Genet. Res., Camb. (1973), **21**, *pp.* 79–86
With 1 *plate*
Printed in Great Britain

Developmental mutants in a homothallic strain of *Physarum polycephalum*

By A. E. WHEALS*

Department of Genetics, University of Leicester, Leicester LE1 7RH, England

(*Received* 20 *November* 1972)

SUMMARY

A derivative line of the homothallic Colonia strain of *Physarum polycephalum* has been isolated which produces plasmodia with high efficiency within clones of amoebae. Using the synergistic effect of ultraviolet light and caffeine, mutants of this line have been isolated which fail to undergo the developmental transition between haploid amoebae and diploid plasmodia (*apt* mutants). They are isolated by selecting for amoebae which fail to produce plasmodia within clones. Complementation tests of four mutants have shown that they are mutants of four different loci and they are recessive to wild-type. A further analysis of one mutant reveals that the *apt-1* locus is unlinked to three other known markers. Crosses of this mutant with heterothallic strains yield progeny which are homothallic indicating that the lesion is not a revertant from a homothallic to a heterothallic mating-type. The use of this system in isolating developmental mutants is discussed.

1. INTRODUCTION

There is an increasing body of evidence to suggest that cell differentiation in eukaryotes is often ultimately based on the regulation of gene activity (Britten & Davidson, 1969). The differentiated state reflects the activation and inactivation of different sets of genes. This does not preclude other kinds of control which almost certainly exist but probably play secondary or modifying roles (Gross, 1969; Sussman *et al.* 1967). However, if there are genetic mechanisms controlling these regulatory processes then genetic analysis should reveal them. Much can be learnt by studying the effect of *specific* mutations on the development of simple eukaryotes which have homogeneous cell types, can be handled by standard microbiological techniques and are amenable to genetic studies.

The Myxomycetes or acellular slime moulds are almost unique in having alternate stable vegetative haploid *and* diploid states of differentation that are morphologically and biochemically dissimilar. Haploid amoebae can, under appropriate conditions, undergo cell and nuclear fusion to form diploid zygotes. These grow by nuclear division without cell division to form the diploid vegetative stage, the plasmodium (Gray & Alexopoulos, 1968). Under conditions of light and starvation

* Present address: Max-Planck-Institut für Biologie, Corrensstrasse 41, 74 Tübingen, W. Germany.

the plasmodium will sporulate, undergoing meiosis and cleavage to yield the haploid amoebal state. The characterization of a homothallic strain of *P. polycephalum* (Wheals, 1970) gave the opportunity for the isolation and analysis of mutations affecting the transition between the haploid amoebae and the diploid plasmodium.

This paper reports the preliminary analysis of four of these mutants. The genetic lesion is one affecting either cell or nuclear fusion or the activation of genes and functions peculiar to the plasmodium.

2. MATERIALS AND METHODS

(i) *Strains.* The Wisconsin strains (*a* and *i*) have been described elsewhere (Dee, 1966). A subline of the homothallic Colonia strain (Wheals, 1970), designated *C5-1*, was used in these experiments. It was derived as a homothallic progeny clone from the parent *C50*. It differs from *C50* in that it produces plasmodia within plaques with 100 % efficiency. When incubated for 4–5 days on agar plates under appropriate conditions, 100 amoebae produce 100 plaques, *all* of which contain incipient plasmodia, unlike *C50* which would only produce plasmodia when the amoebal plaques had developed into a lawn of amoebae.

(ii) *Loci. mt.* Mating type. Heterothallic alleles mt_1 mt_2 etc. (Dee, 1966). Homothallic allele mt_h (Wheals, 1970).

