APPLIED MICROBIOLOGY, Apr. 1973, p. 621-627

Vol. 25, No. 4
Printed in U.S.A.

Quantitative Growth of *Naegleria* in Axenic Culture

WILLIAM D. O'DELL AND A. R. STEVENS

Research Service, Veterans Administration Hospital, and Department of Biochemistry, University of Florida School of Medicine, Gainesville, Florida 32601

Received for publication 4 January 1973

A strain of *Naegleria gruberi*, isolated from a Vero cell culture and designated TS-1, was axenically cultivated in monolayer and mass aerating suspension culture. Cultural conditions for constant growth parameters and high-exponential cell densities were defined. Serum or other supplemental fractions were found essential in both Trypticase-yeast extract-glucose (TYG) and Casitone (CAS)-based media. Monolayer cultures grown in the CAS medium required lower levels of serum to reach maximum stationary densities of amoebae than cultures grown in the TYG medium. Heat-killed (121 C, 10 min) whole cell and cell lysate bacterial fractions were capable of replacing the serum in both the TYG and CAS media. Heat-killed bacterial fractions provided the same levels of growth as attained with serum in TYG medium, whereas the bacterial lysate supported only minimal growth in the same medium. In the CAS medium, both bacterial fractions resulted in the same level of growth which was equal to that obtained in reduced serum content. Strain TS-1 was established in suspension culture with the CAS medium used in monolayer culture. The addition of sheep red blood cells (RBC) or RBC lysate greatly enhanced growth responses. Further modifications resulted in a final medium for suspension culture consisting of Casitone-yeast extract-glucose-vitamin base, supplemented with serum and RBC lysate. This medium supported growth with a mean generation time of 9 h at 30 C and a stationary phase yield of greater than 5×10^6 amoebae per ml.

Varying degrees of success have been reported in the development of a suitable medium for the axenic cultivation of both the pathogenic and free-living strains of *Naegleria* (8). These media range from cell culture debris (8) to a basal salts medium with a bacterial fraction (5). Most of the media contain either peptone, yeast extract, liver extract, or casein derivative bases supplemented with bacterial, serum, or chicken embryo extract fractions (3, 4, 7, 10, 13, 15, 17). Although previous reports have emphasized the thermolabile nature of growth factors, unfortunately only scanty descriptions of the strains and their growth parameters have been given.

Due to the recognized potential virulence of strains of *Naegleria* as the etiological agent in human meningoencephalitis (9, 13), the demand is increasing for improved culture methods and techniques. The present report is of the mass axenic cultivation of a strain of *N. gruberi*, designated TS-1, which was isolated as a contaminant from a Vero cell culture (O'Dell, unpublished results). A description of the strain's biological characteristics and its virulence in mice will be published elsewhere.

MATERIALS AND METHODS

A description of the isolation, identification, and preliminary nutritional work for *N. gruberi*, strain TS-1, grown in monolayer culture will appear elsewhere.

The aerating culture bottles used for the axenic cultivation of strain TS-1 were the same, except for the noted modifications, as those described for *Acanthamoeba castellanii* (12). The modifications included the use of a double air train, the addition of an exhaust air trap, and the elimination of the sampling port (Fig. 1). The bottles were cleaned and siliconized according to Neff et al. (12).

The double air train was made of 25-mm (outer diameter) by 250-mm Pyrex glass tubing. The train was loosely packed with cotton, and both ends were stopped with a single-hole, rubber stopper fitted with a bent glass tube. The stoppers were secured with autoclaved tape (3M Company) and the trains were connected with surgical Latex tubing from the top of the primary train to the bottom of the secondary train. The exhaust air trap was connected to the

