Defined and Semi-defined Media for the Growth of Amoebae of *Physarum polycephalum*

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(Received 26 November 1975; revised 10 January 1976)

SUMMARY

Amoebae of the true slime mould *Physarum polycephalum* were cultured in two fully-defined liquid media containing amino acids, glucose, three vitamins and a buffered salts solution. Absolute requirements were demonstrated for methionine, haematin, thiamine and biotin, all of which were known to be specific requirements of the plasmodial stage. Methods are described for large-scale culture in three semi-defined media.

INTRODUCTION

The true slime mould *Physarum polycephalum* is useful for studying many biological processes, including the mitotic cycle, DNA synthesis, protoplasmic streaming and cell differentiation (reviewed by Hüttermann, 1973; Dee, 1975). However, although the multinucleate plasmodial stage can be cultured on rich synthetic and defined media (Daniel & Baldwin, 1964), and has been the subject of many biochemical studies, very little work has been reported on the nutrition or biochemistry of the other vegetative stage in the life-cycle, the uninucleate amoeba. For genetic work, in which the culture of amoebae is essential, a lawn of living or formalin-killed *Escherichia coli* on agar has generally been used as the culture medium.

Goodman (1972) reported the first soluble liquid medium for *P. polycephalum* amoebae; however, this had disadvantages for large-scale cultures since some constituents were expensive and could not be autoclaved. It was also unsuitable for some experiments because it contained a large quantity of protein [3 % (w/v) bovine serum albumin]. One aim of the present work was to develop methods for culturing amoebae in autoclavable synthetic media in sufficient quantities for comparative biochemical studies of amoebae and plasmodia. Henney, Asgari & Henney (1974) reported growth of *P. flavicomum* amoebae in two partially-defined media which were originally devised for *P. flavicomum* plasmodia (Henney & Henney, 1968). These two media have been used as a basis for investigating the specific requirements of *P. polycephalum* amoebae.

A second aim of our work was to find a fully-defined medium for *P. polycephalum* amoebae so that auxotrophic mutants could be isolated and genetically analysed using amoebae. Mutant isolation and analysis are possible in plasmodia, but are laborious (Cooke & Dee, 1975). A chemically defined medium would also allow the study of aspects of amoebal metabolism, such as the uptake of single amino acids.

METHODS

Amoebal strains. RSD4 is a heterothallic clone, mating-type 1 (*mt*₁) (Haugli, 1971), originally described by Dr F. B. Haugli, University of Tromsø, Norway, and obtained from him. The same strain was cultured in axenic medium by Goodman (1972). CLD is a strain

of the Colonia isolate, which forms plasmodia in amoebal clones under appropriate conditions; it carries allele mt_h at the mating-type locus (Cooke & Dee, 1974). APT1 is a mutant clone isolated after mutagenesis of mt_h amoebae. It fails to form plasmodia in clones due to a mutation ($apt-1^-$) at a locus unlinked to mt (Wheals, 1973).

Culture vessels. For nutritional tests, cultures (1.0 to 1.5 ml) were set up in 100 × 16 mm plastic tubes (supplied sterile with caps by Sterilin, Teddington, Middlesex), and incubated horizontally in stationary racks. Larger cultures were grown in 140 ml medical flats (5 to 10 ml cultures) incubated horizontally, or in conical flasks (25 to 500 ml cultures) on a rotary shaker. Flask capacities were approximately $5 \times$ culture volume. All cultures were incubated at 25 °C in the dark.

Inoculation, subculturing and sampling. Amoebal strains to be grown axenically were freed from live *E. coli* by allowing them to migrate along a streak of formalin-killed *E. coli* (Haugli, 1971) on liver infusion agar (Oxoid liver infusion powder, 1 g l⁻¹; agar 20 g l⁻¹). After two migrations, the amoebae were plated out on a lawn of formalin-killed *E. coli* in order to increase their number, and were then washed off the plates with axenic medium (2 ml/plate) to give a density of approximately 10⁶ cells/ml in the axenic cultures. In newly-established cultures and in subcultures it was necessary to use a large inoculum; if initial cell numbers were less than 5×10^5 cells/ml, no growth was obtained.

For growth estimations, the culture was thoroughly mixed and a small sample transferred with a Pasteur pipette to two chambers on a haemocytometer slide. Duplicate counts were made on each sample.

