An Improved Chemically Defined Medium for Mass Cultures of *Tetrahymena*: Nutrient Uptake and Growth Regulation

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A chemically defined medium for growing mass cultures of *Tetrahymena* was developed. The growth characteristics and the effects of variations in the culture conditions and medium constituents were studied. The results show that *Tetrahymena* can be grown in a completely defined medium with rates and yields nearly comparable to those obtained in non-defined media. In the defined medium, K⁺ but not Na⁺, was indispensable for growth; unlike in media containing proteose peptone, the growth rate was independent of the presence of insoluble Fe³⁺ complexes, but was more dependent on culture temperature. Although *Tetrahymena* has an absolute requirement for preformed purine and pyrimidine bases in addition to several amino acids, its proliferation and growth were primarily under amino acid control.

INTRODUCTION

The ciliate protozoan *Tetrahymena* is currently receiving much attention (Gall, 1984), mainly because it offers several interesting features among which are its highly specific nutritional requirements (Holz, 1973) and the organization (Gorovsky, 1980) and function of its genome, in particular the ribosomal RNA genes (Engberg, 1985; Hufschmid, 1985). In its natural environment, *Tetrahymena* feeds phagotrophically on bacteria, yeasts and other particulate nutrients (Elliott, 1959; Holz, 1973). In the laboratory, however, controlled experimental conditions require cells grown in axenic and completely soluble media. Several such media have been developed, the most successful being those that contain proteinaceous complexes, liver or yeast-extract, and salts (Everhart, 1972). However, these non-defined media are not suitable for biochemical experiments which require efficient labelling of macromolecules and manipulation of the cell's nutritional environment. Therefore, the development of a chemically defined minimal medium capable of supporting reproducible growth with high yields is of great interest.

Kidder & Dewey (1951) were the first to develop a defined medium that was later revised by Elliott *et al.* (1954); however, these media gave poor generation times and final yields. Holz *et al.* (1959, 1962) introduced important modifications which significantly improved the medium but yields remained low and the growth pattern was often irreproducible. A variant of this medium was described by Rasmussen & Modweg-Hansen (1973). Since then these media have not been optimized nor have the parameters of growth of *Tetrahymena* in them been established.

In this paper a systematic study of the medium developed by Holz *et al.* (1962) is described.

METHODS

Chemicals. Guanosine, Tween 80, folic acid calcium salt and L-amino acids, except methionine, were Sigma grade from Sigma. L-Methionine (A-grade) was from Calbiochem. Folic acid, pyridoxamin.HCl, water-free glucose, citric acid and other salts were 'pro analysi'-grade from Merck. Biotin was obtained from Hoffmann-La Roche; DL-6,8-thioctic acid and riboflavin 5'-phosphate sodium salt were from Serva; other vitamins were from Fluka. ¹⁴C-Labelled compounds were from CEA.

Abbreviation: LDS, lithium dodecylsulphate.

Strains. Amiconucleate strains *T. ellioti* (CCAP 1630/Id) and *T. pyriformis* (CCAP 1630/Iz) were obtained from the Culture Collection of Algae and Protozoa, Ambleside, UK. The micronucleate *T. thermophila* strains BIV 1868 and BVII 1868 were a generous gift from Dr Nanney, Department of Zoology, University of Urbana, Illinois 1801, USA.

Preparation of stock solutions. The components and their concentrations in the stock solutions and in the final medium are listed in Table 1. Glass-distilled water was used throughout. The pH of the stock solutions of all amino acids was adjusted to 6.8 with dilute HCl or with KOH if the mixture contained free glutamic acid in place of monosodium glutamate. Amino acid and Tween solutions were filter-sterilized (0.22 µm Swinnex; Millipore). Folic acid was dissolved by adding a few drops of a dilute solution of K_2CO_3 ; a working solution (5 µg ml⁻¹) was prepared by further dilution and was filter-sterilized. Thiocotic acid solution was prepared in absolute ethanol and a working solution (5 µg ml⁻¹) was made by further dilution in 25% (v/v) ethanol. Glucose solutions were filtered (0.45 µm; Millipore) to remove particulate impurities; uracil and guanosine were dissolved by adding a few drops of 2 M-KOH; these solutions were autoclaved at 110 or 120 °C for 20 to 30 min. A 500 × stock solution of a mixture of all the trace elements was adjusted to pH 2 with concentrated HCl. All solutions except glucose, uracil and guanosine were stored protected from light at -20 °C. Soluble Fe³⁺ was a filter-sterilized solution containing 5 mg FeCl₃·6H₂O ml⁻¹. Insoluble Fe³⁺ was prepared as described by Rasmussen *et al.* (1985).