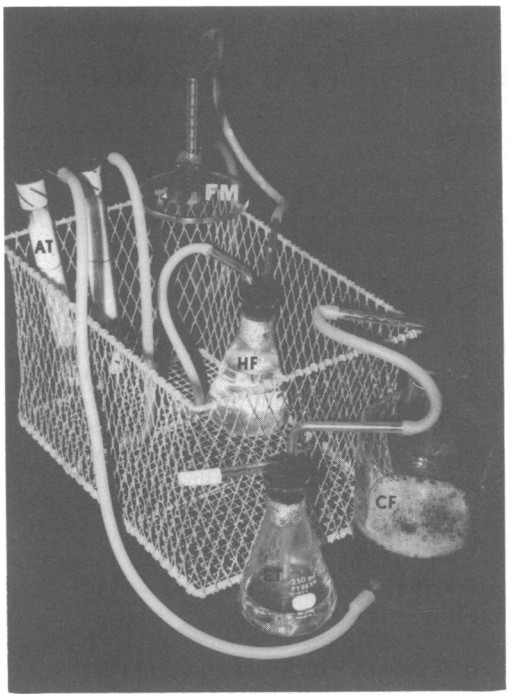

FIG. 1. *Apparatus for mass aerating cultures of Naegleria. Identical set-ups are used for culturing Acanthamoeba strains (Neff; A-1; HN-3). Abbreviations: FM, flow meter; AT, air train, HF, humidifying flask; ET, exhaust air trap; CF, culture flask.*

aerating bottle with surgical tubing in a manner similar to that for the incoming humidifying flask. Exhaust air was bubbled through 1% Formalin or other suitable disinfectant for the pathogenic strains.

Sampling and subculturing were accomplished with 20-gauge, 1.5-inch (3.8-cm) needles and sterile disposable syringes (Fig. 2). Prior to sampling, aeration was discontinued by clamping the incoming tubing. To prevent backflow into the culture bottle of the disinfectant, the tubing leading to the exhaust air trap was clamped during sampling. The Latex tubing connected to the spout of the aspirator bottle was flushed with 95% ethyl alcohol from a washing bottle prior to and after sampling. Aeration was begun prior to final withdrawal of the sampling needle from the tubing. These modifications have been used successfully for more than one year in the routine maintenance of *A. castellanii* (nonpathogenic, Neff strain [12]; pathogenic A-1 and HN-3 strains [9]) in this laboratory with a very minimum of contamination.

Axenic media were prepared by first heating the dry ingredients in approximately 1/10 the final volume of distilled water on a hot plate until dissolved. The concentrated solution was then centrifuged to remove the particulate debris at 17,000 $\times$ *g* for 20 min. Unless otherwise noted, the clarified concentrate was added to a base, designated PAS, which consisted of Page's amoebae saline (14) supplemented with a vitamin mix containing, per liter, 1.0 mg of thiamine hydrochloride (Calbiochem), 0.2 mg of d-Biotin (Sigma Chemical Co.), and 1.0 μg of B_{12} (Sigma) (12, see also 1, 6). The pH was adjusted, if necessary, to 6.8 to 7.0 prior to dispensing 300 ml in 1-liter aspirator bottles and autoclaving (121 C, 15 lb/in^2) for 30 min. Additions to the autoclaved media in aerating bottles (e.g., serum or red blood cell [RBC] lysate) were made through a Swinnex type HA (0.45 μm) filter just prior to subculturing.

Counts for growth curves were done with a Neubauer Bright Line Phase Hemacytometer. Monolayer cultures were shaken prior to sampling for cell counts. Data were compiled from observations on two or more experiments in all instances.

RESULTS

Previous nutritional studies indicated that *N. gruberi*, strain TS-1, could be grown successfully in monolayer culture using a medium consisting of (wt/vol) 0.5% Trypticase (BBL), 0.5% yeast extract (Difco), and 1.0% glucose, designated TYG, and supplemented with 10.0% dialyzed fetal calf serum (DFCS; Gibco) (O'Dell, unpublished results). Fifty milliliters of this medium, in 250-ml Erlenmeyer flasks, supported a maximum yield at stationary phase of about 8 $\times$ 10^5 cells per ml and an average generation time of 10 to 11 h at 37 C (Fig. 3). The cell population at confluency was 2.5 $\times$ 10^5 cells per ml. Attempts to replace the DFCS with calf serum (Flow) or to reduce the DFCS concentration in the TYG medium resulted in decreased yields of cells at stationary phase (Fig. 3). The medium would not support growth in the absence of the serum supplement.

