Hemin Replaces Serum as a Growth Requirement for *Naegleria*

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Four strains of *Naegleria gruberi* were grown axenically without serum. Serum was replaced by hemin or two selected hemoproteins. Aside from the utility of eliminating serum from the culture medium, the present work shows that *Naegleria* does not require intact protein, and establishes a specific micronutrient requirement for this amoebo-flagellate.

The amoebo-flagellate *Naegleria* has attracted attention for studies of cytodifferentiation (5) and the pathogenicity of some strains to man (4). *Naegleria* grows readily on living or dead bacteria, and since 1964 (1) has been adapted to several axenic media, all containing an essential protein component (e.g., whole serum, serum fraction IV-4, bacterial lysate, etc.). The variability between batches of serum and the objective of developing a simplified medium for experimental studies led us to seek a replacement for the protein moiety. The starting point was medium H-4 base, as currently formulated in Balamuth’s laboratory (see below). Exploratory trials showed that lysates prepared from sheep erythrocytes could replace the serum supplement; this led to tests of certain organic-iron complexes present in serum. This report demonstrates that medium H-4 base plus a porphyrin supports excellent axenic growth of *Naegleria*.

**MATERIALS AND METHODS**

*Naegleria gruberi*, strain EG, primarily used in the present study, has been maintained axenically for a decade in Balamuth’s laboratory. It does not exhibit the pathogenic potential reported for the closely related *N. fowleri* characterized by Carter (4). Other strains of *N. gruberi* tested, 1518/1, CR45 and CR33B, were obtained from F. Schuster, Brooklyn College. Stock cultures were maintained on medium H-4 base supplemented with 10% (vol/vol) calf serum. The medium consisted of 1% (wt/vol) proteose peptone (Difco), 0.5% (wt/vol) yeast extract (Difco), 1% (wt/vol) liver extract (Oxoid), 0.03 M glucose, and 10 mM phosphate buffer (pH 6.5). The base was autoclaved as a unit, while supplements were sterilized by passage through membrane filters (Millipore Corp.) of 0.45 μm porosity.

Amoebas were incubated in 5-ml volumes of test media at 28 C in slanted culture tubes (16 by 125 mm outer diameter) for assay of various serum replacements. Where specified, growth was also measured in suspension cultures (10 ml), using silicone-coated, 50-ml Erlenmeyer flasks incubated on a rotary shaker (100 rpm) at 28 C (2). Growth rates and maximum yields were determined by duplicate hemacytometer counts. Prior to making counts, we chilled cultures in ice and agitated to remove amoebas from tube walls and to disperse clumps of amoebas from shaken cultures. Some amoebas in suspension cultures became multinucleate and heterogeneous in size so that growth, expressed as increase in number of amoebas per milliliter, underestimated mass (3). Exponential growth in slanted tubes was limited to low amoeba densities (i.e., less than 10⁵/ml) so that most of the growth curve was represented by a decelerating growth rate. Generation times were determined during early growth (Table 1).

Replacements for the serum supplement in medium H-4 were tested by serial subcultivation in parallel with controls in medium H-4 base alone (i.e., devoid of supplements). The latter supported reduced growth only through two serial subcultures. For those supplements supporting growth, data were obtained (Table 1) from later serial subcultures. Initial subcultures of a test series were made by first gently removing the culture medium while leaving most amoebas adhering to the wall of the tube (i.e., "wall-hangers"). These wall-hangers were then washed twice in phosphate-buffered saline (PBS); 137 mM NaCl, 3 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄ (pH 7.4). Subsequent serial subcultures were inoculated with PBS-washed suspensions which were adjusted to deliver inocula in the range of 0.1 × 10⁴ to 0.5 × 10⁴ amoebas per ml. Supplements (obtained from Sigma Chemical Co., except for catalase which was obtained from Worthington Biochemical Corp.) were dissolved in PBS, filter-sterilized, and dispensed into medium H-4 from concentrated stocks to yield a 10% (vol/vol) dilution of the final medium (20% in the case of hemoglobin). Hemin and hemoglobin, not readily soluble at neutral pH, were dissolved at pH 10.5 before adding to medium H-4, without altering the final pH (pH 6.5). Controls uniformly received 10% (vol/vol) PBS.
Table 1. Growth of Naegleria gruberi (strain EG) in supplemented medium H-4

| Supplement (µg/ml) | Maximum yield (no. organisms x 10⁴/ml) | Generation time (h)* |
|-------------------|----------------------------------------|----------------------|
|                   | 4*                                     | 5*                   | Avg*     |
| Hemin             |                                        |                      |          |
| 1 (shaker)        | 6.20, 5.56                            | 6.23, 5.32           | 5.83     |
| 1                 | 2.36, 2.58                            | 2.13, 2.69           | 2.44     |
| 0.1               | 2.11, 1.82                            | 1.67, 1.27           | 1.72     |
| 0.01              | 0.31, 0.40                            | 0.19, 0.33           | 0.31     |
| Hemoglobin        |                                        |                      |          |
| 100               | 2.03, 2.40                            | 2.09, 2.27           | 2.20     |
| 10                | 1.98, 1.61                            | 1.77, 2.02           | 1.85     |
| 1                 | 0.75, 0.79                            | 0.78, 0.59           | 0.73     |
| Catalase          |                                        |                      |          |
| 40                | 2.28, 2.18                            | 2.25, 2.58           | 2.32     |
| 10                | 0.95, 0.74                            | 0.58, 0.76           | 0.76     |

*— Serial subculture number. Data for duplicate cultures incubated at 28°C.
*— Generation time given as an average of duplicate cultures, was determined at densities of less than 10⁴/ml and counted at 0, 6, 12, and 24 h after inoculation.
*— Incubated on a rotary shaker (100 rpm); multinuclearity prevented an accurate estimate of generation time.

RESULTS

For strain EG, substances replacing the serum supplement in medium H-4 are given in Table 1. The maximum yield in slanted tubes (Table 1) was limited by conditions other than the culture medium, at least for hemin in which shaker incubation significantly increased the maximum yield. The generation time was similar for most concentrations of supplements (i.e., 8 h) in slanted tube cultures. Growth in medium H-4, supplemented with 10% (vol/vol) serum, was similar to growth in medium H-4 supplemented with 1 µg of hemin per ml. (Table 1). The following related substances (obtained from Sigma Chemical Co.) did not support growth of strain EG, each at concentrations of 1 and 10 µg/ml: cytochrome c (type III); chlorophyllin (Na-Cu salt); protoporphyrin-IX-dimethyl ester; 5-aminolevulinic acid HCl. Strains 1518/1, CR45, and CR33B were grown on medium H-4, supplemented with 10 µg of hemin per ml, through six serial subcultures, which indicated that the serum supplement could be replaced for these as well, although in the presence of higher concentrations of hemin.

DISCUSSION

The serum requirement for the growth of N. gruberi in medium H-4 is actually a heme requirement which can also be satisfied by at least two hemoproteins, hemoglobin and catalase (Table 1). Metabolic precursors (i.e., protoporphyrin-IX-dimethyl ester, 5-aminolevulinic acid) and some related compounds did not satisfy this requirement. The absence of adequate usable heme from proteose peptone, yeast extract, and liver extract was unexpected. Obviously, serum still may provide important nutrients in media with low nutrient levels, but the absence of any absolute requirement in axenic cultures for protein or compounds peculiar to serum marks a distinct advance in efforts to identify the specific nutritional requirements of N. gruberi. Finally, technical difficulties in the use of serum (e.g., sterility, antibodies, and other inhibitors) are eliminated.

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