and possibly a basal disc (doesn't look like D.discoideum)
ANU135	D. mucoroides	N.E.N.P.	October,1977	Very short species, possibly a tall D. minutum.
ANU143	unknown species	N.E.N.P.	October,1977	Brown pigmented species of unknown type - possibly a crampon base.
ANU97 ANU103 ANU118 ANU121	P. pallidum	N.E.N.P.	October,1977	Large spores - possibly diploid

* N.E.N.P. - New England National Park

APPENDIX B STRAINS, MEDIA AND CHEMICALS

1. STRAINS

The strains of D. discoideum used in this thesis are described in Tables 21, 22, 23, 24, 25, 26 and 27.

Haploid segregants of DU260 have been classified into phenotypic groups. The morphological features of representative strains from these phenotypic groups are shown in Fig. 10.

Evidence is presented in Fig. 11 to show that the spore shape marker, sprA, is homozygous in DU260.

1.A Nomenclature

Genetic nomenclature in D. discoideum follows the system of Demerec et al., (1966) Genetics 54, 61. In this system each mutation is described by a three letter gene symbol, a capital letter locus code and an isolation number. To be consistent with this system the mating-type locus, formerly called "mating type" (Erdos, et al., (1973b), has been redesignated mat. The respective mating-types are called matA and mata instead of mating type A_1 and mating type A_2. Strain NC4 has been given isolation number 1 (∴ matA1 instead of mating type A_1) and V12 isolation number 2 (∴ mata2 instead of mating type A_2). Other wild isolates are given different isolation numbers to distinguish them from the NC4 and V12 alleles; e.g. WS472 is matA353 (Table 27).

TABLE 21

Phenotype and genotype of haploid segregants from DU260

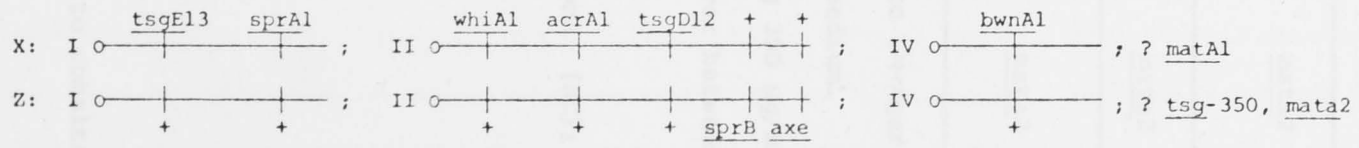
Strain*	Phenotypic Group	Phenotype†									Genotype‡			
		Sp.Col.,	Pig.,	G.Res.,	Meth.,	Sp.Sh.,	Fb.H.,	Ab.,	G.Ra.,	Ax.	Parental Linkage Groups I	II	IV	Mating-ty mat
HU97 HU167 HU169 HU173	A	W	B	NG	R	RD	T	WI	F	NG	X	X	X	matA1
HU107 HU171	B	Y	B	NG	R	RD	T	WI	F	NG	X	X + Z	X	matA1
HU104 HU168 HU170 HU172 HU177	C	W	NB	NG	R	RD	T	WI	MS	NG	X	X	Z	matA1
HU101 HU174 HU175 HU176 HU178	D	W	B	NG	R	RD/TE	MT	N	SL	NG	X	X	X	matA1
HU193 HU195	E	Y	B	NG	S	TE/RD	MT	WI	F	NG	X	Z	X	matA1
HU197	F	Y	B	NG	S	RD/TE	T	N	MS	NG	X	Z	X	matA1
HU196 HU202	G	Y	B	NG	S	TE/RD	S	N	S	NG	X	Z	X	matA1
HU192 HU199	H	Y	B	NG	S	TE/RD	S	WI	MS	NG	X	Z	X	matA1

* Haploid segregants of phenotypic groups A, B, C and D were derived from DU260 by plating the diploid on methanol 2% - SM-agar. Haploid segregants of phenotypic groups E, F, G and H were derived from DU260 by suspending the diploid in axenic medium.

† The symbols used to describe the phenotypes are: fruiting body height (Fb.H.); width of aggregation band (Ab.); growth rate of colony measured as diameter of colony on nutrient agar plate (G.Ra.); spore colour (Sp.Col.); fruiting body pigment (Pig.) growth at restrictive temperature 27°C (G.Res.); methanol (Meth); spore shape (Sp.Sh.); axenic medium (Ax.); sensitive (S); resistant (R); yellow (Y); white (W); brown (B); not brown (NB); growth (G); no growth (NG); round (RD); thin elliptic (TE); round plus elliptical (RD/TE) or (TE/RD) with predominant spore shape as numerator; tall (T); moderately tall (MT); short (S); wide (WI); narrow (N); corona (C); slow (SL); moderately slow (MS); fast (F).

‡ I, II and IV refer to linkage groups one, two and four respectively.

Symbols X and Z represent the linkage groups from parental strains HU1 (matA1) and NP158 (mata2) respectively.