Subcultures were made, after counting, by transferring an appropriate volume of the culture to fresh medium to give a cell density of approximately 10⁶ cells/ml in the new culture. It was not practicable to free the amoebae of old medium by centrifuging and washing before subculturing because the cells grew poorly after such treatment.

Media. The compositions of the defined and semi-defined media used are given in Table 1. Sterilization of all components was achieved by autoclaving (15 min at 121 °C), except for bovine serum albumin (BSA) which was filter-sterilized through a Sartorius membrane filter (pore size 0.45 µm), and beef embryo extract (EE) which was reconstituted in sterile water.

Chemicals. Glass single-distilled water was used throughout. Inorganic chemicals were analytical grade. Other media constituents were: beef embryo extract (Grand Island Biological Co.; supplied by Tissue Culture Services, Slough SL1 6BY); bovine serum albumin, Cohn fraction V (Sigma); Bacto yeast extract (Difco); thiamine-HCl, biotin and amino acids (Sigma); haematin (Koch-Light). Several forms of casein hydrolysate were used with equal success: pancreatic digest of casein, N-Z Case (Sheffield Chemical; supplied by KW-Revai Chemicals, London, WC1V 6EF); Bacto-Casitone (Difco); enzymic casein hydrolysate from milk (Sigma).

RESULTS

Morphology of amoebae in liquid media

Physarum polycephalum amoebae can exist in three different forms, depending on the conditions. When grown on a lawn of *E. coli*, they have typical amoeboid morphology, with pseudopodia and contractile vacuoles. When the food supply is exhausted, they form rounded cysts which remain viable for many months. Amoebae placed in water without nutrients develop flagella and swim actively.

In the liquid media described in this paper, the majority of cells seen during sampling

Table 1. Composition of semi-defined and defined media for *Physarum polycephalum myxamoebae*

Quantities given are for 100 ml of medium. The final pH of all media was 4.7: SD and BTC needed little adjustment; ADM-13 was adjusted to the correct pH with KOH before autoclaving. For modified Goodman medium, the basal salts solution with trace elements solution and glucose was made up to 68 ml, the pH was adjusted and the mixture autoclaved before adding 31.5 ml sterile 10 % (w/v) BSA stock solution and 1.25 ml reconstituted embryo extract.

Medium	Nitrogenous component (mg)		Vitamin-yielding component* (μ g)		Glucose (g)	Basal salts solution† (ml)	Trace elements solution‡ (ml)
Modified Goodman§	Bovine serum albumin	3150	Embryo extract	$\sim 3 \times 10^4$	0.2	10	0.01
SD	Casein hydrolysate	500	Yeast extract	0.5×10^6	0.5	10	0.01
			Haematin	500			
BTC	Casein hydrolysate	500	Biotin	10	0.5	10	0.01
			Thiamine	20			
			Haematin	500			
ADM-13	L-Alanine	2.73	Biotin	10	0.5	10	0.01
	L-Arginine-HCl	2.04	Thiamine	20			
	L-Cysteine	1.30	Haematin	500			
	Glycine	1.53					
	L-Histidine-HCl	0.91					
	L-Isoleucine	0.59					
	L-Leucine	3.54					
	L-Lysine	2.13					
	L-Methionine	0.43					
	L-Phenylalanine	0.73					
	L-Serine	0.79					
	L-Threonine	0.63					
	L-Valine	0.76					
ADM-12	As ADM-13, but lacking L-phenylalanine						
ADM-5	L-Alanine	2.73	Biotin	10	0.5	10	0.01
	L-Arginine-HCl	2.04	Thiamine	20			
	Glycine	1.53	Haematin	500			
	L-Leucine	3.54					
	L-Methionine	0.43					
ADM-4	As ADM-5, but lacking L-leucine						

* Haematin was made up separately: 0.05 g was added to 100 ml of 1 % (w/v) NaOH, autoclaved and 1 ml was added per 100 ml sterile medium.

† Basal salts solution (pH 4.0) (Henney & Henney, 1968). To about 700 ml glass-distilled water (final volume 1000 ml) was added successively, with stirring, (g): citric acid, 29.78; K_2HPO_4 , 33.10; NaCl, 2.50; $MgSO_4 \cdot 7H_2O$, 1.00; $CaCl_2 \cdot 2H_2O$, 0.50.

