Genet. Res., Camb. (1973), **21**, *pp.* 87–101
With 1 *plate*
Printed in Great Britain

Inheritance of plasmodial valine requirement in *Physarum polycephalum*

By JENNIFER DEE, A. E. WHEALS* and C. E. HOLT†

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

(*Received* 20 *November* 1972)

SUMMARY

A defined medium for *Physarum polycephalum* plasmodia has been devised containing glutamic acid, glycine, methionine, biotin, thiamine, glucose, salts and haematin, which supports good growth on agar plates of many different strains. Tests on this medium have revealed a requirement for valine in some plasmodia formed by homothallic progeny (mt_h *apt-1*$^+$) from the cross *a* (mt_1 *apt-1*$^+$) × *APT1* (mt_h *apt-1*$^-$). The valine requirement was also inherited among heterothallic progeny of this cross and its segregation was followed in several heterothallic crosses. To explain the results it is proposed that valine synthesis requires the presence of dominant alleles at either of two unlinked loci and that only plasmodia homozygous for recessive alleles at both loci are valine dependent. In some crosses studied only one pair of alleles is segregating and valine requirement thus provides a useful genetic marker, and the first reported nutritional marker in *P. polycephalum*. The value of crosses with *apt*$^-$ mutants for both the detection and analysis of plasmodial markers is demonstrated and discussed.

1. INTRODUCTION

We have been interested for some years in developing techniques for isolating biochemical genetic markers in *P. polycephalum*. Towards this aim, the homothallic strain Colonia was investigated by Wheals (1970) and methods have recently been developed for the isolation of plasmodial markers in this strain (J. Dee & C. E. Holt, unpublished), using the mutagenic technique of Haugli & Dove (1972).

Although a minimal defined medium for *P. polycephalum* plasmodia was published in 1963 (Daniel *et al.* 1963), it has never been successful with our strains and it has rarely been used in published work from other laboratories. It seems that there may be a need for particular strains to adapt to the medium. For the isolation of nutritional mutants, a minimal defined agar-based medium was required that would support the growth of a wide variety of strains, including

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

† Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachussetts 02139, U.S.A.

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

homothallic plasmodia. Such a medium has been devised and is reported in the present paper. We are indebted to Daniel *et al.* for their work on which our experiments were largely based. The differences in our results are probably due to genetic dissimilarities in the strains, and possibly also to the different method of culture.

Following the development of mutagenic techniques, Wheals has recently been concerned with isolating and analysing developmental (*apt*) mutants in a homothallic strain (Wheals, 1971, 1973). An *apt-1*$^-$ mutant carries the homothallic allele (mt_h) at the mating-type locus, but plasmodia fail to develop from the amoebae because of the mutation (*apt-1*$^-$) at an unlinked locus. When a mt_h *apt-1*$^-$ amoebal strain is crossed with a heterothallic strain (e.g. mt_1 *apt-1*$^+$), a quarter of the amoebal progeny have the recombinant homothallic genotype mt_h *apt-1*$^+$ and therefore develop into plasmodia. These plasmodia are completely homozygous for various combinations of the alleles present in the two parent strains. Thus recessive alleles from the heterothallic parent which are expressed in plasmodia and not in ameobae may be detected in such progeny for the first time. When homothallic plasmodia from a cross *a* (mt_1 *apt-1*$^+$) × *APT1* (mt_h *apt-1*$^-$) were tested on defined medium, some showed a requirement for valine which proved to be inherited. Since this is the first reported nutritional marker in *P. polycephalum*, it has been analysed in some detail and the results form the main subject of the present paper. Some discussion of methods for the further isolation and analysis of plasmodial markers in *P. polycephalum* will also be included.

2. MATERIALS AND METHODS

(i) *Strains.* The homothallic plasmodium *C5-1* used in the tests of defined media was a clone derived homothallically from Colonia (Wheals, 1970). The amoebal strains *a* and *i* ('Wisconsin' strains) have been described previously (Dee, 1966). Progeny amoebal clones of *a* × *i* designated *a.i:1*, *a.i:2* etc. were recently isolated and provided by D. J. Cooke (unpublished). Progeny amoebal strains from the cross *a* × *APT1* were isolated by Wheals (1971, 1973). The full designation of these is *a.APT1:1*, etc., but for convenience the numbers alone are often used in the text and tables.

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

apt-1 (amoebal–plasmodial transition). The recessive allele *apt-1*$^-$ causes failure of plasmodial formation. An amoebal strain carrying mt_h *apt-1*$^-$ fails to form plasmodia but can be cultured as amoebae and crossed with heterothallic *apt-1*$^+$ strains (Wheals, 1971, 1973).

sax (sensitivity to axenic medium). Plasmodia homozygous for the recessive allele *sax*$^-$ die on all axenic media used in this laboratory (SDM, DM-1, etc.; see below) although they grow on the lawn of *Escherichia coli* on which crosses are done (Poulter, 1969).

(iii) *Culture of amoebae and crossing.* Amoebal strains were cultured at 26 °C on

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

liver infusion agar with *E. coli* as described previously (Wheals, 1970, 1973). Crosses were performed on water agar containing 6·5 % liquid SDM (see below) spread with a thick suspension of *E. coli* harvested from overnight broth cultures. Two strains of amoebae were mixed on each plate. Plasmodia usually appeared after 4–7 days incubation at 26 °C and were transferred to SDM agar containing streptomycin (250 μg/ml) to kill the bacteria. If a cross had not given plasmodia after 7 days, 1 ml liquid SDM was added to the agar, allowed to soak in and the plate re-incubated; it was then usually successful in a few days.