Preparation of media. Standard defined medium. For preparing 1 litre of standard medium, the appropriate amounts of each of the inorganic salts and citric acid (Table 1) were dissolved in a small amount of water – potassium phosphate and magnesium sulphate separately, citric acid and calcium carbonate together, and uracil and guanosine together (by addition of a few drops of KOH). Appropriate portions of the vitamin stock solutions (except folic acid) were mixed in about 10 ml water. Each of these solutions was transferred, with stirring, in the following order to about 200 ml water (for medium without amino acids) or of a solution containing all the amino acids except methionine, tryptophan or any other amino acid to be omitted from the medium: citric acid and calcium carbonate, uracil and guanosine, vitamin mixture, 5 ml portion of the trace-element stock solution, magnesium sulphate and finally potassium phosphate. The pH was adjusted to 6.8 with 10 M-KOH and the volume brought to 500 ml; 25 to 100 ml portions were autoclaved at 120 °C for 20 min in screw-capped bottles wrapped in aluminium foil and stored between 5 and 10 °C. This is the standard double-strength basal medium; it is stable for more than 1 year. At the time of setting up cultures, appropriate amounts of the stock solutions of amino acids, folic acid, glucose and Tween were added, and the medium was brought to the final volume with sterile (autoclaved) water.

Non-defined media. Proteose peptone yeast-extract (PPY) and proteose peptone (PP) media were prepared as described previously (Hjelm, 1970; Everhart, 1972; Cuny *et al.*, 1985).

Culture conditions. Stock cultures. The different strains of *Tetrahymena* were maintained at 20 °C as continuously growing stock cultures by frequent subculturing in PP medium in capped tubes.

Experimental cultures. Cultures were grown, unless specified otherwise, at 31 °C in subdued light on a gyratory shaker (90 r.p.m.) in Erlenmeyer flasks of capacity 10 times the volume of culture. Pre-cultures were grown in the respective medium (PPY or defined) to a density of about 8×10^5 cells ml⁻¹ and the working cultures, usually 50 to 200 ml, were seeded with these cells to an initial density of 0.5 to 1.0 × 10³ cells ml⁻¹. Cells were counted in a Coulter counter model F.

Determination of protein, RNA and DNA accumulation. Protein. Accumulation of protein was determined by measuring the incorporation of a labelled amino acid. At intervals, samples were transferred to tubes containing LDS (0.1%, w/v, final concentration); cells lysed immediately. After adding 1 vol. cold 20% (w/v) TCA and mixing, samples were left at 4 °C for 1 h, then heated for 30 min in a water-bath at 90 °C and cooled; the protein precipitate from each sample was collected on a Millipore filter and washed with cold 5% (w/v) TCA. The filters were dried and their radioactivity was determined in a toluene-based scintillation mixture.

RNA and DNA. Almost 99% of uracil incorporated by *Tetrahymena* is in RNA and DNA; therefore, the accumulation of RNA during culture growth was measured directly in a small number of cells as the radioactivity incorporated from [2-¹⁴C]uracil which was continuously present in the culture; at various intervals, two samples were withdrawn and transferred to tubes, one containing LDS (0.1%, w/v, final concentration) and the other an equal volume of 0.6 M-KOH. The cells lysed immediately in both tubes. The KOH-treated sample was kept at 37 °C for 10 h, for alkaline hydrolysis of RNA. The LDS-treated sample was immediately mixed with 1 vol. cold 20% (w/v) TCA and kept at 4 °C for 1 h; the precipitate was then collected on a Millipore filter and washed with cold 5% (w/v) TCA; radioactivity was determined as for the protein samples. This represents the cold-TCA-precipitable total radioactivity in the sample.

The KOH-treated samples were cooled to 4 °C and mixed with 0.1 vol. 3 M-HCl and 1 vol. cold 20% (w/v) TCA. The precipitates formed were processed and radioactivity was measured as described above. This represents the alkali-stable, mostly DNA, radioactivity in the sample. The radioactivity of RNA in the sample was obtained by subtracting the alkali-stable radioactivity from the total TCA-precipitable radioactivity.

Table 1. *Composition of standard defined medium*

The presence of D-isomers of amino acids and a mixture of all four purine and pyrimidine nucleosides or nucleotides is not only unnecessary but does not contribute to optimum growth; therefore, only L-amino acids, guanosine and uracil were included. Tween 80 is not essential for growth, but was added in order to prevent adherence of cells to the inner surface of acid-washed and unsilicized glassware normally used for growing and handling cultures.