‡ Trace elements solution (Henney & Henney, 1968). The following compounds were dissolved successively in 95 ml glass-distilled water, with stirring, (g): citric acid, 5.00; $ZnSO_4 \cdot 7H_2O$, 5.00; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 1.00; $CuSO_4 \cdot 5H_2O$, 0.25; $MnSO_4 \cdot H_2O$, 0.05; H_3BO_3 anhydrous, 0.05; $Na_2MoO_4 \cdot 2H_2O$, 0.05; $CoCl_2 \cdot 6H_2O$, 0.05.

§ Goodman (1972).

|| Henney *et al.* (1974).

were amoeboid, and cysts were formed in cultures reaching a growth plateau. Flagellated cells were seen occasionally, but amounted to less than 0.1 % of the total.

Semi-defined media

Cultures of RSD4 amoebae were grown initially in the medium described by Goodman (1972). Numerous modifications to this medium (Table 1), including the omission of

Fig. 1

Fig. 2

Fig. 1. Growth of RSD4 amoebae in three semi-defined media: ●, modified Goodman; ○, SD; ■, BTC.

Fig. 2. Test for biotin requirement of RSD4 amoebae using avidin in ADM-13 medium. Cells were transferred to the media containing avidin from ADM-13 lacking biotin. ●, Growth in ADM-13; ○, growth in ADM-13 lacking biotin containing avidin ($0.05 \text{ units ml}^{-1}$); ■, growth in ADM-13 containing avidin ($0.05 \text{ units ml}^{-1}$) and excess biotin ($0.4 \mu\text{g ml}^{-1}$).

peptone, liver infusion and chick embryo extract, the substitution of citrate-phosphate for phosphate buffer, the inclusion of a wider range of salts, and alteration of the pH from 6.3 to 4.7, resulted in an improved growth rate and yield (Fig. 1). Despite many attempts, we failed to replace BSA and beef embryo extract in the medium.

Henney *et al.* (1974) reported growth of *P. flavicomum* amoebae in two partially-defined media, and so RSD4 amoebae were inoculated into both these media. They were identical in composition except that one (SD) contained yeast extract, and the other (BTC), biotin and thiamine (Table 1). After a period of several weeks during which growth was extremely slow, good growth rates and yields were obtained in both media (Fig. 1) and these have been maintained through many subcultures. Doubling times were 2 days in SD and 3 to 4 days in BTC. Stock cultures of amoebal strains CLD and APT1 are also maintained currently in both media.

Fully-defined media

Cultures of RSD4 growing in BTC medium were used to provide inocula for tests of fully-defined media. The defined medium, AV-40, developed for *P. polycephalum* plasmodia (Daniel *et al.*, 1963) had already been tested for amoebal culture without success (Goodman, 1972). We found that a slightly modified form of AV-40, lacking phenylalanine and tryptophan, supported some growth of RSD4 amoebae, but this ceased in the fourth subculture.

ADM-12 and ADM-13. The 12 amino acids used in our modified AV-40 medium were combined with the glucose, basal salts, vitamins and trace-elements used in BTC medium.

This new medium (ADM-12) supported growth of RSD4 amoebae through numerous subcultures over a period of seven months. The minimum doubling time observed was $4\frac{1}{2}$ days. The addition of phenylalanine resulted in slightly improved growth and it is now routinely included in the medium, renamed ADM-13 (Table 1). ADM-13 medium, lacking glucose, failed to support growth of RSD4 amoebae.

ADM-4 and ADM-5. In an attempt to simplify the composition of the defined medium, seven amino acids were omitted from ADM-13, leaving alanine, arginine, glycine and methionine, the four present in the minimal medium OV-40 developed for *P. polycephalum* plasmodia (Daniel *et al.*, 1963). This simplified medium (ADM-4) supported growth of RSD4 amoebae through three subcultures. In the fourth subculture growth ceased although the cells appeared healthy and were used to inoculate further test media. Increasing the concentrations of alanine, arginine and glycine in ADM-4 to give a total amino-acid content equivalent to ADM-13 did not improve growth.

The seven amino acids omitted from ADM-4 were then re-introduced in pairs and the media were inoculated with RSD4 amoebae from the fourth subculture in ADM-4. Growth in the medium containing leucine and serine was markedly better than in the other media. Leucine and serine were therefore added singly to ADM-4 and the media inoculated as before with RSD4 amoebae from ADM-4. No growth occurred in the control or in the medium containing serine but the medium containing leucine supported growth for two weeks. Growth in this medium (ADM-5) has now continued through four subcultures.