(iv) *Culture and tests of plasmodia.* Plasmodia were cultured routinely on the semi-defined medium (SDM) (Dee & Poulter, 1970) which was modified from the medium of Daniel & Baldwin (1964). Subculture was by the transfer of plasmodia on agar blocks ($\approx$ 1 cm^2). Tests were on defined medium (DM-1, Table 1) with and without valine. Further details are given in Results. Incubation was at 26 °C except during the development of DM-1, which was tested at 30 °C. Stocks not needed for immediate use were cultured at 23 °C.

(v) *Spore formation, spore plating and isolation of progeny clones.* Spores were obtained from plasmodia as described by Wheals (1970). For the analysis of a cross, spores were plated on liver infusion agar (LIA) as described previously (Wheals, 1970). Amoebae from about 100 plaques were streaked on separate LIA plates for re-cloning. About 100 clones, each derived from a separate spore, were picked and cultured on separate plates or slopes. For analysis of plasmodial markers, the clones were then crossed to appropriate amoebal strains and the resulting plasmodia tested. For the isolation of homothallic (mt_h *apt-1*$^+$) progeny from a cross of the type mt_h *apt-1*$^-$ × mt_1 *apt-1*$^+$, spores were plated on water agar containing 6·5 % SDM, spread with thick *E. coli* suspension, as for crossing. About a quarter of the plaques were clearly homothallic after 5–6 days incubation (see Wheals (1973) for illustration). These plaques were cut out on agar blocks and transferred to separate SDM + streptomycin plates. The isolates were not re-cloned so a small proportion may have been mixed clones. If *sax*$^-$/*sax*$^-$ plasmodia were present, these grew on the spore plates but died when transferred to SDM.

(vi) *Storage of strains.* Plasmodia usually formed sclerotia on test plates when these were left at room temperature after incubation. These were easily peeled off the medium, dried in sterile Petri dishes and stored in screw-cap bottles. Amoebae were stored as streaks on LIA + *E. coli* plates at 4 °C or on LIA + *E. coli* slopes at room temperature.

3. RESULTS

(i) *The defined medium*

The formula and preparation of our defined medium (DM-1) is shown in Table 1. Development of the medium was based on the OV-40 defined medium of Daniel *et al.* (1963) but a different amino acid composition was introduced because OV-40 had previously failed to support growth of plasmodia in several laboratories. The amino acids in OV-40 were arginine, alanine, glycine and methionine. Daniel *et al.* (1963) discovered that methionine was the only absolute amino acid require-

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

ment in all media. Alanine was required in the minimal medium and was supplied in excessive amount, presumably providing a source of organic nitrogen. Glycine and arginine were not essential but both greatly stimulated growth.

In our tests we used the homothallic plasmodium, *C5-1*. All tests were done on agar-based medium and concentrations given below refer to the final concentrations in the agar medium. Growth on plates was recorded after 3–4 days incubation at 30 °C. In early tests, we found poor growth on a medium containing vitamin-free, salt-free casamino acids and greatly stimulated growth when glycine was added. We found negligible growth on a medium with the amino acid composition of OV-40. We then made a defined medium containing the four amino acids used by Daniel but we also added glutamic acid because this is a major constituent of casein (about one-fifth the total weight). Growth on this medium was sufficient for us to use it as a basis for further modification of the amino acid composition. In a series of tests, we reached the following conclusions:

(*a*) *Alanine* was not essential and at concentrations between 500 and 1250 mg/l. was not stimulatory.

(*b*) *Arginine* was neither essential nor stimulatory (100–500 mg/l.). The plasmodia tended to look orange and unhealthy in the presence of arginine.

(*c*) *Glutamic acid* was stimulatory with a broad optimum concentration between 1000 and 2000 mg/l. Glutamine was a satisfactory alternative, but not significantly better, and it was not used in the final medium since it is more expensive and requires filter-sterilization. In the absence of both glutamic acid and glutamine there was no growth. In our medium glutamic acid is presumably the source of organic nitrogen.