Component	Stock solution concn	Final medium concn	Component	Stock solution concn	Final medium concn
L-Amino acids	(mg ml ⁻¹)	(mg l ⁻¹)	Inorganic salts	(mg ml ⁻¹)	(mg l ⁻¹)
Alanine	3.75	150	K ₂ HPO ₄ ·3H ₂ O		650
Arginine	7.50	300	MgSO ₄ ·7H ₂ O		500
Asparagine-H ₂ O	5.00	200	CaCO ₃		75
Sodium glutamate	10.00	400			
Glutamine	2.50	100	Trace elements		
Glycine	5.00	200	Fe(NH ₄) ₂ (SO ₄) ₂ ·6H ₂ O	2.840	14.20
Isoleucine	5.00	200	ZnSO ₄ ·7H ₂ O	0.900	4.50
Leucine	5.00	200	MnSO ₄ ·4H ₂ O	0.242	1.21
Lysine.HCl	5.00	200	Co(NO ₃) ₂ ·6H ₂ O	0.100	0.50
Methionine	2.25	90	CuSO ₄ ·5H ₂ O	0.060	0.30
Phenylalanine	3.75	150	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.020	0.10
Proline	5.00	200			
Serine	3.75	150	Vitamins		(µg l ⁻¹)
Threonine	5.00	200	Nicotinic acid	0.90	900
Tryptophan	3.75	150	Calcium D-pantothenate	1.50	750
Valine	3.75	150	Sodium riboflavin 5'-phosphate	0.90	450
			Pyridoxal.HCl	0.50	50
Pyrimidine/purine			Pyridoxamin.HCl	0.50	50
Uracil	12.50	37.5	Biotin	0.05	1
Guanosine	20.00	100.0	Thiamin.HCl	1.00	500
			Folic acid	1.00	20
Other organics		(g l ⁻¹)	DL-6,8-Thioctic acid	1.00	20
Glucose	500.00	10.0			
Tween 80	50.00	1.0			
Citric acid		0.6			

RESULTS AND DISCUSSION

Growth characteristics of Tetrahymena in the standard medium

When cells from late exponential cultures were seeded into fresh standard medium, exponential growth started immediately after seeding even with as few as 500 cells ml⁻¹ and ended abruptly when the maximum culture density was reached, such that the overall growth pattern was a fairly long exponential phase immediately followed by stationary phase (see Fig. 2a). There was no cell death even up to 20 h in the stationary phase cultures. The abrupt ending of the exponential phase was, however, the result of specific nutrient depletion (see below). A comparison of the growth rates and maximum yields in PPY and defined media showed that these parameters varied with strain as well as with the nature of the culture medium. Thus, the doubling time of the amiconucleate (*pyriformis* and *elliotti*) strains was 220 min in both media, but that of the micronucleate (*thermophila*) strains was 220 min in defined medium and 165 min in PPY medium; also, whereas the amiconucleate strains reached maximum densities of 2×10^6 and the micronucleate strains of 4.2×10^6 cells ml⁻¹ in PPY medium, they both reached only about 1.2×10^6 cells ml⁻¹ in defined medium. Nevertheless the values of these parameters in the defined medium represent significant improvements over those reported so far with mass cultures of *Tetrahymena* in similar media (Everhart, 1972). Some of the factors that affect the growth parameters in the defined medium were examined in the *thermophila* strain BIV.

Fig. 1

Fig. 1. Population doubling time in defined and non-defined media. *T. thermophila* was inoculated into fresh PPY medium (○) or standard defined medium (●) and cultured at temperatures from 20.5 to 30.5 °C.

Fig. 2

Fig. 2. Dependence of cell growth on the concentration of amino acids in the culture medium. *T. thermophila* was grown in (a) standard medium, (b) standard medium containing 0.1 times (○) or 4 times (●) the normal concentration of all the seventeen amino acids, (c) standard medium with lysine initially at the normal (●) or one-tenth the normal (○) concentration and after restitution, at the point indicated by arrow, with either one-tenth (△) or nine-tenths (▲) of the normal concentration.