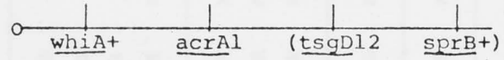


The symbols used to describe the mutant loci of X and Y are: temperature-sensitive for growth at the restrictive temperature, 26.8±0.3°C (tsgE & tsgD); round spore shape (sprA); thin elliptical spore shape (sprB); white spore head (whiA); resistance to methanol (acrA); brown pigment (bwnA).

TABLE 22

Phenotype and genotype of haploid segregants from DP72

Strain*	Phenotypic Group	Phenotype†										Genotype‡ Parental Linkage Groups		
		Sp. Col.,	Pig.,	G. Res.,	Meth.,	Sp. Sh.,	Fb. H.,	Ab.,	G. Ra.,	Co.,	Ax.,	II	VII	Mating-type
X37										S	NG	X	Z	mata2
X49	A	W	NB	NG	R	E	MS	A	S	S	G	X	Z	
X39	B	W	NB	NG	R	E	T	C	F	R	NG	X	X	mata2
X42		Y	NB	NG	R	E	T	NC	F	ND	ND	(X+Z)	N.D.	mata2
X44	C	Y	NB	NG	R	E	T	C	F	R	NG	(X+Z)	X	

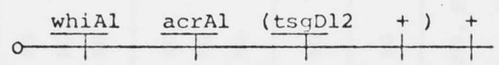
* Haploid segregants X37, X39 and X42 were derived from DP72 by plating approximately 2 x 10^4 diploid amoebae on to SM-agar containing 2% methanol (v/v). Haploid segregant X49 was derived from DP72 by suspending diploid ameobae in axenic medium. Haploid segregant X44 was derived from DP72 by plating approximately 2 x 10^4 diploid amoebae on to SM-agar containing 350 µg/ml cobaltous chloride. Both X42 and X44 were originally kept because they are recombinant for l.g.II, showing a cross-over between whiA and acrA i.e. 

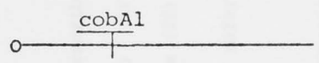
.

† The symbols used to describe the phenotypes have been defined in Tables 21 & 23, except for corona (C); no corona (NC); absent (A) ; not done (N.D.).

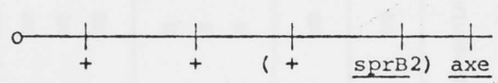
‡ II and VII refer to linkage groups two and seven respectively.

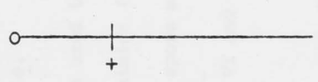
Symbols X and Z represent the linkage groups from parental strains X23 (matA1) and NP84 (mata2) respectively.

X : II 

; VII 

;? matA1

Z : II 

; VII 

;? mata2, tsg-350

The symbols used to describe the mutant loci of X and Z have been defined in Table 21, except cobA1 (resistance to cobaltous chloride).

TABLE 23

Phenotype and genotype of haploid segregants from DU454

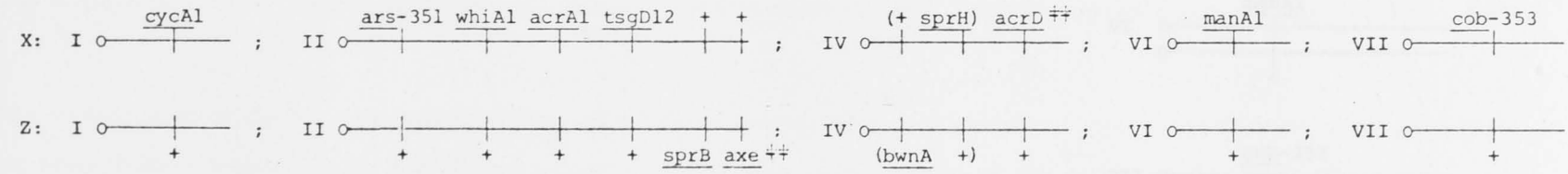
Strain*	Phenotype†											Genotype‡					
												Parental Linkage Groups					Mating-type
	Sp.Col.,	Pig.,	G.Res.,	Meth.,	Co.,	Cy.,	Ar.**,	Fb.H.,	Ab.,	Sp.Sh.,	Ma.	I	II	IV	VI	VII	
HU299	W	NB	NG	S††	R	R	S	T	WI	E^{xx}	A						
HU301	W	NB	NG	R/S††	R	R	S	T	WI	R	A						
HU315	W	NB	NG	S††	R	R	S	T	WI	E^{xx}	A	X	X	X	X	X	matA1
HU320	W	NB	NG	S††	R	R	S	MS	WI	E^{xx}	A						
HU300	W	B	NG	R/S††	R	R	R	T	WI	E	A						
HU305	W	B	NG	S††	R	R	R	MT	WI	E	A						
HU314	W	B	NG	S††	R	R	R	MT	WI	E	A	X	X	Z	X	X	matA1
HU321	W	B	NG	S††	R	R	R	MS	WI	E	A						
HU308	W	NB	NG	R	S	R	S	T	WI	R	A						
HU312	W	NB	NG	R	S	R	S	T	WI	R	A						
HU316	W	NB	NG	R	S	R	S	T	WI	R	A	X	X	X	X	Z	matA1
HU318	W	NB	NG	R	S	R	S	MT	WI	R	A						
HU311	W	B	NG	R	S	R	R	MS	WI	E	A						
HU313	W	B	NG	R	S	R	R	MS	WI	E	A	X	X	Z	X	Z	matA1
HU319	W	B	NG	R	S	R	R	MS	WI	E	A						
HU317	Y	NB	NG	R	S	R	S	MT	WI	R	A	X	X+Z	X	X	Z	matA1
HU329	Y	NB	NG	S	R	R	S	MT	WI	TE	A	X	Z	X	X	X	matA1