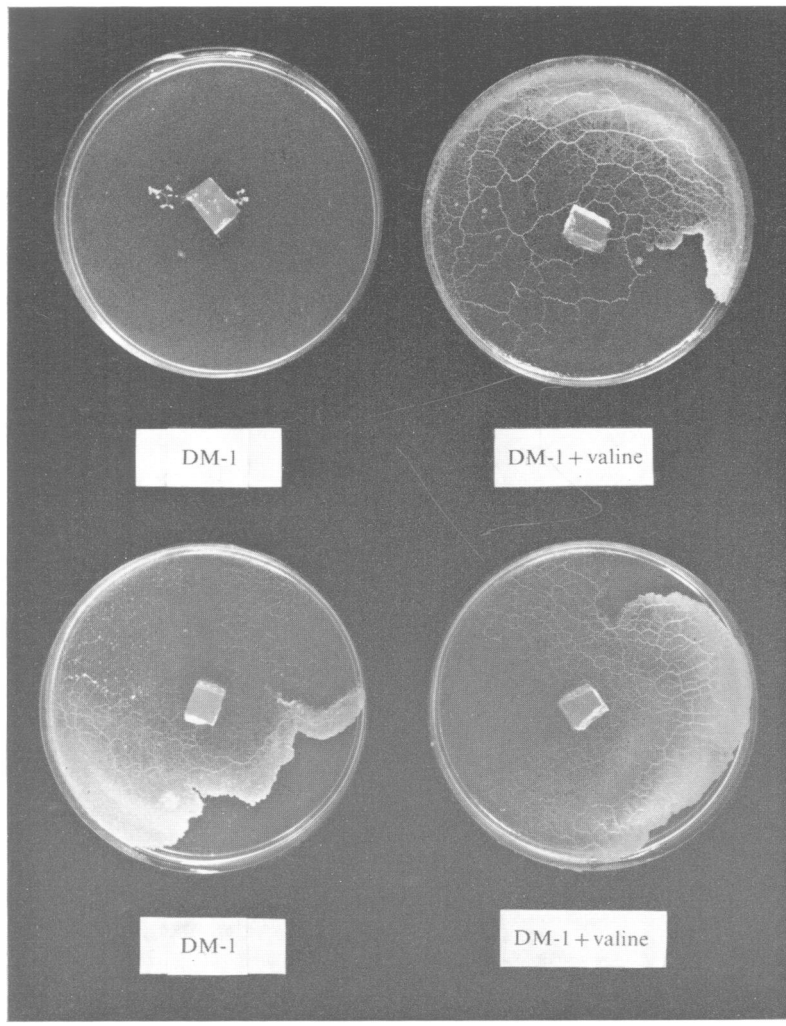
(*d*) *Glycine* was highly stimulatory at a concentration of 250 mg/l. Increasing this gave no significant improvement.

(*e*) *Methionine* was essential. The optimum concentration was 50 mg/l. and halving or doubling this concentration was unfavourable.

As a result of these tests, which were by no means exhaustive, a medium containing glutamic acid, glycine and methionine as the only amino acids was adopted. This medium (DM-1) has maintained very good growth of many different strains at temperatures between 26 and 31 °C (Pl. 1). The glucose concentration of DM-1 was decreased during tests from 5 g/l. (as in SDM) to 2 g/l. since it was found that although this led to decreased growth rate, the plasmodium appeared healthier (less orange) and the final yield was only slightly decreased. The two vitamins, biotin and thiamine, found to be essential by Daniel *et al.* (1963) were included throughout, and salts and haematin concentrations were the same as in SDM. The final yield from a DM-1 plate is of course considerably less than the yield from SDM which contains peptone and more glucose.

The optimum concentrations of the amino acids for liquid culture have not yet been determined. In liquid medium in which all the constituents are at twice the concentration used in DM-1-agar, microplasmodia of a homothallic strain have been found to grow satisfactorily with a doubling time of approximately 30 h.

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press



Appearance of a valine-requiring (top) and a valine-independent (bottom) plasmodial strain after their second successive transfer on DM-1 and DM-1 + valine. Each plate was inoculated with an agar block from a previous plate of the same medium (originally inoculated from SDM) and incubated 4 days at 26 °C. The valine-requiring strain has not grown on DM-1 but has remained alive and migrated for several days.

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

Table 1. *Preparation of DM-1 (defined medium)*

(*a*)		Final concentration in DM-1 agar (mg/l.)	(mM)
	L-Glutamic acid	1500·0	10·0
	Glycine	250·0	3·0
	L-methionine	50·0	0·3
	Biotin	2·5	.
	Thiamine	20·0	.
	KH_2PO_4	1000·0	.
	$CaCl_2.2H_2O$	450·0	.
	$MgSO_4.7H_2O$	300·0	.
	$FeCl_2.4H_2O$	30·0	.
	$ZnSO_4.7H_2O$	17·0	.
	Citric acid.H_2O	1770·0	.
	Disodium EDTA	112·0	.

The above components are dissolved in distilled water at *twice* the concentrations shown and the pH adjusted to 4·6 with NaOH. This liquid medium is autoclaved (15 lb/in.^{2} for 20 min) in 250 ml quantities and stored in the dark at room temperature.

(*b*) Haematin solution: 100 ml 1 % NaOH; 0·05 g haematin (Koch-Light). Sterilized by autoclave. Stored at 5 °C.

(*c*) For DM-1 agar, constituents are mixed as follows just before plates are poured:

	Vol. (ml)	Concentration in DM-1 agar (mg/l.)
Liquid medium ((*a*) above)	250	See above
Glucose (20 % (w/v) solution)	5	2×10^3
Haematin (solution (*b*))	2·5	2·5
2·25 % (w/v) water agar (melted)	250	—

(ii) *Identification of valine-requiring strains*

Seven recombinant homothallic plasmodia derived from the cross $a \times APT1$ (see Introduction) in the course of work by Wheals (1971, 1973) were tested for growth on DM-1. Two of the strains grew but five did not, and further tests were carried out to identify the growth requirement. The parent strain $a \times APT1$ grew on DM-1 and was included in all tests as a control. A mixture of 14 amino acids added to DM-1 supported growth of the 'requiring' strains and tests in which these amino acids were omitted one at a time suggested the requirement was for valine. This was confirmed by tests in which the amino acids were added to DM-1 one at a time. Four of the 'requiring' strains grew only on DM-1 + valine. The response was clearer for some strains than for others. Neither isoleucine nor leucine added to DM-1 supported growth. The fifth strain would not grow repeatably on any DM-1 supplemented medium. The optimum valine concentration was near 162 μg/ml (approx. 1·5 mM) and this was used in all subsequent tests. The two strains which grew on DM-1 grew well also on DM-1 + valine. Growth of requiring and non-requiring strains on DM-1 and DM-1 + valine is illustrated in Pl. 1.