Since the objective of the present study was to obtain maximal quantities of cells with

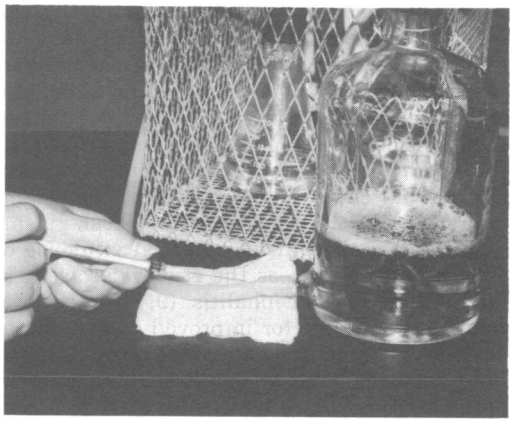

FIG. 2. *Sampling an aerating culture of Naegleria with sterile disposable syringe and needle.*

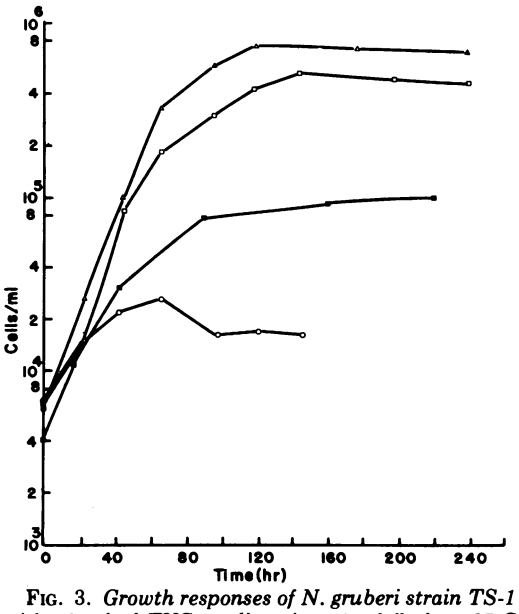

FIG. 3. *Growth responses of N. gruberi strain TS-1 with 50 ml of TYG medium in 250-ml flask at 37 C supplemented with various serum fractions. Symbols: O, 1% dialyzed fetal calf serum (DFCS); □, 5% DFCS; △, 10% DFCS; ■, 10% calf serum. See Results for medium composition.*

TABLE 1. *Maximum stationary phase populations for media with various supplemental fractions*[a]

Fraction[b]	TYG	2% CAS
10.0% Dialyzed fetal calf serum	8×10^5	8×10^5
1.0% Dialyzed fetal calf serum	2×10^4	3×10^5 to 4×10^5
10.0% Calf serum	1×10^5	NT[e]
Autoclaved *E. coli*[c]	7×10^5 to 8×10^5	4×10^5
E. coli lysate[c]	2×10^4	3×10^5
Fraction IV 4	NG[d]	NG
None	NG	NG

[a] Values given as cells per milliliter in 250-ml monolayer flasks containing 50 ml of media at 37 C. See Results for media composition.

[b] Sources: dialyzed fetal calf serum, Gibco; calf serum, Flow; fraction IV, Pentex.

[c] 1.0 ml of a 0.13% (wt/vol) wet-weight suspension or an equivalent of lysate.

[d] NG, No growth.

[e] NT, Not tested.

constant growth parameters and not primarily to define nutritional requirements, additional work was carried out in monolayer cultures in an attempt to promote a higher level of growth. Cerva (7) reported successful axenic cultivation of pathogenic strains of *Naegleria* in monolayer cultures in a medium consisting of only 2% Casitone in distilled water and supplemented with serum, but previous attempts to culture TS-1 in this medium had resulted in only moderate growth. However, efforts to replace the TYG components with Casitone led to a modified Casitone medium (designated 2% CAS) that provided growth comparable to that in the TYG medium. Such a modification consisted of the preparation of 2% Casitone in PAS with vitamins.