* Haploid segregants HU299, HU320 and HU321, were selected from DU454 by plating approximately 3 x 10^4 diploid amoebae on to SM-agar containing 350 µg/ml cobaltous chloride. Haploid segregants HU305, HU308, HU316, HU317, HU318, HU319 and HU329 were selected from DU454 by plating approximately 3 x 10^4 diploid amoebae on to SM-agar containing 500 µg/ml cycloheximide. Haploid segregants HU300, HU301 and HU311 were selected from DU454 by plating approximately 2 x 10^4 diploid amoebae on to SM-agar containing 20 µg/ml ben late. Haploid segregants HU312, HU313, HU314 and HU315 were derived from DU454 by plating approximately 40 diploid amoebae on to SM-agar containing 20 µg/ml ben late.

† The symbols used to describe the phenotypes have been defined in Table 21 except for additional phenotypes which are: cobalt (Co); cycloheximide (Cy); arsenate (Ar); partial resistance (R/S); moderately tall (MT); α-mannosidase (Ma); absent (A); thin elliptical spore shape (TE).

‡ I, II, IV, VI and VII refer to linkage groups one, two, four, six and seven respectively; linkage group III was not scored and V was unmarked.

Symbols X and Z represent the inherited linkage groups from parental strains HU227 (matA1) and HU89 (mata2) respectively.



The symbols used to describe the mutant loci of X and Z are: resistance to cycloheximide (cycA); resistance to arsenate (ars) white spore head (whiA); resistance to methanol (acrA); thin elliptical spore shape (sprB); round spore shape (sprH); resistance to acriflavin (acrD); brown pigment (bwnA); α-mannosidase positive (manA); dominant resistance to cobalt (cob-353).

xx the spore shape is elliptical despite the presence of sprH, due to an unmapped spore shape suppressor mutation.

†† methanol sensitivity here results from the presence of an unmapped acrA suppressor.

‡‡ these markers (plus tsg-350) were not scored.

** Anomalous results were obtained for ars due to the presence of ars-351 suppressor mutation(s).

TABLE 24

Phenotype and genotype of illegitimate diploids

Diploid	Parents	Phenotype*												Genotype†	
		Sp.Col.,	Pig.,	G.Res.,	Sp.Sh.,	Fb.H.,	Ab.,	Meth.,	Co.,	Cy.,	Ar.,	Ax.,	G.Ra.	Mapped markers	unmapped markers
DP72	X23 x NP84 (matA1) (mata2)	Y	NB	G	E	T	C	S	S	ND	ND	NG	F	II whiA1 acrA1 (tsgD12 +) / + + (+ sprB2)	tsg-350
														VII cobA1 / +	mata2
DU260	HU1 x NP158 (matA1) (mata2)	Y	NB	G	RD	T	N	S	ND	ND	ND	NG	MF	I tsgE13 sprA1 / tsgE? sprA1	
														II whiA1 acrA1 (tsgD12 +) / + + (+ sprB2)	tsg-350
															mata2
														IV bwnA1 / +	
DU454	HU227 x HU89 (matA1) (mata2)	Y	NB	G	E	MS	WI	S	S	S	S	N.D.	MF	I cycA1 / +	
														II ars-351 whiA1 acrA1 (tsgD12 +) / + + + (+ sprB2)	
														III tsgA1 / +	
														IV (+ sprH351) acrD369 / (bwnA1 +) +	matA1
														VI manA1 / +	
														VII cob-353 / +	

* The symbols used to describe the phenotypes have been defined in Table 21 & 23.

† I, II, III, IV, VI and VII refer to linkage groups one, two, three, four, six and seven respectively.

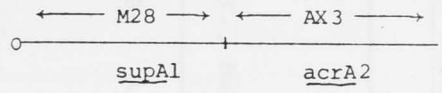
The symbols used to describe the mutant loci have been defined in Tables 21 & 23.