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

(iii) *Segregation of valine requirement among homothallic progeny of* a × APT1

Since a valine requirement had been identified among the homothallic progeny of *a* × *APT1*, spores from this cross were plated again and 245 homothallic plaques were isolated on to SDM plates. It was expected that 50% of the plasmodia would die on SDM, being *sax*⁻/*sax*⁻ since *sax*⁻ is carried by *a* (Poulter, 1969). In fact, 144 could not be cultured on SDM, so that it is possible that some were lost due to other lethal factors. The 101 plasmodia which grew on SDM were tested on DM-1 and 78 grew well for two successive transfers on DM-1. These were not tested on DM-1 + valine. The 23 plasmodia which did not maintain satisfactory growth on DM-1 were tested on DM-1 + valine and 10 grew well, seven showed

Table 2. *Valine requirement among homothallic progeny of* a × APT1

Growth on SDM	Growth on DM-1	Growth on DM-1 + valine	No. homothallic strains			
			1st set	2nd set	Total	
+	+	Not tested	2	78	80	
+	−	+	4	10	14	21
+	−	(+)	0	7	7	
+	−	−	1	6	7	
			7	101	108	

+ = Maintained growth. (+) = Grew in some tests. − = Did not maintain growth.

improved growth and six failed to grow. Tests on these 23 plasmodia were repeated many times but it remained difficult to classify some of them satisfactorily. Some strains showed very poor, slow growth on all media. For some strains, it was found that when the plasmodium was first transferred to DM-1 from SDM, growth was moderate, but on subsequent transfers from DM-1 to DM-1 growth failed. The initial growth seemed to be due to a 'carry-over' effect of nutrient pools in the plasmodium itself and not simply in the SDM-agar block, since transfers made on filter-paper disks gave the same result. A few of the plasmodia showed valine requirement very clearly, showing no growth at all when transferred to DM-1 and very good growth on DM-1 + valine.

The total results for homothallic progeny of *a* × *APT1* are shown in Table 2. Of 108 plasmodia tested, 28 failed to maintain good growth on DM-1 and 21 of these showed improved growth on valine. The number of plasmodia carrying the genotype for valine requirement is therefore probably between 21 and 28.

(iv) *Progeny of a homothallic valine-requiring plasmodium*

Spores were plated from one of the homothallic plasmodia which had shown a clear valine requirement. Fifteen homothallic plasmodia derived from single spores were tested on DM-1 and DM-1 + valine and all failed to grow on DM-1. Fourteen of them grew on DM-1 + valine but one failed on this medium also. Since the parent plasmodium had been in culture for many months when the spores were

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

taken, there had been an opportunity for aberrant nuclei to accumulate in it and this may account for the result. Since the majority of progeny were valine requirers and no non-requirers were found, no further investigation was done.

Table 3. *Genotypes of progeny from the cross* a × APT1 *and derivation of plasmodia used in tests*

Parent strains { *a* (mt_1 *apt-1*$^+$) / *APT1* (mt_h *apt-1*$^-$)

Genotypes of progeny amoebae*	Possible backcrosses		Possible selfing
	× *a*	× *APT1*	
1. mt_1 *apt-1*$^+$	No	Yes†	No
2. mt_h *apt-1*$^-$	Yes†	No	No
3. mt_h *apt-1*$^+$	No	No	Yes†
4. mt_1 *apt-1*$^-$	No	No	No

* Types 1 and 2 were also crossed with one another (Table 5). Type 1 was later crossed with mt_2 strains (Tables 6, 7). Type 3 cannot be crossed because they self so readily. Type 4 cannot be crossed with *APT1* because both strains are *apt-1*$^-$.

† Plasmodia derived in these ways were used in tests of valine requirement (see Results and Tables 2 and 4).

Table 4. *Plasmodia from backcrosses of* a × APT1 *progeny tested on DM-1 and DM-1 + valine*

Backcross		Results of testing plasmodia			
		Growth on DM-1			
Progeny genotype	Parent	Valine stimulated*	Not valine stimulated	No growth on DM-1	Total
1. mt_1 *apt-1*$^+$ × *APT1* (mt_h *apt-1*$^-$)		3†	7	0	10
2. mt_h *apt-1*$^-$ × *a* (mt_1 *apt-1*$^+$)		5‡	3	0	8

* 'Valine stimulated' strains showed significantly faster growth on DM-1 + valine than on DM-1 in at least some tests (see text).

† Strain nos. *9*, *12*, *35* (cf. Table 5).

‡ Strain nos. *1*, *7*, *8*, *54*, *55* (cf. Table 5).