The major difference noted in the cell's growth in the 2% CAS medium was in response to the concentration of serum (Table 1 and Fig. 4). Stationary population densities equivalent to that acquired in TYG-10% serum could be obtained in 2% CAS medium containing only 5% serum; increasing the serum level to 10% was without effect on the stationary yield. Reduction of serum level to 1.0% resulted in only a twofold decrease in stationary density in the 2% CAS medium compared to the 40-fold de-

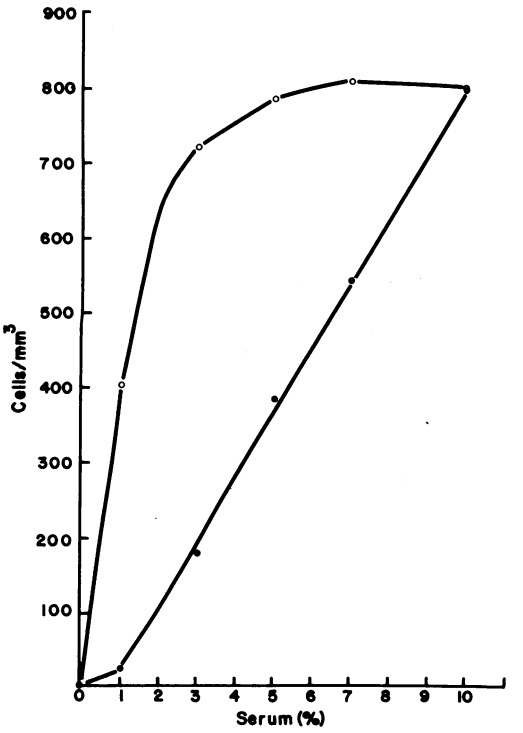

FIG. 4. *Maximum stationary phase population in monolayer cultures of strain TS-1 grown either in TYG (●) or in 2% CAS (O) at various serum concentrations. See Results for medium composition.*

crease in TYG-1% serum medium. In the complete absence of serum, however, the 2% CAS medium could not support growth of TS-1.

A comparison of the increased growth response of TS-1 to increasing serum concentrations for both the TYG and 2% CAS media in monolayer cultures is shown in Fig. 4. As can be seen, the final population density at stationary phase in TYG medium was roughly proportional to the amount of serum in the medium. Similar results have been obtained in studies of the serum requirement in the growth of higher cell types (19, 21). However, the final stationary population density attained in 2% CAS was not directly related to the serum concentration above 1.0%. This observation implied that some other factors may become growth limiting in the Casitone-based medium.

Subsequent studies were performed to attempt total replacement of the serum requirement with supplemental fractions in either the TYG or CAS-based media. A logical substitution for testing was suggested from previous studies which indicated that TS-1 could be successfully cultivated with an autoclaved bacterial fraction (O'Dell, unpublished results). For the present study, *Escherichia coli* strain NRS-I (obtained from M. M. Brent) was grown in fluid Brain Heart Infusion (Difco) at room temperature on a reciprocal platform shaker. The cells were harvested aseptically by centrifugation (15,000 $\times$ *g*, 10 min) and washed twice in sterile PAS. The cells were suspended in sufficient PAS to make a wet-weight (wt/vol) 0.13% suspension and autoclaved at 121 C for 10 min.

As noted in Table 1, whole-cell, autoclaved bacteria could totally replace the serum supplement in the TYG medium but could only provide stationary densities in the 2% CAS medium equivalent to that obtained in the presence of 1.0% serum. Since *Naegleria* will engulf dead bacteria (16), the whole-cell supplement could be acting in either a nutritional or another capacity, e.g., stimulation of phagocytosis thereby resulting in the increased uptake of medium components. Thus, to further elucidate the role of bacteria in the replacement of the serum, a bacterial lysate was tested for growth of TS-1 in either TYG or CAS-based medium. A cell lysate of *E. coli* was prepared by disruption of the cells at 14,000 lb/in^2 in a French pressure cell (Aminco Industries). The lysate was centrifuged in the cold at 17,000 $\times$ *g*, and the supernatant was membrane-filtered (Millipore Corp., Swinnex type HA, 0.45 μm pore size) before addition to the cultures. Although the lysate supported an equivalent level of growth in CAS medium as the whole cell supplement, it provided only limited growth in the TYG medium.