TABLE 25

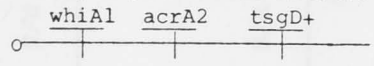
Basic haploid strains

Strain	Parent or Source*	Genotype†													
		whi	bwn	tsg	cyc	acr	cob	spr	ebr	mat	man	axe	ars	ben	bsg
NC4	(1)	+	+	+	+	+	+	+	+	A1	+	+	+	+	+
V12	(2)	+	+	+	+	+	+	B2	+	a2	+	+	+	+	+
X2	(3)	+	A1	A1	+	+	+	+	+	A1	+	A1,B1	+	+	+
X9	(3)	A1	A1	D12	A1	A1	+	+	+	A1	+	+	+	+	+
X23	(4)	A1	+	D12	+	A1	A1	+	+	A1	+	+(B1?)	+	+	+
XM1	(5)	A1	+	D12	A1	(A1,D369)	+	H351	+	A1	A1	+	+	+	+
TS12	(6)	A1	+	D12	A1	+	+	+	+	A1	+	+	+	+	+
NP7	(7)	+	+	C7	+	+	+	+	+	A1	+	(A1,B1?)	+	+	+
NP12	(3)	+	+	B3	+	A2	+	+	+	A1	+	A1,B1	+	+	+
NP84	(4)	+	+	-350	+	+	+	B2	+	a2	+	-350	+	+	+
NP158	NP84	+	+	-350	+	+	+	B2	-350	a2	+	-350	+	+	+
HM3	(8)	-900	+	-901	-900	+	+	+?	+	a2	+	+	+	+	+
M28	(6)	+	A1	E13	+	+	+	A1	+	A1	+	+	+	+	+
HPS83	(9)	+	+	A1	A1	D369	+	H351	+	A1	A1	A1,B1	+	+	+
HU1	DP1	A1	A1	D12,E13	+	A351	+	A1	+	A1	+	+	+	+	+
HU32	(10)	+	A1	A1	+	+	-354	+	A1	A1	+	A1,B1	+	+	+
HU77	DU220	A1	+	B3	A1	A2	+	+	+	A1	+	(?)	+	+	+
HU89	(11)	+	A1	-350	+	+	+	B2	+	a2	+	+	+	+	+
HU128	(12)	+	A1	A1	A1	A352	+	+	+	A1	+	B1	+	+	+
HU156	HU89	+	A1	-350	+	+	-364	B2	+	a2	+	+	+	+	+
HU180	HU128	+	A1	A1	A1	A352	+	+	+	A1	+	B1	-355	+	+
HU184	HU156	+	A1	-350	+	-356	-364	B2	+	a2	+	+	+	+	+
HU208	DU383	+	A1	C7	+	A352	+	+	+	A1	+	B1(?)	-355	+	+
HU214	DU348	A1	+	D12,E13	+	A1	-353	A1	+	A1	+	+	-351	+	+
HU232	DU431	+	+	E13,H	+	+	-353	A1	+	A1	+	A1 B1(?)	-351	-351	A5

* (1) Raper, 1935; (2) Erdos et al., 1973b; (3) Williams et al., 1974a; (4) Mosses et al., 1975; (5) Free, Schimke & Loomis, 1976; (6) Katz & Sussman, 1972; (7) Kessin et al., 1974; (8) V12-M2, received from R. Kay; (9) Welker & Deering, 1978; (10) Williams, 1978; (11) HU89 is a haploid segregant obtained from a cross between NP158 & HU1 (2.3.C); (12) Williams & Barrand, 1978. The important feature of this strain is that it has a recombined linkage group II i.e.



Parent strains : DP1 = M28 x TS12 (This lab); DU220, diploid with mitotic crossover on L.G. II obtained by plating DP32 = NP12 x TS12 on meth 2% - SM-agar ∴ L.G. II of HU77 =



N.B. tsgD has been crossed out; DU348 = HU154 (Williams & Barrand, 1978) x X55 (Ratner & Newell, 1978); DU383 = HU180 x NP7; DU431 = M1-46 (Free, Schimke & Loomis, 1976) x HU214.

† The symbols used to describe the genotype are: whiA: white spore; whi+: yellow spore; bwnA: formation of brown pigment; bwn+: no brown pigment formed; tsg-: temperature sensitive for growth; tsg+: growth at the restrictive temperature; cycA: growth in the presence of cycloheximide (500 µg/ml); acr: growth in the presence of acriflavin (100 µg/ml) or methanol 2% (acrA only); cob: growth in the presence of cobaltous chloride (350 µg/ml); sprB: thin elliptical spore shape; sprH: round spore shape; sprA: round spore shape; ebr: growth in presence of ethidium bromide (35 µg/ml); mat: mating-type; manA: absence of α-mannosidase; axe: growth in axenic medium; ars: growth in the presence of arsenate (1.5 mg/ml); ben: growth in the presence of ben late (600 µg/ml); bsg: unable to grow on Bacillus subtilis.

In this table + denotes wild-type and the different letters denote different mutant loci. Where a mutation hasn't been mapped to a particular locus - precedes the isolation number.