(v) *Crosses with amoebal progeny of* a × APT1

The different genotypes with respect to *mt* and *apt-1* which occur in amoebal progeny from the cross *a* × *APT1* are shown in Table 3. For a full description of how these genotypes were identified see Wheals (1973). As indicated, the genotypes mt_1 *apt-1*$^+$ and mt_h *apt-1*$^-$ can be backcrossed to the appropriate parents and also crossed with one another. Plasmodia derived in these ways were tested on DM-1 and DM-1 + valine, with the following results:

Backcrosses with APT1. Ten mt_1 *apt-1*$^+$ strains were backcrossed with *APT1* (mt_h *apt-1*$^-$). The plasmodia all grew on DM-1. Three of them showed stimulated growth on DM-1 + valine in some tests (Table 4) but this was not completely repeatable.

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

Backcrosses with a. Eight mt_h *apt-1*$^-$ strains were backcrossed with *a* (mt_1 *apt-1*$^+$). The plasmodia all grew on DM-1. Five of them showed stimulated growth on DM-1 + valine in some tests, but this was not completely repeatable (Table 4).

Crosses between progeny amoebae. The eight mt_h *apt-1*$^-$ strains were crossed with the 10 mt_1 *apt-1*$^+$ strains. Valine requirement was found in 10 of the 80 resulting plasmodia, as shown in Table 5.

Table 5. *Valine requirement among plasmodia produced by crossing* a × APT1 *progeny together*

	mt_h *apt-1*$^-$ strains							
mt_1 *apt-1*$^+$ strains	*1*	*8*	*23*	*54*	*55*	*3*	*7*	*42*
12	−	−	−	−	−	+*	+*	+*
35	−	−	−	−	−	+*	+*	+*
9	+*	+*	+*	+*	+	+	+	+*
20	+*	+*	+	+*	+*	+*	+*	+
31	+*	+*	+*	+*	+*	+	+	+
37	+*	+*	+	+*	+*	+*	+	+
51	+*	+	+*	+*	+	+	+	+*
52	+*	+*	+	+*	+	+	+	+
57	+*	+*	+*	+*	+*	+	+	+*
70	+*	+*	+	+	+	+	+	+

− = Valine requirement, i.e. no growth on DM-1, growth on DM-1 + valine.
+ = No valine requirement, i.e. growth on DM-1.
+* = 'Valine stimulation', i.e. growth on DM-1, faster growth on DM-1 + valine in at least some tests (see text).

Method of testing. For tests of these 80 plasmodia, the method was standardized as follows. When the plasmodia were growing well on SDM, but before growth was complete, subcultures were made by transferring 1 cm² agar blocks to SDM, DM-1 and DM-1 + valine (1 plate of each). After 3 days incubation, growth was scored, the area, thickness and appearance of the plasmodia being taken into account. Growth was usually complete on SDM but not on the other plates by this time. On the same day, subcultures were made from each plate to 1 or 2 plates of the same medium (only one SDM plate was used, two each of the others). If growth was very poor on a DM-1 plate in the first test, extra plates of DM-1 were inoculated from DM-1 + valine or SDM for the second test. Scoring was after 4 days and repeated up to 7 days. Where necessary, further tests were done in the same way.

The 80 crossed plasmodia were much easier to score than the homothallics and without exception could be unambiguously classified. The 10 valine-requiring strains all failed completely on DM-1 and grew well on DM-1 + valine. The other strains grew well on DM-1, although a few showed poor growth when first transferred to DM-1 from SDM and then recovered and grew well when subcultured to DM-1. Among these strains, however, there were some variations in their response to DM-1 + valine which suggested various degrees of 'valine stimulation'. For example, some which grew poorly on DM-1 in the first transfer from SDM

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

grew well in the first transfer to DM-1 + valine. In the next transfer growth was good on both media. Other strains showed significantly faster growth on valine in one or both tests, reaching a diameter of 7–8 cm on DM-1 + valine and 3–4 cm on DM-1 after 3 days incubation. Since these responses were not clearly repeatable and were not carefully investigated, only a general indication of 'valine stimulation' is shown in Table 5, and this should not be taken as a definitive classification.

(vi) *Plasmodia derived from* a × i

Thirty-nine clones of amoebae (*a.i:1, 2* etc.) derived from the cross $a \times i$ were backcrossed to the appropriate parent. The 19 plasmodia produced by backcrossing mt_2 strains to *a* (mt_1) all grew on DM-1. Of 20 plasmodia produced by backcrossing mt_1 strains to *i* (mt_2), 19 grew on DM-1 and 1 failed. This one strain failed also on DM-1 + valine and there was no indication of the inheritance of valine requirement in these progeny.

(vii) *Crosses between progeny of* a × APT1 *and* a.i *amoebae*

The results shown in Table 5 indicated that two of the mt_1 *apt-1*$^+$ strains (*12* and *35*) carried factors determining valine requirement. In order to follow the inheritance of these factors in a completely heterothallic cross, these two strains were crossed with four mt_2 strains (*a.i:25, 27, 39, 68*) which had previously

Table 6. *Results of the cross* a.APT1:12 × a.i:27

Parent strains { *a.APT1:12* (mt_1 *apt-1*$^-$) / *a.i:27* (mt_2 *apt-1*$^+$) }

(*a*) Mating-types and valine requirement among progeny

	Plasmodia tested on DM-1 and DM-1 + valine*			
	Valine requirement	No valine requirement	Not classified	Totals
mt_1	18	30	0	48
mt_2	19	18	8	45
Totals	37	48	8	93

* mt_1 progeny classified by crossing with *a.APT1:54* (mt_h *apt*$^-$). When crossed with *a.i:27* all 48 mt_1 strains gave non-requiring plasmodia. mt_2 progeny classified by crossing with *a.APT1:54* and *a.APT1:12*; where plasmodia from both crosses were obtained, the results from both agreed. Eight strains could not be classified because they failed to cross with *a.APT1:54* and gave *sax*$^-$/*sax*$^-$ plasmodia when crossed with *a.APT1:12*.