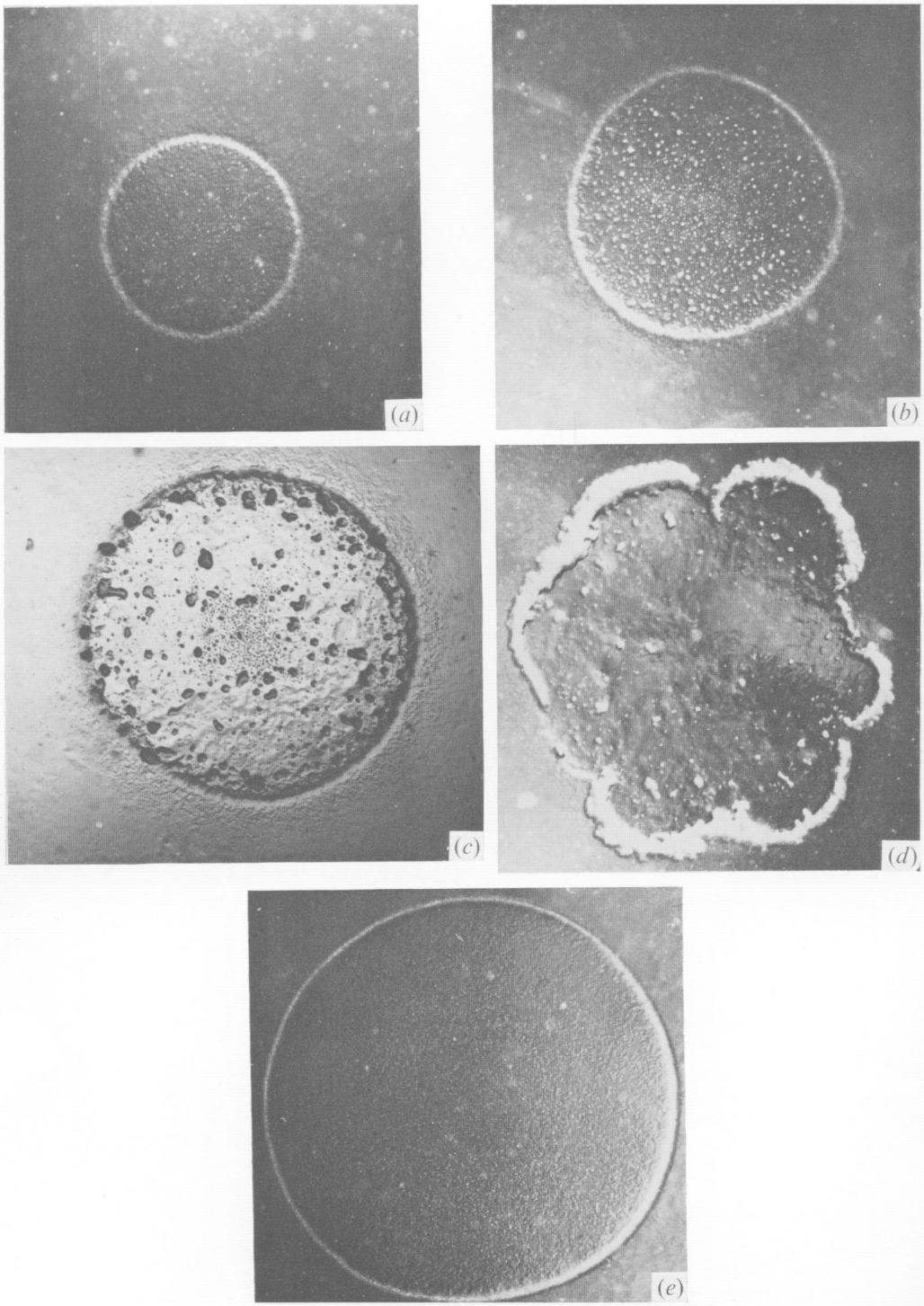
sax. Sensitivity to axenic medium. Plasmodia homozygous for the recessive alleles sax^- fail to grow on SDM (see below) and other axenic media. Growth will continue using *Escherichia coli* as food source (Poulter, 1969).

f and *n*. Fusion type. Somatic cell fusion can occur between plasmodia to form homo- or heterokaryons. The system has now been extensively studied (Poulter, 1969; Poulter & Dee, 1968). There are two unlinked loci, *f* and *n*, each having two alleles among the isolates mentioned in this paper. For fusion to occur there must be identity at both loci. However n_2 is dominant to n_1, so that a plasmodium of genotype f_1n_2/f_2n_2 will fuse with an identical plasmodium and with one of genotype f_1n_1/f_2n_2.

(iii) *Cultural conditions.* Amoebae were cultured on liver infusion agar (see below) at 26° with *E. coli*. Plasmodia were grown at 26° on semi-defined medium (SDM) as modified by Dee & Poulter (1970). Spore formation, spore plating and the isolation of clones have been described elsewhere (Wheals, 1970).