TABLE 26

Phenotype and genotype of well marked strains

Strain‡	Parent*	Phenotype†												Genotype Linkage Groups						
		Sp.Col.,	Pig.,	G.Res.,	Sp.Sh.,	Fb.H.,	Ab.,	Meth.,	Co.,	Cy.,	Ar.,	Ma.,	Be.,	I	II	III	IV	V	VI	VII
HU225	DU403	W	NB	NG	E	MS	WI	R/S	R	R	R	A	R/S	cycA1	whiA1 acrA1 tsgD12 ars-351	tsgA1	-	-	manA1	cob-353
HU226	DU405	W	NB	NG	R	MS	WI	R	R	S	R	A	R	tsgE13 ben-351 sprA1	whiA1 acrA1 tsgD12 ars-351	tsgA1	-	-	manA1	cob-353
HU227	DU405	W	NB	NG	R	MT	WI	R/S	R	R	R/S	A	R/S	cycA1	whiA1 acrA1 tsgD12 ars-351	tsgA1	sprH 351	-	manA1	cob-353
HU234	DU428	W	NB	NG	R	MT	WI	R	R	R	S	A	R/S	cycA1	whiA1 acrA1 tsgD12	tsgA1	sprH 351	-	manA1	cob-353
HU235	DU429	W	NB	NG	R	MT	WI	R	R	R	R/S	A	R/S	cycA1	whiA1 acrA1 tsgD12 ars-351	tsgA1	sprH 351	-	manA1	cob-353

* DU403 and DU405 = HU214 (Table 25) x HPS83 (Table 25); DU428 and DU429 were selected as mitotic cross-over diploids for linkage group I from DU403 on SM-agar containing 500 µg/ml cycloheximide.

† The symbols used to describe phenotype have been defined in Tables 21 and 23, except for growth on 600 µg/ml ben late (Be).

‡ All strains constructed in this laboratory.

TABLE 27

Phenotype and mating-type of American wild isolates of *D. discoideum*

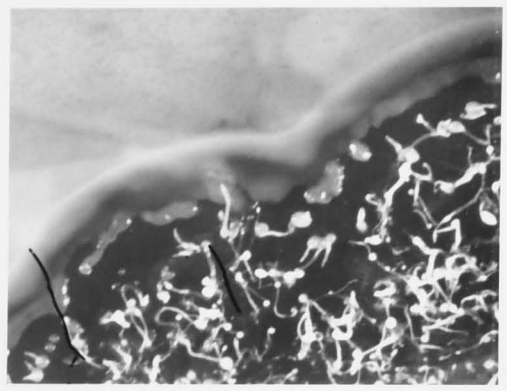
Strain*	Phenotype†				Mating-type‡
	Sp. Col.,	Sp. Sh.,	Fb. H.	Ab	
WS472	PY	HE	T	C	*matA* 353
WS195-6	PY	HE	T	C	*matA* 354
WS205-4	PY	HE	T	C	*matA* 355
WS583	PY	HE	T	C	*matA* 352
WS5-1	PY	HE	T	C	*matA* 356
WS10	PY	DE	T	WI	*matA* 373
WS585	PY	DE	MT	C	*matA* 374
WS655	PY	HE	MT	WI	*matA* 357
WS7	PY	HE	T	C	*mata* 358
WS582	PY	HE	T	C	*mata* 351
WS656	PY	HE/o	T	C	*mata* 359
AC4	PY	HE/o	MS	WI	*matA* 360/*mata* 361
Za-3a	PY	HE	T	ND	*matA* 362/*mata* 363
WS57-6	Y	HE	T	ND	*mat-* 364
WS269a	Y	HE	T	ND	*mat-* 365
WS380b	Y	HE	T	ND	*matA* 366
WS584	DY	HE	S	WI	*mat-* 367
WS526	DY	HE	MS	ND	*mat-* 368
WS216-2	Y	HE	T	ND	*matA* 369/*mata* 370
WS112b	Y	HE	T	C	*matA* 371/*mata* 372
HU182	PY	HE	MT	C	*matA* 374
HU188	PY	HE	T	WI	*matA* 373

These American wild isolates were received from Professor K.B. Raper. They were derived independently of each other.

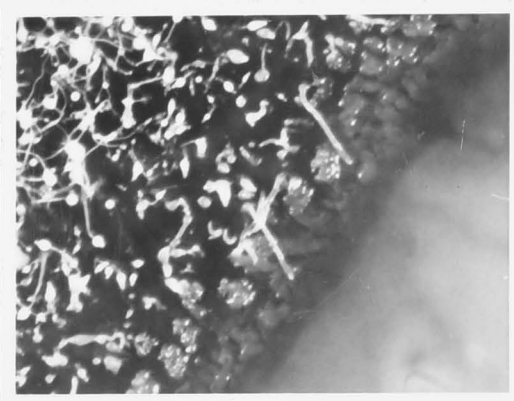
The symbols used to describe the phenotypes are: spore colour (Sp. Col.); spore shape (Sp. Sh.); fruiting body height (Fb. H.); aggregation band (Ab); pale yellow (PY); yellow (Y); dark yellow (DY); haploid elliptical (HE); diploid elliptical (DE); haploid elliptical & slightly oval (HE/o); tall (T); moderately tall (MT); moderately short (MS); short (S); corona (C); wide but no corona (WI); not determined (ND).