Effect of Fe³⁺ and folic and folinic acids on growth rate

Fe³⁺ salts and folic and folinic acids have been shown to promote growth of *Tetrahymena* under different culture conditions (Rasmussen & Kludt, 1970; Orias & Rasmussen, 1976; Suhr-Jessen & Orias, 1979). The standard medium was supplemented with Fe³⁺, either as soluble FeCl₃ or as insoluble hydroxo-Fe³⁺ in colloidal or particulate form (Rasmussen *et al.*, 1985). The doubling rate, however, remained the same whether the culture was supplemented with Fe³⁺ or not. Adding ferric-EDTA in place of FeCl₃ also had no effect. When the culture temperature was raised from 28.5 to 31.5 °C, the doubling time decreased by about 60 min, and the presence of Fe³⁺ in any form had no further influence. The iron requirement is satisfied by 36 μM-Fe present in the standard medium. Similarly, addition of more folic acid either alone or along with Fe³⁺ did not alter the doubling rate significantly. Either folic or folinic acid at a concentration of 45 nM can support normal growth; increasing the concentration of folic acid alone or in the presence of folinic acid did not improve the growth rate.

Effect of temperature on growth rate in the non-defined and defined media

The number of cell divisions per hour (μ) was determined as a function of culture temperature for the *thermophila* strains. Results in Fig. 1 (a) show that, at 20.5 °C (the minimum temperature tested) μ was higher in PPY medium than in defined medium and, as the temperature was raised, μ increased in both media; however, beyond 31.5 °C, it declined in PPY medium but continued to increase, though at a slower rate, in defined medium. The maximum culture yield nevertheless remained the same at all temperatures tested (not shown). Thus, as has been reported for different strains of *Tetrahymena* in various media (Everhart, 1972; Cameron, 1973), temperature stimulated the growth rate in both defined and non-defined media. The degree of stimulation, however, was significantly higher in the defined medium.

Fig. 3. Relation between cell growth and the concentration of a single essential amino acid in the culture medium. *T. thermophila* was cultured in defined medium containing L-[U-¹⁴C]lysine [2.35 kBq ml⁻¹ (a) or 1.97 kBq ml⁻¹ (b)]. ●, No. of cells; ▲, net protein synthesis [¹⁴C]lysine incorporated into protein); △, lysine uptake (percentage of the initial amount). (a) Standard medium with normal concentration of lysine (1.37 μmol ml⁻¹); (b) 0.178 μmol lysine ml⁻¹. Arrow indicates culture stage corresponding to cessation of net protein synthesis.

Growth response to variations in concentration of the essential components in standard defined medium

When the initial concentration of vitamins, guanosine, uracil and glucose was increased or more of these components added, either individually or collectively, to a culture that had ceased growing, there was no change, but, when the concentration of the amino acids was varied, there was a profound effect. Thus, addition of a mixture of amino acids to a culture that had entered stationary phase in the standard medium led to resumption of cellular proliferation within 2 h (not shown). The standard medium is therefore limiting specifically in the essential amino acids. A fourfold increase in the initial concentration of all the amino acids resulted in a slight decrease in the doubling time but caused a substantial rise in the maximum population density (Fig. 2a, b) which reached about 3.6 to 4×10^6 cells ml⁻¹ instead of 1.2×10^6 cells ml⁻¹ in unsupplemented standard medium, a value as high as that in PPY medium; a tenfold decrease, however, resulted in a prompt cessation of growth at 2×10^5 cells ml⁻¹ (Fig. 2b). A similar growth response was also observed when the concentration of a single essential amino acid was reduced (Fig. 2c). Thus, on reduction of the amount of lysine to one-tenth its normal concentration, the doubling rate was unaltered but cellular proliferation ceased as soon as the culture reached 2×10^5 cells ml⁻¹; when the same amount of lysine was added at this stage, growth resumed but ceased again after just one more doubling of the population; if instead, the full complement of lysine was restored, the culture reached the same final density as in the complete medium (Fig. 2c). Similar results were also observed with other essential amino acids tested, e.g. valine, threonine, methionine and phenylalanine. Interestingly, as shown in Figs. 3(a, b) and 4(a), cell proliferation as well as net accumulation of RNA always ceased as soon as a little over 50% of the initial concentration of the essential amino acid had been consumed. Therefore, the abrupt cessation of growth cannot be attributed to complete depletion of amino acids.

