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Growth of Moth Cells in Suspension in Hemolymph-free Medium

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Only a few insect cells have been established in culture. Grace reported the successful culture of mosquito cells (T. D. C. Grace, Nature **211**: 366, 1966) and moth cells (T. D. C. Grace, Nature **195**:788, 1962). This paper describes the growth of Grace's moth cells in suspension in a medium free from insect hemolymph. Hemolymph was always required previously for successful growth of these cells.

The established moth cell line of Grace, *Antheraea eucalypti*, was obtained from J. L. Vaughn (Insect Pathology Pioneering Research Laboratory, Beltsville, Md.), who had been growing the cells in 5-ml quantities of Grace's lepidoptera medium with insect hemolymph in 30-ml Falcon plastic flasks (Falcon Plastics, Los Angeles, Calif.) incubated statically at 26 to 28 C. Cultures had been maintained by replacing half of the medium at the time of weekly feeding. In our laboratory, these cells were placed in our medium. We also used a weekly feeding schedule; however, complete changes of medium were made by centrifuging the culture flasks at 1,000 rev/min for 5 min, pouring off all the spent medium, and replacing it with fresh medium. Cultures were divided when sufficient growth (confluency) of cells had occurred. Excellent growth of these cells, which takes place both on the surface of the flasks and floating free in the medium, occurred within 1 month of placing them in the medium described in this report (Table 1).

The medium, which was designed arbitrarily for growing primary cells from insect tissue, was prepared as follows: all ingredients (except methylcellulose, serum, and antibiotics) were dissolved in distilled water and sterilized by filtering through a 0.22-μ membrane filter. Methyl-cellulose (sterilized by autoclaving as a 2% solution), serum (prefiltered), and antibiotics (as 200$\times$ solutions) were added aseptically to the desired concentrations. Methylcellulose, although not previously used in insect cell media, was employed because of experience with mammalian cell cultures.

Experiments had indicated that *A. eucalypti* cells would at least survive on the rotary shaker at 26 to 28 C under conditions (bottle size, fluid volume, shaker speed) normally employed for mammalian cells (S. C. Nagle, Jr., et al., Proc. Soc. Exptl. Biol. Med. **112**:340, 1963). Growth, however, as determined by cell counts with a hemocytometer, occurred in suspension cultures only after cultures were placed in 250-ml Falcon plastic flasks on a New Brunswick Gyrotory shaker operating at 60 rev/min with 25 ml of medium and a temperature of 26 to 28 C. Media from these cultures were changed weekly by pouring the cells into 50-ml conical centrifuge tubes and centrifuging for 5 min at 1,000 rev/min. Cells were resuspended in fresh medium and returned to plastic flasks.

A growth curve for cells growing in suspension was determined by daily counting the number of cells in replicate flasks. Results, plotted in Fig. 1, show that a maximal cell population of 2.2×10^6 per ml was obtained in 23 days.

The moth cells are differentiated from mammalian cells by several characteristics. Morphologically, *A. eucalypti* cells are of many shapes, even in the suspension system described here, where the cells never attached to the flask surface; HeLa and L cells are always rounded. Established cells of *A. eucalypti* in culture in our laboratory have a chromosome number greater than 200; HeLa and L-cell chromosome numbers are most often 60 to 80. The physical and nutritional conditions described in this report for the moth cells will not allow growth of our HeLa or L cells; similarly, the moth cells will not survive under physical and nutritional conditions used for growth of HeLa and L cells (chemically defined medium, 35 C incubation, 124 to 128 rev/min shaker speed, etc.).

The results described in this paper are of significance primarily for two reasons: (i) growth of insect cells in suspension culture was demonstrated and measured, and (ii) growth of these cells has continued for approximately 1 year in a medium completely free from insect hemolymph

TABLE 1. *Invertebrate medium, modified no. 10*

Component	Concn	Component	Concn
	mg/liter		*mg/liter*
L-Alanine	200	Sodium acetate	50
L-Arginine·HCl	400	Sodium pyruvate	100
L-Aspartic acid	300	$NaH_2PO_4 \cdot H_2O$	200
L-Cysteine·HCl	100	KCl	2500
L-Glutamic acid	400	$CaCl_2 \cdot 2H_2O$	400
Glycine	400	$MgCl_2 \cdot 6H_2O$	400
L-Histidine·HCl	200	$MgSO_4 \cdot 7H_2O$	400
L-Isoleucine	150	NaCl	4500
L-Leucine	300	Malic acid	250
L-Lysine·HCl	300	α-Ketoglutaric acid	250
L-Methionine	60	Succinic acid	100
L-Phenylalanine	150	Fumaric acid	100
L-Proline	150	Sucrose	5000
L-Serine	300	Fructose	1000
L-Threonine	150	Glucose	5000
L-Tryptophan	100	Trehalose	1000
L-Tyrosine	100	Ascorbic acid	100
L-Valine	150	D-Biotin	1
L-Glutamine	500	Choline·Cl	1
L-Asparagine	300	Folic acid	1
Aseptic Additions		Niacinamide	1
Methylcellulose, 15 centipoises	500	Ca pantothenate	2
Streptomycin	100	Pyridoxal·HCl	1
Penicillin	100,000 units/L	Thiamine·HCl	1
Nystatin	50,000 units/L	*i*-Inositol	1
Fetal bovine serum to 10%		Riboflavine	0.1
		B_{12}	0.002
		KOH to *p*H 6.5	

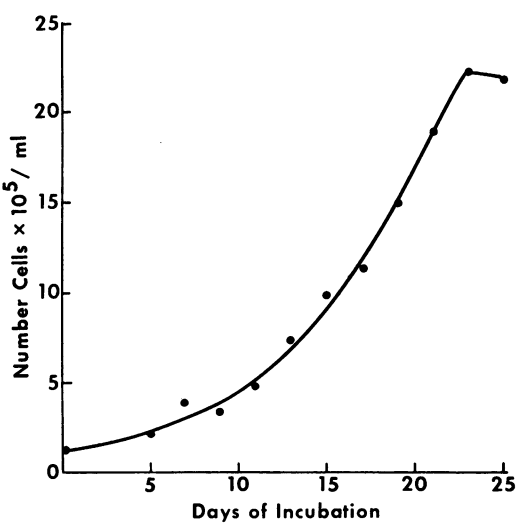

FIG. 1. *Growth of Antheraea eucalypti cells in suspension cultures.*

with no apparent diminution of growth. Because most workers believe hemolymph to be indispensable for growth of insect cells, the requirement for this substance in their nutrition should be reconsidered in view of the present results. It may be that methylcellulose is the single most important medium component for growth of these cells without hemolymph.

Experiments are in progress that will determine the specific medium requirements for *A. eucalypti* qualitatively and quantitatively. Studies of this type are greatly enhanced through the use of the suspension system described. Preliminary studies indicate that Grace's established line of *Aedes aegypti* cells will also grow in a medium similar to that described in this report.

While this manuscript was being prepared, a paper appeared (C. E. Yunker, J. L. Vaughn, and J. Cory, Science **155**:1565, 1967) that described the growth of Grace's moth cells in Grace's medium free from hemolymph but containing bovine and egg factors. Maximal growth of unshaken cultures (about 9.5 × 10^5/ml) was less than that reported here, but their work was the first to show the nonessential nature of hemolymph.

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