Studies on higher cell types have indicated that the alpha globulins are the biologically active fraction in serum (20). A similar observation was made by Balamuth (3) in nutritional studies of *N. gruberi*. Therefore, growth of strain TS-1 was examined in TYG or CAS base supplemented with an alpha 4-globulin fraction. Serum fraction IV-4 (Pentex) was prepared according to the method of Fulton (10). As noted in Table 1, replacement of serum with the globulin fraction failed to support the growth of TS-1 in either medium. The biological activity of other alpha globulins remain to be tested.

Results of the above studies indicated to us that factors other than nutrients might be limiting the growth of TS-1 in monolayer cultures, e.g., surface area, medium depth, etc. To overcome such limitations, growth of TS-1 was next examined in an aerating suspension system which had been considerably advantageous in obtaining high levels of growth of strains of *Acanthamoeba* (12, 18). Figure 5 shows the major steps in the evolution of an axenic medium suitable for the mass aerating cultivation of strain TS-1. Initially the 2% CAS-10% DFCS was used; however, it was noted that under the conditions of aerating culture, 10% DFCS resulted in considerable foaming. For this reason, 1% serum, added just prior to subculturing with a Swinnex type HA (0.45 μm) filter, was used routinely. The curve designated 2% CAS in Fig. 5 was the same medium as described above for the monolayer cultures. Maximum yield at stationary phase was just slightly lower than that obtained in the monolayer cultures with equivalent concentrations of serum. However, as noted in Fig. 5, this medium allowed only a continuously decreasing rate of growth rather than a constant logarithmic growth. Additionally, it was noted that the cells became vacuolated and variable in their growth response after an extended period of cultivation in the 2% CAS medium at 1.0 cfh air flow. Increasing the concentration of Casitone to 4.0% and decreasing the air flow to 0.7 cfh brought the cells back to a more consistent growth response. Nevertheless, it became apparent that the Casitone medium alone was not capable of supporting strain TS-1 for extended periods (i.e., greater than 3 months) with constant parameters of optimal growth.

Addition of a few drops of a 2% sheep RBC suspension to cultures of pathogenic *Naegleria* has been shown to result in enhanced growth of pathogenic *Naegleria* (S. L. Chang, personal

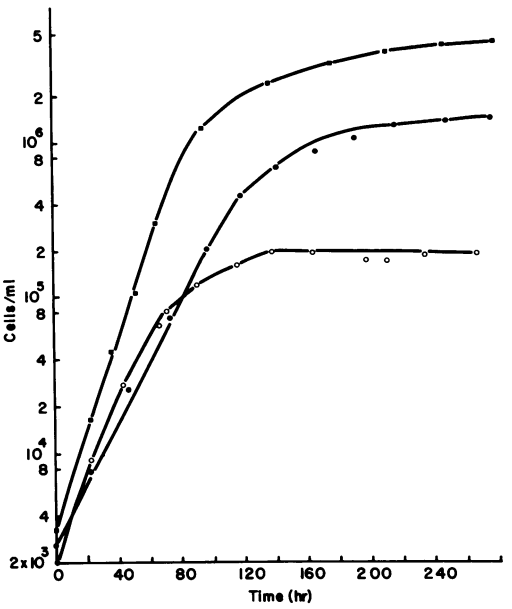

FIG. 5. *Growth curves of N. gruberi strain TS-1 grown in aerating culture flasks at 30 C with various media. See text for details of media compositions and apparatus. Symbols:* ○, *2% CAS;* ●, *CY;* ■, *CYM.*