(iv) *Plasmodium formation.* In heterothallic crosses the two amoebal strains to be crossed were mixed on a 2 % agar plate with a thick suspension of bacteria, which were spun down and resuspended from an overnight broth culture. The underlying medium was either liver infusion agar (1 g Oxoid liver infusion powder: 1 l., 2 % agar) or dilute SDM (DSDM agar) made by adding 65 ml of liquid SDM to 1 l. of 2 % agar. For homothallic strains, amoebae were inoculated at low density together with a thick bacterial suspension onto DSDM agar and spread evenly. After plasmodia had formed they were transferred to SDM agar containing streptomycin (250 μg/ml).

(v) *Plasmodial fusion tests.* These have been fully described and illustrated elsewhere (Poulter & Dee, 1968).



Amoebal plaques growing on liver infusion agar in a lawn of bacteria. (*a*) After 3 days: encysted central area and growing edge. (*b*) After 4 days: larger bodies visible in the plaque are incipient plasmodia. (*c*) After 5 days: the plasmodia are fusing and also ingesting the amoebae as a food source. (*d*) After 6 days: the plasmodia have now fused to give a few large plasmodia which are migrating away from the plaque. (*e*) After 7 days: amoebal clone *APT1* still consists of a smooth lawn of amoebae with no plasmodia visible. (All × 15.)

(*Facing p.* 81)

(vi) *Mutagenesis.* The method used was a slight modification of that of Haugli & Dove (1972). Amoebae, together with a thick bacterial suspension, were inoculated and spread over a 9 cm Sartorius membrane filter (pore-size 0·45 μm) resting on a filter paper supported by a stainless-steel wire mesh grid in a covered Petri dish. The membrane filter was underlaid with 20 ml of liver-infusion solution. After incubation, when the cell density had reached approximately 2×10^6 cells per filter, the amoebae were irradiated with ultraviolet light at a dose of 1500 erg/mm^2, giving about 2% survival. The cells were allowed to continue growing for 24 h (to allow segregation of mutants) in the presence of 0·025% caffeine (to inhibit DNA repair enzymes).

3. RESULTS

(i) *Isolation of mutants*

Exponentially growing cultures of *C5-1* amoebae were mutagenized, incubated, washed off the membrane filters and plated at appropriate dilutions on DSDM agar. The prospective amoebal-plasmodial transition mutants were detected by the failure of isolated amoebal plaques to form plasmodia within clones. After 6 days incubation amoebal plaques had developed containing small plasmodia (Pl. 1*a–d*). The potential mutant clones appeared as homogeneous circular plaques (Pl. 1*e*) in a background of irregular plaques. All plaques failing to produce plasmodia were cloned and retested. In a total of 5×10^5 amoebae, four clones of independent origin were isolated which failed to form plasmodia. They were designated *APT1* to *APT4*, since they had failed to undergo the amoebal-plasmodial transition.

All four strains are stable and in the case of *APT1*, no revertants have been detected in approximately 10^7 cells. The four amoebal strains all grew at normal rates and showed no morphological difference from *C5-1* when examined by phase-contrast microscopy.

(ii) *Complementation tests*

The four strains were crossed with each other in all pairwise combinations and plasmodia formed in all cases; thus the strains behave as recessive mutants and fall into four complementation groups. The growth rate of these plasmodia was slower than that of the *C5-1* line.

(iii) *Crosses with wild-type strains*

Crosses of *APT* strains with *C5-1* were not possible since *C5-1* forms homothallic plasmodia very readily. *APT1* and *APT2* were crossed with two heterothallic strains *a* and *i* (mt_1 and mt_2) and plasmodia appeared in all crosses. To prove that these were truly hybrid plasmodia and not the result of an induction of homothallic plasmodia of the *APT* strains under the influence of the surrounding heterothallic strains, the fusion behaviour of these plasmodia was tested (Table 1). The results indicated that the plasmodia were hybrid. Although the occurrence of heterokaryons between heterothallically and homothallically produced plasmodia was not eliminated, the analysis of the progeny of the $a \times APT1$ plasmodium confirmed the presence of heterozygous diploid nuclei.

(iv) *Analysis of the progeny of* a × APT1

Seventy amoebal clones arising from an $a \times APT1$ cross were picked at random. After recloning they were each crossed with a, with i and also tested for homothallic plasmodium formation by plating on DSDM agar. Each was classified, if possible, for *mt*, *f* and *sax*, and for the putative locus *apt-1*.