Demonstration of requirements

Methionine. Methionine is the only essential amino acid for *P. polycephalum* plasmodia (Daniel *et al.*, 1963). Cultures of RSD4 amoebae were inoculated in ADM-12 lacking methionine. In the fourth subculture growth ceased, indicating that methionine is also an absolute requirement of amoebae.

Vitamins. Since RSD4 amoebae grew through many subcultures in BTC and ADM-12, in which the only vitamins were biotin, thiamine and haematin, it was concluded that the amoebae, like the plasmodia (Daniel *et al.*, 1963) required no other vitamins.

Requirement for thiamine and haematin had previously been indicated by experiments in which amoebae were fed on *E. coli* pre-treated with hot trichloroacetic acid and washed free of acid-soluble materials. The treated bacteria alone failed to support growth of amoebae but growth was restored when thiamine and haematin were added (C. E. Holt, L. Davidow & J. Dee, unpublished results).

Haematin requirement was demonstrated by inoculating RSD4 amoebae into SD medium and SD medium lacking haematin; growth ceased in the second subculture in the absence of haematin.

Thiamine requirement could not be demonstrated clearly until a defined medium was developed. Amoebae of strain RSD4 grew through seven subcultures in BTC medium lacking thiamine although yields were lower than in controls. When amoebae from the seventh subculture without thiamine were transferred to ADM-13 medium lacking thiamine, growth ceased immediately. It was concluded that the casein hydrolysate in BTC medium contained sufficient thiamine to support some growth.

Biotin dependence of RSD4 amoebae could not at first be demonstrated in either BTC medium or ADM-13. The cells grew at the same rate as in controls through six subcultures in BTC medium with no added biotin, and through a further two subcultures in ADM-13 lacking biotin. The cells were then transferred to ADM-13, without added biotin, containing

Table 2. *The effect of ferrous ion concentration on the growth of RSD4 amoebae in SD medium*

In each subculture, RSD4 amoebae were grown for 7 days in SD medium containing $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ at concentrations of 6.0×10^{-8} M (SD control) or 2.5×10^{-4} M (high Fe^{2+}).

Subculture	Medium	$10^{-6} \times$ Initial cell no. (cells/ml)	$10^{-6} \times$ Final cell no. (cells/ml)	No. of doublings
1st	SD control	3.6	10.0	1.4
	high Fe^{2+}	4.1	9.1	1.1
2nd	SD control	1.4	9.0	2.6
	high Fe^{2+}	1.6	7.6	2.2
3rd	SD control	1.4	11.0	3.0
	high Fe^{2+}	1.5	6.9	2.1

Table 3. *The effect of pH on growth of RSD4 amoebae in SD medium*

RSD4 amoebae were grown in SD medium for 7 days at various pH values. The results shown are from two separate experiments: the first over the range pH 3.5 to 5.0, the second pH 4.5 to 6.5.

Initial pH		$10^{-6} \times$ Initial cell no. (cells/ml)	$10^{-6} \times$ Final cell no. (cells/ml)	No. of doublings	Final pH
Expt 1	Expt 2				
3.5		2.1	1.6	—	3.7
4.0		1.9	1.6	—	4.0
4.5		1.5	3.9	1.30	4.5
	4.5	1.1	3.3	1.58	5.3
4.7		1.4	4.0	1.43	4.7
5.0		1.2	3.9	1.63	5.3
	5.0	0.8	2.3	1.45	5.5
	5.5	1.0	2.7	1.30	5.7
	6.0	0.9	1.9	1.04	5.9
	6.5	0.8	1.6	1.00	6.2

avidin (0.05 units ml^{-1} ; Sigma) which should have removed any residual biotin from the medium. Growth ceased in the second subculture (Fig. 2). The inhibitory effect of avidin was prevented in cultures having $0.4 \mu\text{g}$ biotin ml^{-1} . (1 unit of avidin is defined by the supplier as the amount which binds $1 \mu\text{g}$ of biotin.)