(*b*) Valine requirement and *sax* among mt_2 progeny†

	Valine requirement	No valine requirement	Not classified	Totals
mt_2 *sax*$^+$	11	11	0	22
mt_2 *sax*$^-$	8	7	8	23
Totals	19	18	8	45

† Only mt_2 progeny were classified for *sax* since *a.APT1:12* (mt_1) carried the recessive allele *sax*$^-$ and the other strains used in test crosses were *sax*$^+$.

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

given non-requiring plasmodia when backcrossed with *a* (vi). Progeny from 4 crosses were analysed (Tables 6, 7) and the four crossed plasmodia (*a.APT1:12* × *a.i:27*, etc.) were also tested for valine requirement. All four plasmodia grew on DM-1 and were stimulated by valine. Progeny of the cross *a.APT1:12* × *a.i:27* were analysed in full, to detect recombination between valine requirement and mating-type (Table 6). A total of 93 progeny clones were isolated of which 48 were mt_1 and 45 mt_2. Each of these was backcrossed to the appropriate parent and the resulting plasmodia were tested for valine requirement. All 93 clones were also crossed with *a.APT1:54* (mt_h *apt-1*$^-$), a strain which was thought to carry valine

Table 7. *Segregation of valine requirement among the progeny of four crosses between* a × APT1 *progeny and* a.i *strains*

		Plasmodia tested on DM-1 and DM-1 + valine*			
Cross	Parent strains	Valine requirement	No valine requirement	Not classified†	Totals
1	*a.APT1:12* (mt_1 *apt-1*$^-$) *a.i:27* (mt_2 *apt-1*$^+$)	37	48	8	93
2	*a.APT1:12* *a.i:68* (mt_2 *apt-1*$^+$)	41	45	1	87
3	*a.APT1:35* (mt_1 *apt-1*$^-$) *a.i:25* (mt_2 *apt-1*$^+$)	38	49	1	88
4	*a.APT1:35* *a.i:39* (mt_2 *apt-1*$^+$)	38	49	3	90
Pooled results		154	191	13	358

* All progeny strains classified by crossing with *a.APT1:54* (mt_h *apt-1*$^-$).

† Includes strains which showed doubtful growth on DM-1 and two strains (from crosses 3 and 4) which failed to grow on DM-1 and DM-1 + valine.

requirement because of the results in Table 5, and these plasmodia were tested. Tests for valine requirement followed the procedure described in section (v) above and the plasmodia were again easy to classify. Most of the strains which showed poor growth on DM-1 at the first subculture failed completely in the second while maintaining good growth on DM-1 + valine. Valine stimulation was noticed in some strains, but in many tests growth on both media was so fast that stimulation could not easily be scored. The results showed segregation of valine requirement: valine non-requirement which did not deviate significantly from 1:1 in either mating type. This suggests the segregation of one pair of alleles unlinked to mating-type. The crosses with *a.APT1:54* provided a satisfactory indication of the segregation of valine requirement among the progeny. The results agreed entirely with the expectation that *a.APT1:12* and *54* carried the same genotype for valine requirement, since every progeny clone which crossed with both these strains gave plasmodia of the same phenotype (i.e. requiring or non-requiring) with both. None of the crosses with *a.i:27* gave rise to valine-requiring plasmodia, although half of the mt_1 strains were shown to carry valine requirement by their

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

crosses with *a.APT1:54*. The mt_2 progeny were also classified for *sax*$^-$ since it was discovered that *a.APT1:12* carried *sax*$^-$ when half of the backcrosses to this strain produced plasmodia which failed to grow on SDM. The results indicated the absence of linkage between *sax* and the genes for valine requirement and confirmed the absence of linkage between *sax* and *mt* previously observed (Poulter, 1969).

The three other crosses (Table 7) were analysed in an attempt to detect different genotypes for valine requirement among the *a.i* strains. The progeny were not classified for mating type but were crossed only with *a.APT1:54* and the resulting plasmodia were tested for valine requirement by the usual procedure. Valine requirement segregated in every cross and the results do not deviate significantly from 1:1 in any set of progeny. In the pooled data, the deviation from 1:1 is significant at the 5% level, there being a slight shortage of valine requirers.