Table 1. *Fusion behaviour of plasmodia formed by crossing* APT1 *or* APT2 *strains with heterothallic amoebal strains*

Cross in which plasmodium arose	Expected fusion-type of plasmodium if hybrid	Expected fusion-type of plasmodium if derived from *APT* strain only	Observed fusion-type
$a \times APT1$	f_1n_1/f_2n_1	f_2n_1/f_2n_1	f_1n_1/f_2n_1
$a \times APT2$	f_1n_1/f_2n_1	f_2n_1/f_2n_1	f_1n_1/f_2n_1
$i \times APT1$	f_2n_2/f_2n_1	f_2n_1/f_2n_1	f_2n_2/f_2n_1
$i \times APT2$	f_2n_2/f_2n_1	f_2n_1/f_2n_1	f_2n_2/f_2n_1

Table 2. *Analysis of progeny of plasmodium* a × APT1

(*a*) Segregation of *mt*

Phenotype of progeny clones: Within clones	Forming plasmodia: With *a*	Forming plasmodia: With *i*	No. showing phenotype	Deduced genotype of amoebal clone
.	.	+	31	mt_1
.	+	+	17	mt_h
+	+	+	18	
+	.	+	3	(mt_h)
	Contaminated		1	.
		Total	70	

(*b*) Segregation of *apt-1* and recombination with *mt*

Genotype of clone	No. in class	Total *apt-1*⁻	Total *apt-1*⁺
mt_1 *apt-1*⁺	14	.	14
mt_1 *apt-1*⁻	17	17	.
mt_h *apt-1*⁺	18	.	18
mt_h *apt-1*⁻	17	17	.
		34	32

(*a*) *Mating-type locus*. A clone carrying mt_1 should not form plasmodia within clones or when crossed with a (mt_1). Thus, clones which only formed plasmodia when crossed with i (mt_2) were classified as mt_1. Clones which carried the mt_h allele should form plasmodia when crossed with a (mt_1) or with i (mt_2). However, if they also carried the *apt-1*⁻ allele (like the parent *APT1*) they would not form

plasmodia in clones. Amoebae which produced plasmodia in clones were classified as recombinant mt_h *apt-1*$^+$ amoebae.

The results are displayed in Table 2*a*. The segregation of mt_1 from mt_h was not significantly different from 1:1. The three clones which unexpectedly produced plasmodia in clones and with *i* are probably mt_h clones which have not produced plasmodia with *a* either because of insufficient inoculation or adverse environmental conditions.

(*b*) apt *locus*. Two classes of amoebae that were not mt_1 were detected (Table 2*a*) – (1) those that produced plasmodia with *a*, with *i* and within clones were classified as mt_h *apt-1*$^+$; (2) those that crossed with *a* and with *i* but did not produce plasmodia in clones were classified as mt_h *apt-1*$^-$. In addition, the $a \times APT1$ progeny clones which were mt_1 were backcrossed to *APT1* (mt_h *apt-1*$^-$) (J. Dee & C. E. Holt, personal communication). Those that failed to produce plasmodia were classified as mt_1 *apt-1*$^-$; those that did produce plasmodia were classified as mt_1 *apt-1*$^+$. The results of this classification are given in Table 2*b*.

The segregation of *apt-1*$^-$ from *apt-1*$^+$ was almost exactly 1:1 and free recombination occurred between *mt* and *apt*.

(*c*) *Fusion loci*. The plasmodia which arose in clones and those derived from the crosses with *a* and *i* were tested for fusion-type against tester plasmodia. Plasmodia produced when mt_1 clones were crossed with *i* (mt_2) were always hybrid, containing the n_2 allele of amoebal strain *i*. They fell into 2 groups: f_1n_1/f_2n_2 when *i* (f_2n_2) crossed with an f_1n_1 amoebal clone (15 clones), and f_2n_1/f_2n_2 when *i* crossed with an f_2n_1 amoebal clone (16 clones).

Plasmodia formed by mt_h clones were more difficult to analyse. The homothallic plsamodia formed in clones of mt_h amoebae fell into two groups: f_1n_1/f_1n_1 and f_2n_1/f_2n_1 having arisen from mt_h f_1 n_1 and mt_h f_2 n_1 amoebae (3 and 12 clones) respectively. Amoebal clones giving rise to these homothallic plasmodia also produced identical plasmodia when mixed with *a* and *i* indicating that these plasmodia had also arisen homothallically.