communication). Subsequent studies in which the concentration of Casitone was varied and the medium was enriched with yeast extract, glucose, and RBC led to a modified CAS medium for use in the suspension cultures. The medium consisted of (wt/vol) 1.0% Casitone (Difco), 0.5% yeast extract (Difco), 1.0% glucose, vitamins, 1.0% DFCS, and the addition of 0.3% (vol/vol) of a sheep blood/Alsevers solution (BBL). This medium was designated CY, and a typical growth curve is shown in Fig. 5. As noted, the medium led to a 10-fold increase in the cell density and resulted in a more constant logarithmic type growth and a generation time of approximately 9 h at 30 C.

The whole sheep RBC would normally undergo lysis in the CY medium within 6 to 12 h after addition and usually resulted in cultures with considerable debris. Thus attempts were made to replace the whole blood cells with a lysate. The lysate was prepared by adding the blood/Alsevers solution to twice its volume of distilled water and centrifuging at 36,000 × *g* for 30 min to remove the RBC ghosts. The supernatant fluid was then filtered under a vacuum through a membrane filter (Millipore type SM; 5.0 μm pore size). The filtered lysate was frozen until required. Prior to subculturing, the lysate was added to the culture medium through a Swinnex type HA (0.45 μm) filter to a concentration of 1.0% (vol/vol). The sheep RBC lysate supported growth identical to that of the whole sheep RBC. Moreover, the lysate generally gave cultures much freer from fibrous debris than did the whole RBC.

A final attempt was made to further improve the growth of TS-1 in aerating suspension cultures. Fulton (11) described the axenic cultivation of *Naegleria* strains in a medium containing 0.3 mM L-methionine. The final curve in Fig. 5, designated CYM, represents the improved growth response of strain TS-1 in the CY medium described above and further supplemented with 0.6 mM DL-methionine and RBC lysate. The methionine and RBC lysate additions provided an extended period of exponential growth, a fivefold increase in stationary densities, and a slightly faster generation time than obtained in CY medium. The CYM medium was prepared and autoclaved as described in Materials and Methods; the serum and RBC lysate were added just prior to subculturing. The air flow rate was adjusted to 0.7 cfh for a 300-ml culture in a liter aspirator flask, and the culture was incubated at 30 C.

N. gruberi strain TS-1 trophozoites from the CYM medium tended to be slightly rounded upon initial sampling, but became more typically monopodial and actively moving after being allowed to settle and attach to the counting chamber. Settled trophs, from exponentially growing cultures, appeared typical with slight vacuolization and were only rarely multinucleated. With increasing culture age and increased cell density (e.g., greater than 2×10^6 to 3×10^6 cells per ml), exobuds (10) became more common and the degree of debris increased. These exobuds were small non-nucleated bits of cytoplasm, most often hyaline rather than granular.

Encystment occurred spontaneously in older stationary phase cultures in the complete CYM medium, but rarely exceeded 30% mature typical cysts. Spontaneous encystment in any of the other media was a rare event and then most often only abortive cysts were formed. Typical biflagellate cells frequently appeared in the complete CYM and the CY media when the stationary phase growth was above 2×10^6 cells per ml. When present, these flagellates accounted for less than 1.0% of the total population. They appeared spontaneously 1 to 2 days after initiation of stationary phase and persisted only a day or so. They were, however, not an invariable feature of the suspension cultures.

DISCUSSION

The Casitone medium supported the highest level of growth of *N. gruberi*, strain TS-1, in

monolayer cultures under the conditions of decreased serum content. Neither this medium nor the TYG could support growth in the complete absence of serum or other similar fraction (Table 1). Maximum cell density for either medium under optimum conditions was 8×10^5 cells per ml or between one and two doublings past confluency. Additional evidence of the differences in the nutritional value of the two media can be found in Fig. 4.

N. gruberi, strain TS-1, grew best with dialyzed fetal calf serum, moderately well with normal calf serum, and not at all with normal bovine serum (O'Dell, unpublished results). These findings are consistent with those of others (2) for nonpathogenic strains of *Naegleria*. Pathogenic *Naegleria* are not as exacting in their serum requirements and grow well with a number of different sera (E. C. Nelson, personal communication).