4. DISCUSSION

(i) *Inheritance of valine requirement in the cross* a × APT1

The results shown in Table 5 clearly indicate the presence of recessive alleles in certain amoebal progeny of *a* × *APT1* which cause valine requirement in homozygous plasmodia. From these results alone, one might postulate the segregation of a single pair of alleles concerned with valine requirement. This hypothesis is untenable, however, since none of the backcrosses between the same progeny strains and the parents *a* or *APT1* produced valine-requiring plasmodia (Table 4). We therefore propose that recessive alleles at two loci *valA* and *valB* must be involved, *a* having the genotype *valA*$^-$ *valB*$^+$ and *APT1* being *valA*$^+$ *valB*$^-$. If the dominant allele at either locus is sufficient to allow synthesis of valine, only the doubly homozygous plasmodia *valA*$^-$/*valA*$^-$, *valB*$^-$/*valB*$^-$ will require valine and this genotype will not be found in any of the backcrossed plasmodia. The two loci could be involved in the same enzymic step or in alternate pathways (but see (iv) below). If the two loci are unlinked, the double recessive genotype *valA*$^-$ *valB*$^-$ will be found in a quarter of the amoebal progeny and consequently in a quarter of the homothallic plasmodia. The amoebal progeny which were crossed (Table 5) provide too small a sample to test this hypothesis, but among the homothallic progeny the proportion of valine requirers is not significantly different from a quarter (see Results (iii) and Table 2). The homothallic progeny however are selected for the recombinant genotype mt_h *apt-1*$^+$ and linkage of the valine loci to either of the selected markers could affect the frequency of requirers observed. To test the 2-gene hypothesis it was therefore necessary to follow the segregation of valine requirement in other crosses.

(ii) *Crosses between* a × APT1 *progeny and* a.i *strains*

If *a* is *valA*$^-$ *valB*$^+$ as proposed and *i* is either *valA*$^-$ *valB*$^+$ or *valA*$^+$ *valB*$^+$, no valine requirers will be found when *a.i* progeny are backcrossed to *a* or *i*, as was observed (3, (vi)). On our hypothesis, *a.APT1:12* and *35* were both *valA*$^-$ *valB*$^-$

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

(from Table 5) but the *a.i* strains with which they were crossed (3 (vii), Tables 6, 7) could have been *valA*$^-$ *valB*$^+$ or *valA*$^+$ *valB*$^+$. We hoped to detect these two genotypes among the *a.i* strains used since the proportion of valine-requiring progeny in the two types of cross would be one half and one quarter respectively, assuming no linkage. (The possibility of close linkage between *valA* and *valB* can be disregarded, since this would disagree with the segregation in homothallics.) Since half of the progeny were requirers in each cross, it seems likely that all the crosses were of the type *valA*$^-$ *valB*$^-$ × *valA*$^-$ *valB*$^+$. This result might have been obtained by chance even if *i* was *valA*$^+$ *valB*$^+$ since only four *a.i* strains were tested. However it is also possible that both *a* and *i* are *valA*$^-$ *valB*$^+$ since these clones were derived from spores of the same plasmodium ('Wisconsin isolate') originally obtained from Wisconsin (Dee, 1966). The *APT1* strain was derived from the Colonia isolate (Wheals, 1970) and is probably distantly related to *a* and *i*. We have disregarded the possibility that *i* and the *a.i* progeny are *valA*$^+$ *valB*$^-$ (though this would also give 1 : 1 segregation in the crosses) because it seems unlikely that *valA*$^-$ and *valB*$^-$ are both present in the Wisconsin isolate.

Another model which will explain all the results is that a single gene involved in valine synthesis (*val*$^+$) was translocated during the history of the Wisconsin or Colonia isolate so that it lies on different chromosomes in the Wisconsin strains and in *APT1*. The plasmodium *a* × *APT1* would carry two copies of the same allele of *val*$^+$ but, being on different chromosome pairs, they would not segregate at meiosis. In fact, a quarter of the amoebal progeny would be expected to receive no copies of *val*$^+$, and these would give valine-requiring plasmodia when selfed or crossed to other strains of the same type. Other progeny crossed together would give plasmodia with 1–4 copies of *val*$^+$ and these might show variations in valine response (stimulation). All the results in Tables 2, 4 and 5 agree with these expectations. When a strain (e.g. *a.APT1:12* or *35*) carrying no copies of *val*$^+$ is crossed with an *a.i* strain, which carries one, valine requirement would always segregate 1 : 1 among the progeny. In fact, it would be associated with the segregation of a small deletion. This would account for the results in Tables 6 and 7. One possible objection to this model, however, is that unless the initial translocation was very small, the deletion might be expected to be lethal.

It is not possible on the present evidence to choose between these models but there is no doubt that in the progeny of *a* × *APT1* two pairs of factors are segregating. In the crosses between *a.APT1:12* and *35* and the *a.i* strains, the segregation of valine requirement and non-requirement is clearcut and repeatable and agrees with the expectation for a single pair of factors. We therefore consider that valine requirement provides a useful genetic marker.

The results indicate no linkage between the valine locus (*valB*) segregating in these crosses and *mt* or *sax*, but linkages between *valA* and other loci cannot of course be excluded.