‡ Each of the wild isolate strains were tested for mating-type and given individual isolation numbers to indicate their independence. The homothallic and bisexual strains have been assigned both mating-type alleles (each with separate isolation numbers), even though this postulate has not been proved. Asexual strains have been given an isolation number only.

Parental Strains

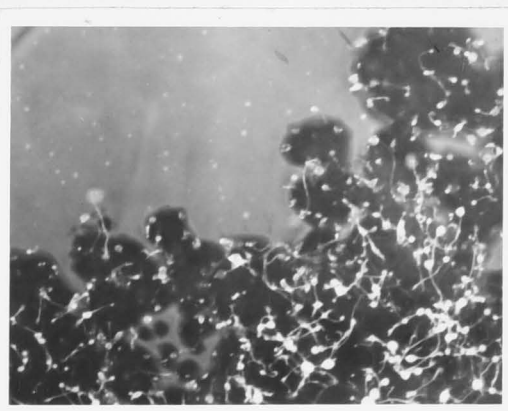


HU1



NP158

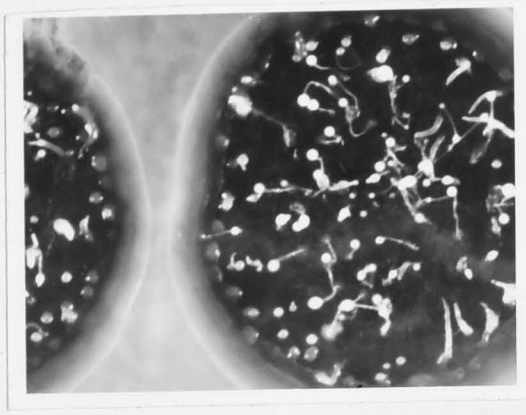
Diploid



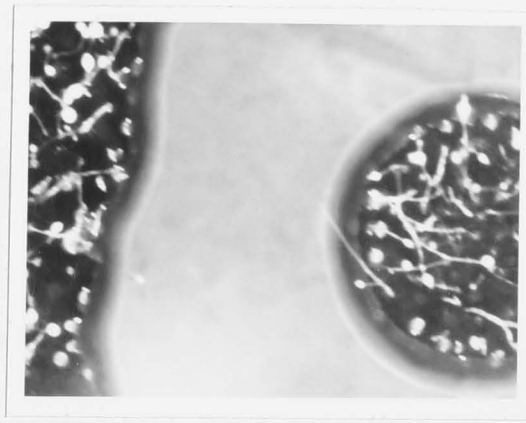
DU260

Fig. 10 Morphological features of diploid DU260 and its parental strains, HU1 and NP158. Representative haploid segregants of DU260 are shown over the page. The phenotypic group of each haploid segregant is shown in parenthesis. Full details of the phenotype and genotype of the segregants are given in Table 21. The colonies were photographed as described in 2.2.G. (Mag. x2).

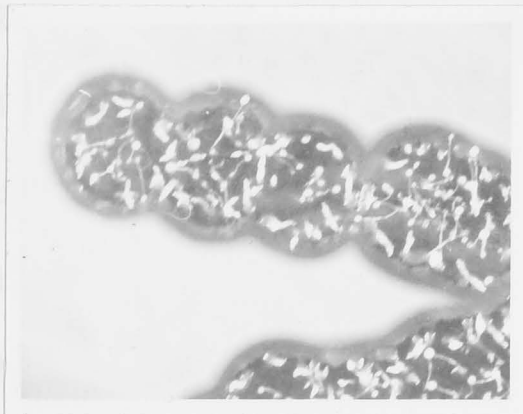
Haploid Segregants



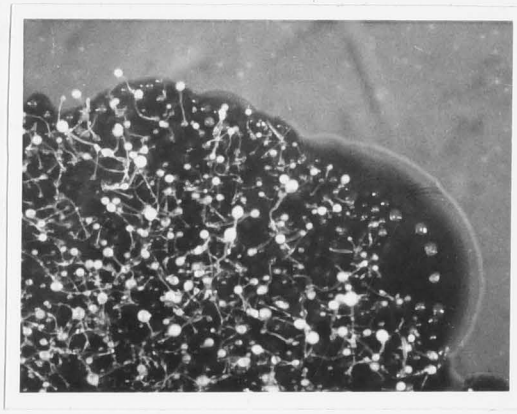
HU167(A)



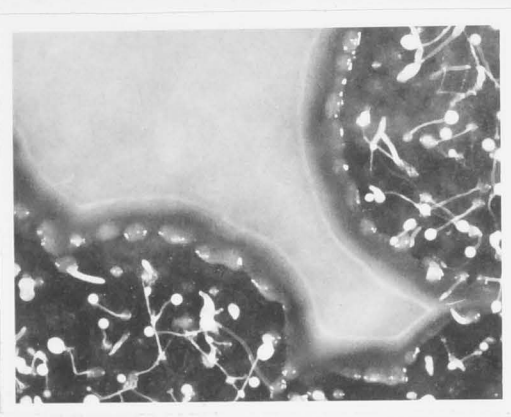
HU107(B)