Many of the mt_h amoebal clones carried the *sax*$^-$ allele and they could therefore not be subcultured onto SDM and fusion-tested. However, although they would also form homozygous plasmodia when crossed with *a* (*sax*$^-$), they could form vigorous plasmodia if crossed with *i* (*sax*$^+$). These plasmodia could thus be fusion-tested. They all fell into two groups, f_1n_1/f_2n_2 when *i* ($mt_1f_2n_2$) was crossed with mt_h f_1n_1*sax*$^-$ amoebal clones (13 clones), and f_2n_1/f_2n_2 when *i* was crossed with mt_h f_2n_1 *sax*$^-$ amoebal clones (3 clones). Four mt_h *sax*$^-$ clones could not be classified for fusion type since thick bacterial growth prevented the development of the hybrid plasmodia formed by crossing with *i*.

The segregation of *f* alleles was 31:31.

(*d*) sax *locus*. Clones carrying mt_1 could only form plasmodia with *i* (mt_2 *sax*$^+$) and were thus either heterozygous or homozygous for *sax*$^+$ and could not be scored for this marker. Amoebal clones carrying mt_h and *sax*$^-$ would produce plasmodia homozygous for *sax*$^-$ either by homothallic plasmodium production or by crossing with *a* (mt_1 *sax*$^-$) and could thus be scored by subculturing the

plasmodium onto SDM. The *sax* genotype deduced for a clone from homothallic plasmodia and from plasmodia formed on cross plates with *a* was consistent throughout. The segregation of sax^+ from sax^- was 15:20.

(*e*) *Linkage.* No linkage was detected between *mt*, *f* and *apt*. However, confirming previous observations, linkage was detected between *f* and *sax*, the recombination frequency of 18 % being not significantly different ($P = 0{\cdot}10$) from the value of 12 % obtained in the analysis of progeny of the cross $a \times i$ (Poulter, 1969).

(*f*) *Crosses of* a × APT1 *progeny.* The above analysis allowed the classification of all the mt_1 clones for *f* and *apt*, and all the mt_h clones for *f*, *sax* and *apt*. The classification was confirmed by selecting clones of amoebae for pairwise crossing and comparing the predicted with the observed behaviour. In all cases the prediction was confirmed. For example, $mt_h f_1$ sax^- $apt\text{-}1^-$ amoebae crossed with $mt_h f_1$ sax^+ $apt\text{-}1^-$ amoebae did not produce plasmodia, $mt_h f_1$ sax^- $apt\text{-}1^-$ amoebae with $mt_h f_2$ sax^- $apt\text{-}1^+$ amoebae produced plasmodia which showed the sax^- phenotype, and $mt_h f_1$ sax^+ $apt\text{-}1^-$ amoebae with $mt_h f_2$ sax^- $apt\text{-}1^+$ amoebae produced plasmodia which were heterozygous for *f*.

4. DISCUSSION

The absence of revertants in 10^7 cells indicates that the *APT* strains contain very stable inherited characters. The results of the complementation tests and crosses with wild-type strains indicate that they are mutants of four separate genes, but some caution will need to be exercised in interpreting complementation data.

First, there is the possibility that these results indicate reversion of mt_h to different mating-types which can now cross with each other. Two pieces of evidence argue against this. The analysis of $a \times APT1$ progeny shows the *apt-1* mutation to be in a different locus from *mt*. Also, mutation of heterothallic strains from one mating-type to a different one is a very rare event. Formation of plasmodia in clones of a heterothallic mating-type can often be ascribed to other reasons (Collins & Ling, 1968). Secondly, a negative result could merely reflect the failure of the two strains to undergo cell fusion even though they were mutants of different genes. This problem has not yet arisen.

In a number of crosses involving the Wisconsin amoebae (*a* or *i*) and the Colonia amoebae (*C5-1* or *APT1*), the resulting plasmodium has shown both expected and anomalous fusion behaviour. Normally the fusion behaviour has been consistent with the hypothesis that the plasmodium was hybrid and therefore heterozygous for fusion loci (see Table 1). However, occasional microscopic fusions and lethal interactions (Carlile & Dee, 1967) have been seen between, for example, $a \times APT1$ plasmodia (f_1n_1/f_2n_1) and f_2n_1/f_2n_1 tester plasmodia. Two possible explanations of this anomaly are presented. Firstly, there are modifying genes in the Colonia line which can affect the expression of the fusion loci. Secondly, the $a \times APT1$ plasmodium is in reality a heterokaryon of f_1n_1/f_2n_1 nuclei and f_2n_1/f_2n_1 nuclei, the former being the majority. This situation could possibly have arisen if the $apt\text{-}1^-$