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

(iii) *Valine stimulation*

If the valine-requiring plasmodia shown in Table 5 are homozygous for both recessive alleles *valA*$^-$ and *valB*$^-$, then the other plasmodia in this table consist of a number of different genotypes which carry 3, 2, 1 or none of the recessive alleles. Plasmodia formed from the amoebal strains *12*, *35*, *1*, *8*, *23*, *54*, *55* will carry at least two recessive alleles. Valine stimulation was observed among more of the non-requiring plasmodia derived from these strains (36/46) than among the other non-requiring plasmodia shown in Table 5 (6/24). Also among the backcrosses (Table 4), most of the stimulated plasmodia involved the same strains. It is suggested that a partial requirement for valine is found in strains carrying 2 or 3 recessive alleles. This would not be surprising since it is well known that organisms heterozygous for alleles causing enzyme deficiencies often produce less enzyme than wild-type homozygotes. A preliminary analysis of 30 homothallic progeny of a backcrossed plasmodium *a.APT1:1* × *a* that showed marked stimulation has supported this hypothesis. Half of these homothallics were valine-requiring as expected if the genotype of the parent plasmodium was *valA*$^-$/*valA*$^-$, *valB*$^-$/*valB*$^+$ (K. R. Gladwell, unpublished). Valine stimulation was also found in the plasmodia formed by crossing *a.APT1:12* and *35* with the *a.i* strains and these are also believed to carry three recessive alleles. Much more work is necessary before the occurrence or significance of valine stimulation can be understood. It is possible that several distinct phenotypes are involved, and no attempt has been made in the present study to distinguish these.

(iv) *Valine biosynthesis*

The pathways of valine, isoleucine and leucine biosynthesis are closely interrelated in those organisms in which they have been elucidated (Meister, 1965). The immediate precursor of valine is α-oxo-isovaleric acid which is also an intermediate in leucine biosynthesis. Since our valine requiring strains do not require leucine, one might conclude that only the final step in valine synthesis is affected by the mutation(s). However, one would then expect the valine-requiring strains to require isoleucine also since the final step in valine synthesis is catalysed by either of two transaminases, one of which is the sole catalyst of the final step in isoleucine synthesis. Since no isoleucine requirement is found one must conclude that the pathways in *P. polycephalum* differ in some respects from those in organisms previously investigated.

(v) *The use of homothallic strains in genetic analysis of* P. polycephalum

The discovery of homothallic strains in *P. polycephalum* is obviously of great importance in allowing the isolation of recessive mutations in plasmodia following mutagenesis of amoebae, and work on these lines is proceeding in several laboratories. However, the work reported here suggests another line of investigation which may also be helpful in the search for genetic markers. Homothallic mt_h *apt-1*$^+$ clones can be isolated from any cross between a heterothallic amoebal strain and

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

an mt_h *apt-1*$^-$ strain and recessive alleles can be detected in the plasmodia. In outbreeding organisms, there is often an accumulated load of recessive deleterious alleles at many different loci in natural populations which can only be detected by inbreeding programmes. Crosses with *apt*$^-$ mutants, followed by isolation of homothallic plasmodia, gives a neat and easy method for such investigations in *P. polycephalum*. This method could be useful both in investigating the genetic structure of wild populations and in isolating naturally occurring mutations useful in the laboratory. A number of different independent isolates are in culture which can be tested in this way.

In our analysis of the homothallic progeny of $a \times APT1$, the allele *sax*$^-$, which is carried by *a* (Poulter, 1969) caused the death on SDM of half of the homothallics and other lethal factors probably operated at this stage also, since more than half died (3 (iii)). Among the survivors, a quarter failed to grow on DM-1, some due to valine requirement and some apparently carrying other requirements. Since the homothallic plasmodia were presumably homozygous for many different combinations of genes from two parents rather distantly related to each other, it is not surprising that many showed poor growth and were difficult to classify. Thus, homothallics do not provide good material for a clear demonstration of the inheritance of biochemical markers, although they may be valuable for their initial detection. The subsequent genetic analysis of such markers may be done by crosses such as we have used. As we have shown, *apt*$^-$ mutants are useful in such crosses also, having the particular advantages that they cross with amoebae of all mating-types and that in every cross they give rise to homothallic progeny among which the segregation of recessive alleles affecting plasmodia can easily be determined.

We wish to thank Mrs Jennifer Foxon and Miss Eileen Pott very warmly for much excellent technical assistance. We are also grateful to Mr C. A. Beasley for photography, to Dr G. Turnock for helpful suggestions during the development of the defined medium, to Mr D. J. Cooke for some strains and to Mr K. R. Gladwell for some preliminary results. One of us (C. E. H.) was in receipt of a Special Fellowship from the National Institutes of Health (U.S.A.) and this is acknowledged with thanks. Part of this work was supported by a Science Research Council grant (No. B/SR/9722) which is also acknowledged with thanks.

REFERENCES

DANIEL, J. W., BABCOCK, K. L., SIEVERT, A. H. & RUSCH, H. P. (1963). Organic requirements and synthetic media for growth of the Myxomycete *Physarum polycephalum*. *Journal of Bacteriology* **86**, 324–331.

DANIEL, J. W. & BALDWIN, H. H. (1964). Methods of culture for plasmodial myxomycetes. *Methods in Cell Physiology* vol. 1 (ed. D. M. Prescott), pp. 9–41. New York: Academic Press.

DEE, J. (1966). Multiple alleles and other factors affecting plasmodium formation in the true slime mould *Physarum polycephalum*. *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.

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

MEISTER, A. (1965). *Biochemistry of the Amino Acids*. New York: Academic Press.

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press

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

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

WHEALS, A. E. (1971). Mutants affecting plasmodium formation in a homothallic strain of *Physarum polycephalum*. Ph.D. Thesis, University of Leicester.

WHEALS, A. E. (1973). Developmental mutants in a homothallic strain of *Physarum polycephalum*. *Genetical Research*, **21**, 79–86.

https://doi.org/10.1017/S0016672300013240 Published online by Cambridge University Press