Iron inhibition

Specific inhibition of amoebal growth by ferrous ions was originally indicated by tests carried out on *P. polycephalum* amoebae fed on formalin-killed *E. coli* on agar supplemented with solutions of various inorganic salts (Holt & Dee, unpublished results). Thus we suspected iron inhibition in the present work when growth ceased in the fourth subculture in AV-40, which has a much higher ferrous ion content than SD and BTC. When amoebae were grown in SD medium supplemented with ferrous ions at the level used in AV-40, reduced yields were obtained in three successive subcultures (Table 2).

Optimum pH

The optimum pH for growth was determined in SD medium by adjusting the citrate-phosphate buffer to give pH values in the range 3.5 to 6.0. Best growth was obtained between pH 4.5 and 5.0 (Table 3).

Large-volume cultures in enriched media

Large-scale cultures of RSD4 have been successfully grown in modified Goodman medium, yielding sufficient quantities of amoebae for comparative studies of plasmodial and amoebal ribosomal RNA (Hall, Turnock & Cox, 1975). Large-volume cultures (500 ml) have now also been grown in SD medium in 2 l conical flasks which had indentations to increase aeration. After adding 50 ml extra water to allow for evaporation, the medium was autoclaved in the flask which was plugged with a foam bung and covered with aluminium foil. Each flask was inoculated with a 50 ml culture of RSD4 amoebae in SD medium (approximately 10^7 cells/ml) and incubated on a rotary shaker (70 rev./min). Total yield after 6 days was 5.4×10^9 cells. Calculations based on published data indicate that this number of cells should yield 3.25 mg DNA (Mohberg & Rusch, 1971) and 215 mg protein (J. Mohberg, personal communication). In the large-volume SD cultures, the amoebae tended to form clumps but this was not observed in modified Goodman medium. Recent experiments have indicated that clumping may be eliminated by altering the composition of the buffered salts solution.

DISCUSSION

Although the minimal requirements of *P. polycephalum* amoebae have not yet been defined, it is clear that they will not differ greatly from those of the plasmodia. So far, the only absolute requirements identified in amoebae are for the same three vitamins and the one amino acid (methionine) which are specifically required by plasmodia. This result was unexpected in view of the repeated failures in our own and other laboratories to culture the amoebae on any of the defined or semi-defined media developed for *P. polycephalum* plasmodia. These failures, together with the obvious morphological and functional differences between the two stages and differences in gene expression revealed by genetic studies (Dee, 1975), led us to suppose that the nutritional requirements of amoebae might be different from and more complex than those of plasmodia. The only evidence so far of a requirement in amoebae which is not present in plasmodia is the stimulation of growth by leucine. However, it is not yet clear if this indicates an absolute requirement for leucine nor whether this requirement demonstrated in RSD4 will be shown by other strains. Work is in progress to define the specific requirements of several strains of amoebae including RSD4 and CLD.

Henney & Asgari (1975) have recently cultured both amoebae and plasmodia of *P. flavicomum* in a defined minimal medium containing only three vitamins and three amino acids. They attributed their success in culturing amoebae on defined media, after many previous failures, to the gradual adaptation of the cells to nutritionally simpler media and the use of large inocula (about 10^6 cells/ml) in the initial cultures. There was evidence that *P. polycephalum* RSD4 amoebae underwent a process of adaptation when first cultured in SD medium since there was an initial period of several weeks during which growth was very poor. Similar lag periods were observed in several other strains when first cultured in axenic media. The use of large inocula was also important in *P. polycephalum*, since initial cell numbers smaller than 5×10^5 cells/ml frequently failed to give viable cultures.

Physarum polycephalum amoebae cultured on a lawn of *E. coli*, on the surface of agar or moist filter paper at 25 °C, feed by phagocytosis and multiply with a doubling time of 6 to 8 hours – a growth rate similar to the fastest observed in plasmodia on rich axenic media. Amoebal doubling times in liquid axenic media are considerably longer. Preliminary attempts to culture amoebae on semi-defined agar media without bacteria have not been

successful. For the isolation of nutritional mutants in amoebae, growth on agar-based fully defined media will be essential. However, with the liquid defined media already available, it is possible to test for nutritional requirements in amoebae carrying mutations identified in auxotrophic plasmodia (Cooke & Dee, 1975).

This work was supported by a Science Research Council grant (no. B/RG/49103) which we acknowledge with thanks. We also wish to thank Professor C. E. Holt and Mr L. Davidow for permission to quote unpublished results and our colleagues, in particular Dr Joyce Mohberg, for helpful discussion.

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