The concentration of the essential factor(s) supplied by the serum apparently determines, within the range examined, the cell density at stationary phase for the TYG medium. This relationship between serum concentration and cell density did not hold true for the Casitone medium. With this medium, serum concentrations greater than 5% resulted in no increase in cell density which indicates that other factor(s), nutrient or otherwise, were growth limiting.

Considering the results of the studies in which bacterial fractions were substituted for the serum, it would appear that at least two separate factors may be supplied by the serum that stimulate the growth of TS-1. One of these factors may be present normally in the TYG medium but not actively taken up by the cells. The addition of whole-cell bacteria to the TYG medium could stimulate uptake of this media component in addition to providing the second factor also offered by the serum. The fact that the whole-cell bacteria cannot support the level of growth in the CAS medium equivalent to that obtained with high concentration of serum would in turn imply the absence of the component present in the TYG medium. Thus, cells growing in CAS-based medium obtain both factors from the serum supplement. The manner by which the serum factors act in promoting growth of TS-1 cannot be deduced from the present studies. However, information accumulating from studies on higher cell types indicates that there may be a variety of biological effects by serum factors that are involved in promoting cell multiplication (20).

The growth response demonstrated with the autoclaved whole-cell bacteria preparation in the present studies is in contrast to that reported by others for cultivation of *Naegleria* strains. Bacteria killed at temperatures above 100 C were inactive in supporting growth (8, 15). Conceivably this difference may be explained by the use of different types of bacteria. *Aerobacter aerogenes* was used mainly by other workers. Nevertheless, our success with autoclaved *E. coli* in culturing TS-1 leaves open to question the reported absolute thermolabile nature of essential nutrients for cultivation of *Naegleria* (8, 15).

Although many aspects of the studies on monolayer growth of TS-1 remain to be examined further, the results obtained greatly facilitated our subsequent efforts to mass cultivate TS-1. Introduction of suspension culture and additional modifications in the 2% CAS-based medium led to a 10-fold increase in cell density over that acquired in the monolayer cultures. The addition of sheep blood/Alsevers lysate and methionine stimulated growth considerably, but neither would replace the serum supplement. These additives obviously supplied a factor(s) which enhanced cellular growth but which was either essential and present before in minimal quantities or nonessential and absent. That is, these factors enhance growth but are not in the category of essential nutrients. Neither Fulton (11) nor Chang (personal communication) attempt to explain the enhancement abilities of the additives, and we can offer no explanation for their potentiating effect on growth of TS-1 at this time.

The use of the aerating suspension cultures greatly facilitates handling and sampling amoebae strains. Moreover, the experimental addition of reagents for chemotherapy studies, for biochemical studies and labeling experiments, and for nutritional studies can be simply performed. The constant parameters of growth and the ease with which large numbers of cells can be obtained in defined periods of growth should finally allow critical cytological, biochemical, and physiological definition of *Naegleria* strains.

Naegleria gruberi strain TS-1 was isolated as an accidental contaminant from a Vero cell culture. The strain demonstrates all the properties of a pathogenic strain, but recent studies by us indicate that it is of low virulence or avirulent in mice. Nevertheless, characteristics of the strain (i.e., its ability to proliferate at temperatures up to 42 C, to produce cytopathic effect in tissue culture, etc.) make it useful as a control in studies involving the pathogenic strains. The pathogenic strains of *Naegleria*, obtained from E. C. Nelson, are presently being maintained in our laboratory, and work is in progress to adapt these strains to similar aerating culture conditions.

ACKNOWLEDGMENTS

This study was supported by designated research funds from the Veterans Administration, project No. 5148-01; Public Health Service grants AI-09608 from the National Institutes of Health awarded to A. R. Stevens (Larkin), Principal Investigator; and AI-52313-01 from the National Institutes of Health awarded to W. D. O'Dell, Postdoctoral Fellowship.