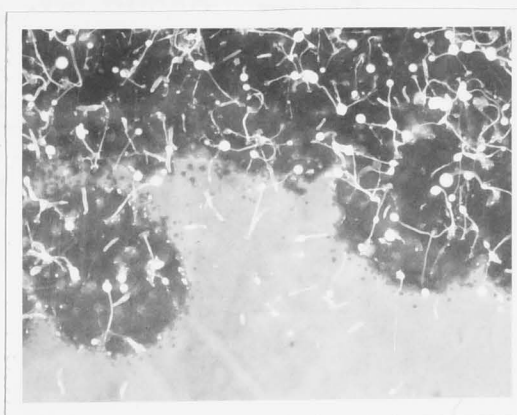
HU104(C)



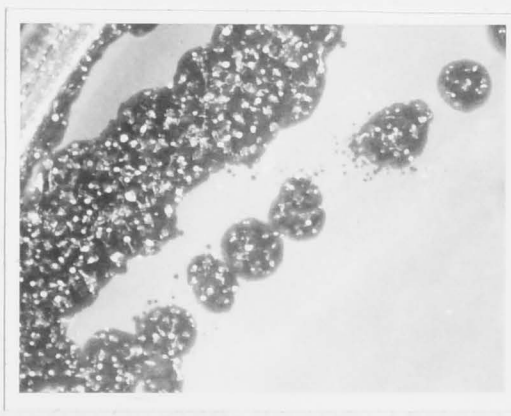
HU178(D)



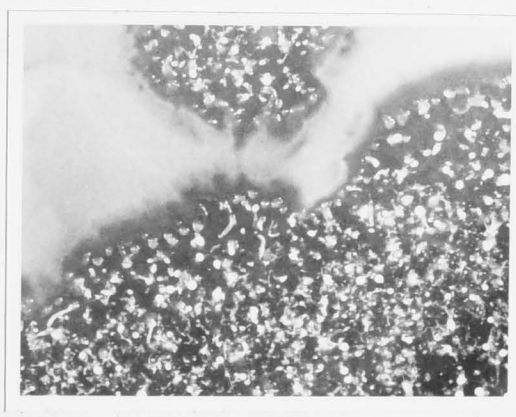
HU195(E)



HU197(F)



HU196(G)



HU199(H)

Fig. 11 Evidence to show that sprA is homozygous in diploid DU260

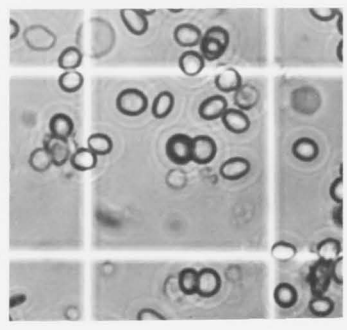
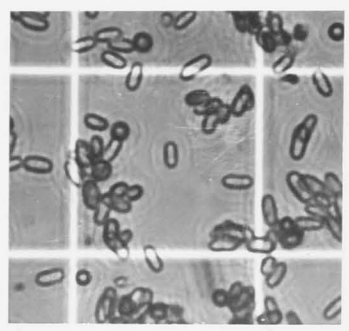
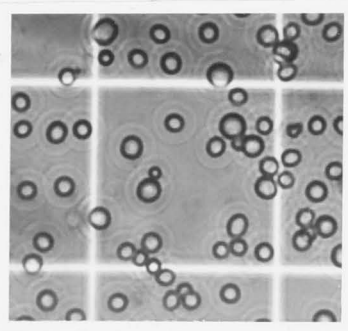
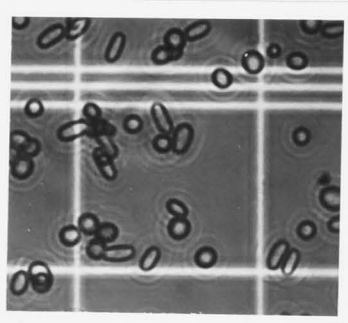
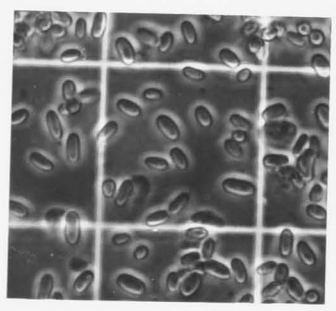
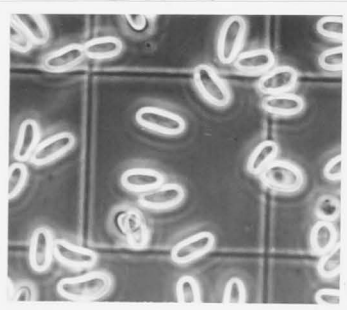
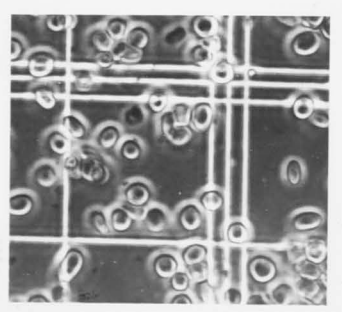
Haploids HU1 and NP158 are the parental haploids of diploid DU260. Haploid HU101 is a haploid segregant of DU260 and was selected on SM-agar containing 2% methanol (∴ sprB+). Haploid HU197 is a haploid segregant of DU260 and was selected in axenic medium. It is yellow and methanol sensitive and therefore is expected to carry sprB. The spore shape of HU197 is a mixture of round spores and elliptical spores. Further crosses have proved that HU197 contains both sprA and sprB. Diploid DU531 was constructed by crossing HU197 x HU77. Strain HU77 is a haploid containing neither sprA nor sprB. DU260 is heterozygous at sprB since sprB haploids (e.g. HU197) and sprB+ haploids (e.g. HU101) can be segregated. All DU260 haploid segregants contain sprA.