Growth was controlled by the presence of amino acids, since cell proliferation was relatively insensitive to variation in the concentration of uracil or guanosine, even though the organism has an absolute requirement for these nucleic acid precursors. When growth ceased in standard

Fig. 4. Relation between cell growth and the pyrimidine concentration in the culture medium. *T. thermophila* was grown in the standard defined medium containing $[2\text{-}^{14}\text{C}]\text{uracil}$ (3.7 kBq ml^{-1}). ●, No. of cells; ▲, net accumulation of RNA ($[^{14}\text{C}]\text{uracil}$ incorporated into total RNA); △, uracil uptake (percentage of the initial amount of uracil consumed). (a) Standard medium with normal concentration of uracil ($335 \mu\text{mol ml}^{-1}$); (b) $45 \mu\text{mol uracil ml}^{-1}$. Arrow indicates culture stage corresponding to cessation of uracil uptake and net RNA synthesis in (a), and to total depletion of uracil and arrest of RNA accumulation in (b).

medium, because the amount of amino acids became inadequate, the medium still contained almost 50% of the initial amount of uracil (Fig. 4a). But, if the initial concentration of uracil had been reduced so that it became limiting before that of the amino acids, the cells grew normally and consumed all the uracil; then, only net accumulation of RNA ceased but both protein accumulation and cellular proliferation continued through almost two generations in the absence of uracil (Fig. 4b). This is not surprising since exponentially growing cells, when shifted to a pyrimidine-free medium, can continue to grow and divide more than twice in 24 h (Cameron, 1965).

Effect of Na^+ , K^+ and Tris on cell growth

The standard medium designed for optimum growth contained 2.4 mM-Na^+ present as monosodium glutamate, and about 11 mM-K^+ added as potassium phosphate and hydroxide. For pulse-chase experiments or for provoking nutrient starvation in cultures grown in non-defined media, the cells were generally shifted to dilute Tris buffers with or without NaCl. In order to determine the effect of these ions on cell growth, exponentially growing cells were harvested, washed and recultured either in the standard medium or in the same medium in which K^+ was replaced by Na^+ . The cells shifted to standard medium grew normally and reached stationary phase in about 15 h, whereas those shifted to medium containing Na^+ in place of K^+ failed to reach the maximum population density even after 25 h but showed normal growth if the medium also contained KCl (not shown). Tris inhibited growth when present in standard medium, and its effect, though resembling that of Na^+ , could not be reversed by K^+ .

In order to study whether K^+ alone is sufficient for optimum growth, the Na^+ in the standard medium was replaced by K^+ so that the medium now contained 13.5 mM-K^+ and perhaps traces of Na^+ . Fig. 5(a) shows that, in the absence of Na^+ but in the presence of 13.5 to 33.5 mM-K^+ , the growth rate was the same as in standard medium; NaCl added to 1 to 10 mM to medium containing 13.5 mM-KCl did not alter the growth rate (not shown), but concentrations $> 10 \text{ mM}$

Fig. 5. Effect of different concentrations of K^+ and Na^+ on cellular growth in the standard defined medium and the obligatory requirement of K^+ for optimum growth. Cells of *T. thermophila* from late exponential phase cultures grown in the defined medium were harvested and washed, and recultured as follows: (a) Na^+ -free standard defined medium (13.5 mM- K^+) supplemented with 0, 10 or 20 mM-KCl (●; all gave the same results), or with 20 (△) or 30 (▲) mM-NaCl; (b) standard medium (11 mM- K^+ , 2.4 mM- Na^+) (●) or K^+ -free standard medium with 20 (□) or 30 (△) mM- Na^+ , or after addition of 20 mM- K^+ , at the point indicated by arrow, to cultures containing 20 (■) and 30 (▲) mM-NaCl alone.

retarded growth significantly. Fig. 5(b) shows that while Na^+ cannot sustain optimum growth, K^+ , either alone or in the presence of Na^+ , can. In the standard medium containing Na^+ in place of K^+ , growth was greatly retarded with less than four generations in 50 h; however, addition of KCl at any stage, resulted in resumption of normal growth after 2 h.

The results presented in this paper suggest that *Tetrahymena* depends on membrane transport for the uptake of nutrients from the defined medium, since in the defined medium nutrients occur as low molecular mass substrates that do not stimulate the formation of digestive vacuoles that are normally required for rapid growth (Rasmussen, 1974). The growth response to variations in culture temperature, to the nature of monovalent cations and to the concentration of amino acids present indicates that transport is not by simple diffusion but is carrier-mediated and dependent on the presence of K^+ . A significant observation on the regulation of growth by amino acids is that nutrient uptake as well as cell growth and proliferation are under stringent control. Interestingly, the cells manifest such a stringent response in a growing culture as soon as the concentration of even a single essential amino acid declines to a critical threshold which is insufficient to support one more doubling of the population, at which point they arrest net accumulation of both protein and RNA.

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