LITERATURE CITED

1. Adam, K. M. G. 1959. The growth of *Acanthamoeba* sp. in a chemically defined medium. J. Gen. Microbiol. **21**:519–529.
2. Anderson, K., and A. Jamieson. 1972. Primary amoebic meningoencephalitis. Lancet **1**:902.
3. Balamuth, W. 1964. Nutritional studies on axenic cultures of *Naegleria gruberi*. J. Protozool. **11**:19–20.
4. Balamuth, W., and D. Outka. 1962. Cultivation of the amoebo-flagellate, *Tetramitus rostratus*, in a chemically defined medium. Nature (London) **193**:698–699.
5. Balamuth, W., and G. Visvesvara. 1970. Differential growth requirements of several genera of small free-living amoebae. J. Protozool. **17**:10.
6. Band, R. N. 1961. Biotin, a growth requirement for four soil amoebae. Nature (London) **192**:674.
7. Cerva, L. 1969. Amoebic meningoencephalitis: axenic culture of *Naegleria*. Science **163**:576.
8. Chang, S. L. 1960. Growth of small free-living amoebae in various bacterial and in bacteria-free cultures. Can. J. Microbiol. **6**:397–405.
9. Culbertson, C. G. 1971. The pathogenicity of soil amoebas. Annu. Rev. Microbiol. **25**:231–254.
10. Fulton, C. 1970. Amebo-flagellates as research partners: the laboratory biology of *Naegleria* and *Tetramitus*. *In* D. M. Prescott (ed.), Methods in cell physiology, vol. 4. Academic Press Inc., New York.
11. Fulton, C. 1972. Early events of cell differentiation in *Naegleria gruberi*. Synergistic control by electrolytes and a factor from yeast extract. Develop. Biol. **28**:603–619.
12. Neff, R. J., S. A. Ray, W. F. Benton, and M. Wilborn. 1964. Induction of synchronous encystment (differentiation) in *Acanthamoeba*, p. 55. *In* D. M. Prescott (ed.), Methods in cell physiology, vol. 1, Academic Press Inc., New York.
13. Nelson, E. C., and M. N. Jones. 1970. Culture isolation of agents of primary amebic meningoencephalitis. J. Parasitol. **56**:248.
14. Page, F. C. 1967. Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. J. Protozool. **14**:499–521.
15. Schuster, F. L. 1961. Axenic cultivation of *Naegleria gruberi*. J. Protozool. **8**:19.
16. Schuster, F. L. 1963. An electron microscope study of the amoebo-flagellate, *Naegleria gruberi* (Schardinger). I. The amoeboid and flagellate stages. J. Protozool. **10**:297–313.
17. Schuster, F. L., and G. Svihla. 1968. Ribonucleoprotein-containing vesicles in cysts of *Naegleria gruberi*. J. Protozool. **15**:752–758.
18. Stevens, A. R., and P. F. Pachler. 1972. Discontinuity of 26S rRNA in *Acanthamoeba castellani*. J. Mol. Biol. **66**:225–237.
19. Temin, H. M. 1966. Studies on carcinogenesis by avian sarcoma viruses. III. The differential effect of serum and polyanions on multiplication of uninfected and converted cells. J. Nat. Cancer Inst. **37**:167–175.
20. Temin, H. M., R. W. Pierson, Jr., and N. C. Dulak. 1972. The role of serum in the control of multiplication of avian and mammalian cells in culture, p. 50–83. *In* G. H. Rothblat and V. J. Cristofalo (ed.), Growth, nutrition and metabolism of cells in culture, vol. 1. Academic Press Inc., New York.
21. Todaro, G. J., Y. Matsuya, S. Bloom, A. Robbins, and H. Green. 1967. Stimulation of RNA synthesis and cell division in resting cells by a factor present in serum, p. 87–98. *In* V. Defendi and M. Stoker (ed.), Growth regulating substances for animal cells in culture. Wistar Inst. Symp. Monogr. No. 7. Wistar Inst. Press, Philadelphia.