It is evident from DU531 that neither sprA nor sprB is fully expressed when in the same strain. However, in DU260, round spore shape (sprA) is fully expressed. This finding, together with the fact that all haploid segregants of DU260 contain sprA, indicates that, in DU260, sprA is homozygous.

* Spores on a haemacytometer slide were photographed as described in 2.2.G. The bar represents 10μ. ⌙

† The symbols used to describe spore phenotype are: round spore (R); thin elliptical spore (TE); mixture of round and thin elliptical spores (R/TE); elliptical-oval spores (E/O); elliptical spores (E).

‡ I and II refer to linkage groups one and two respectively. Only the spore shape markers of each strain have been shown; round spore shape (sprA); thin elliptical spore shape (sprB); wild-type (+).

Strain	Spore Shape*	Spore Phenotype†	Genotype‡ I	Genotype‡ II
HU1		R	sprA	+
NP158		TE	+	sprB
HU101		R	sprA	+
HU197		R/TE	sprA	sprB
HU77		E	+	+
DU531		E/O	sprA +	sprB +
DU260		R	sprA sprA	sprB +

2. MEDIA

The composition and preparation of the culture media and solutions in this study are listed below.

For drug supplemented media, the required amount of sterile drug solution was added to sterilised SM-agar after autoclaving and just before pouring. The drug plates prepared in this way were (final concentration shown in parenthesis): methanol (2% v/v); cycloheximide (500 µg/ml); ethidium bromide (35 µg/ml); sodium arsenate (1.5 mg/ml); ben late (600 µg/ml or 20 µg/ml); acriflavin (100 µg/ml).

Composition of culture media and solutions			Preparation
SM-agar			
Difco Bacto Peptone	10.0	g	Sterilise by autoclaving for 20 min at 15 lbs/sq. in. Allow to cool to approx. 60°C before pouring.
Glucose	10.0	g	
$Mg\ SO_4 \cdot 7H_2O$	1.0	g	
$K\ H_2PO_4$	2.2	g	
K_2HPO_4	1.0	g	
Oxoid Yeast Extract	1.0	g	
Calbiochem agar	11.0	g	
Distilled water	1.0	l	
LP-agar			
Difco Bacto Peptone	10.0	g	As for SM-agar.
Lactose	10.0	g	
Difco agar	15.0	g	
Distilled Water	1.0	l	

Axenic medium		Preparation
Oxoid Bacteriological Peptone	14.3 g	
Oxoid Yeast Extract	7.15 g	
Glucose	15.4 g	Sterilise by autoclaving for 20 min at 15lbs/sq.in. adjust to pH7.0 with NaOH
$Na_2HPO_4 12H_2O$	1.28 g	
KH_2PO_4	0.48 g	
Dihydro streptomycin sulphate	0.25 g	
Distilled water	1.0 l	

Hay agar		
Old weathered & dry grass	10.0 g	Autoclave grass in 1 litre water for 5 min at 20lbs/sq.in. Add filtrate of boiled grass to salts and agar. Autoclave for 20 min. at 15lbs/sq.in. Pour into petri plates.
KH_2PO_4	1.5 g	
$Na_2HPO_4 12H_2O$	0.75 g	
Calbiochem agar	12.0 g	
Water	1.0 l	

Bonners Salt solution (S.S.)		
NaCl	0.6 g	
KCl	0.75 g	Sterilise by autoclaving for 20 min. at 15lbs/sq.in.
$CaCl_2$	0.3 g	
Distilled water	1.0 l	

LPS agar		
KCl	1.5 g	
$MgCl_2 \cdot 6H_2O$	0.5 g	
KH_2PO_4	5.45 g	As for SM-agar
Dihydro streptomycin sulphate	0.5 g	
Difco agar	15.0 g	

H_2O agar		Preparation
Dihydro streptomycin sulphate	0.25 g	
Difco agar	15.0 g	As for SM-agar
Distilled water	1.0 l	

3. CHEMICALS

DU PONT: ben late (50% benomyl).

SIGMA : acriflavin (neutral); cycloheximide; dihydrostreptomycin sulphate; ethidium bromide.

UNIVAR : lactose; glucose; methanol; sodium arsenate.

All other chemicals were of the highest reagent grade available.

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