mutation allowed the formation of diploid zygotes but prevented their further development. If the fusion loci were not expressed in these 'blocked' zygotes heterokaryon formation could occur between plasmodia of different fusion-type. Poulter (1969) has shown that when heterokaryons are made between strains containing different fusion alleles, ambivalent fusion behaviour can occur when the alleles are in a critical ratio of approximately 9:1. Outside of this value the heterokaryon mimics either a heterozygote or homozygote of the predominating nuclear type. Although this anomalous fusion behaviour was unexpected, it in no way affected the analysis of the progeny of the $a \times APT1$ plasmodium and no excess of f_2 progeny was detected.

The results of the analysis of the $a \times APT1$ cross indicated normal recombination and segregation of *mt*, *f* and *sax* alleles. Moreover, the data agree well with the hypothesis that *apt-1*$^-$ is a recessive nuclear gene mutation which segregates normally from wild-type and is unlinked to all three loci previously mentioned. The analysis above has not eliminated the possibility that the *apt-1*$^-$ mutation is a deletion, perhaps of a considerable portion of a chromosome.

The results show the feasibility of isolating and analysing genetically a novel class of developmental mutants – those affecting the transition from a vegetative haploid phase to a vegetative diploid phase. Linkage and complementation studies can be easily performed to locate the genes and determine their functional relationships. Genetic analysis does not distinguish between mutants affecting cell fusion, nuclear fusion and plasmodial gene activation. Such distinctions can be made using a variety of techniques including time-lapse photomicrography of fusing cells, microspectrophotometry to measure nuclear DNA content and immunology to detect the appearance of new plasmodial specific surface antigens. Thus, although primarily concerned with gene activation, studies of *apt* mutants should reveal some of the processes underlying cell and nuclear fusions.

I should like to thank Dr Jennifer Dee for valuable discussions and much help during the preparation of this manuscript, Dr F. B. Haugli for making freely available much unpublished work, and Mrs J. Foxon for excellent technical assistance. I am also indebted to the late Mr G. Asquith for some of the photography. This work was supported by the award of a Research Scholarship from the Medical Research Council.

REFERENCES

Britten, R. & Davidson, E. H. (1969). Gene regulation for higher cells: a theory. *Science* **165**, 349–357.

Carlile, M. J. & Dee, J. (1967). Plasmodial fusion and lethal interaction between strains in a Myxomycete. *Nature, London* **215**, 832–834.

Collins, O. R. & Ling, H. (1968). Clonally-produced plasmodia in heterothallic isolates of *Didymium iridis*. *Mycologia* **60**, 858–868.

Dee, J. (1966). Multiple alleles and other factors affecting plasmodium formation in the true slime mould *Physarum polycephalum* Schw. *Journal of Protozoology* **13**, 610–616.

Dee, J. & Poulter, R. T. M. (1970). A gene conferring actidione resistance and abnormal morphology on *Physarum polycephalum* plasmodia. *Genetical Research* **15**, 35–41.

Gray, W. D. & Alexopoulos, C. J. (1968). *Biology of the Myxomycetes*. New York: Ronald Press.

GROSS, S. R. (1969). Genetic regulatory mechanisms in the Fungi. *Annual Review of Genetics* **3**, 395–424.

HAUGLI, F. B. & DOVE, W. F. (1972). Mutagenesis and mutant selection in *Physarum polycephalum*. *Molecular and General Genetics* **118**, 109–124.

POULTER, R. T. M. (1969). Senescence in the Myxomycete *Physarum polycephalum*. Ph.D. Thesis, University of Leicester, England.

POULTER, R. T. M. & DEE, J. (1968). Segregation of factors controlling fusion between plasmodia of the true slime mould *Physarum polycephalum*. *Genetical Research* **12**, 71–79.

SUSSMAN, M. W., LOOMIS, W. F., ASHWORTH, J. M. & SUSSMAN, R. R. (1967). The effect of actinomycin D on cellular slime mould morphogenesis. *Biochemical and Biophysical Research Communications* **26**, 353–359.

WHEALS, A. E. (1970). A homothallic strain of the Myxomycete *Physarum polycephalum*. *Genetics* **66**, 623–633.