# Growth of L-M Strain Mouse Cells in a Chemically Defined Medium.* (27464)

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A number of chemically defined media have been described for cultivation of animal cells *in vitro*(1-15). Some of these media, such as CMRL-858 reported by Healy and associates(7), NCTC-109 described by Evans and co-workers(3) and medium MB 752/1 of Waymouth(9) will support the continued proliferation of selected cell strains without supplementation. These media contain from 40 to 70 components consisting of most of the known amino acids, vitamins, purines, pyrimidines, and co-factors.

Eagle(16) determined the minimal nutritional requirements for growth of L strain and HeLa cells *in vitro*. On the basis of his findings he formulated a basal medium composed of 12 essential amino acids, glutamine, 8 vitamins, and glucose in a balanced salt solution—a total of 28 components. This medium when supplemented with small amounts of either whole or dialyzed serum permits the continued rapid growth of a variety of animal cell strains as well as primary isolates.

This report deals with the continuous growth of L-M strain mouse cells in Eagle basal medium *without* supplements. The 12 amino acids, 8 vitamins and glutamine are incorporated at twice the concentration originally described by Eagle.

*Materials and methods.* The L-M cell(17), derived in this laboratory from L strain clone 929(18,19) is routinely maintained in medium 199 of Morgan *et al.*(12) supplemented with 0.5% peptone† (199-peptone). It was first adapted to grow in Eagle basal medium in April 1959 and has been maintained in this medium continuously since that time; the cell strain is now in the 105th serial passage.

In this study, stationary cultures were grown as monolayers in 200-ml serum dilution bottles. Ten ml of growth medium at pH 7.2 and containing between 2 and 3 $\times$ $10^5$ cells per ml was used as the initial inoculum. The medium was changed after 3 days, and the cells were harvested routinely after 7 to 10 days, by scraping with a rubber policeman. The cells were dispersed by trituration, diluted with fresh medium, and replanted.

For the purpose of growth curve analysis, 2 to 3 cultures of a replicate series were harvested every 24 hours and cell counts were made individually on each culture. A 1-ml sample was removed from each culture; 0.5 ml of which was used for determining cell number with an electronic cell counter,‡ and the remaining 0.5 ml was used for viability determinations by the erythrosin B dye exclusion technic(20).

Suspension cultures were grown in 250-ml Erlenmeyer flasks and were agitated on a rotary shaker with a 1-inch radial stroke at 110 rpm. The cells used were obtained from 6- to 7-day-old monolayer cultures harvested in the manner previously described. An initial inoculum of approximately 2.5 $\times$ $10^5$ cells per ml in a total volume of 100 ml of medium containing 0.12% methylcellulose§ was added to each flask. Methylcellulose was added hoping to prevent cell clumping and to prevent cell damage resulting from agitation. A 1-ml sample was removed every 24 hours for cell number and cell viability determinations. The cultures were harvested after 6 to 7 days.

The morphology of L-M cells growing in 199 peptone and in 2$\times$ Eagle basal medium was studied from coverslip cultures. These cultures were prepared by growing cells in Leighton tubes on 9 $\times$ 22 mm coverslips, one of which was removed at each 24-hour interval, fixed in 80% ethanol and subsequently

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† Bacto-Peptone, Difco Labs., Detroit, Mich.

‡ Model A, Coulter Electronics, Hialeah, Fla.

§ Metho-Cel 15CPS, Reagent Grade, Dow Chemical Co., Midland, Mich.

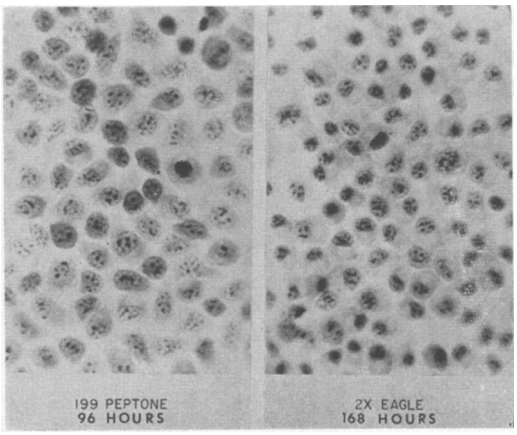



FIG. 1.

stained by the May-Grünwald-Giemsa technic(20).

*Results.* During the adaptation of L-M strain cells to grow in Eagle basal medium, the following observations were made: (1) the first passage of L-M cells in Eagle medium indicated no appreciable decrease in viable cells nor marked change in morphology, as evidenced by microscopic observations and the viability staining procedure; (2) chromosomal analysis of cells in 199 peptone and in Eagle medium indicated no difference in either the number or configuration of chromosomes(17); (3) it was possible, at any time, to take cultures grown in 199 peptone, and demonstrate immediate attachment and proliferation of the cells in Eagle medium; the converse was also true.

As seen in Fig. 1, L-M cells growing in 199 peptone or in Eagle medium appear similar in gross morphology. When examined at comparable time intervals after planting, cells in Eagle medium possess a somewhat smaller and denser staining nucleus than cells in 199 peptone. However, comparison of cells taken at comparable points in the population growth cycle in the 2 media (96-hr culture in 199 peptone and 168-hr culture in Eagle medium) shows a striking similarity in cell morphology and staining characteristics. Few giant cells were seen in cultures grown in Eagle medium and in this respect they resembled cells grown in 199 peptone.

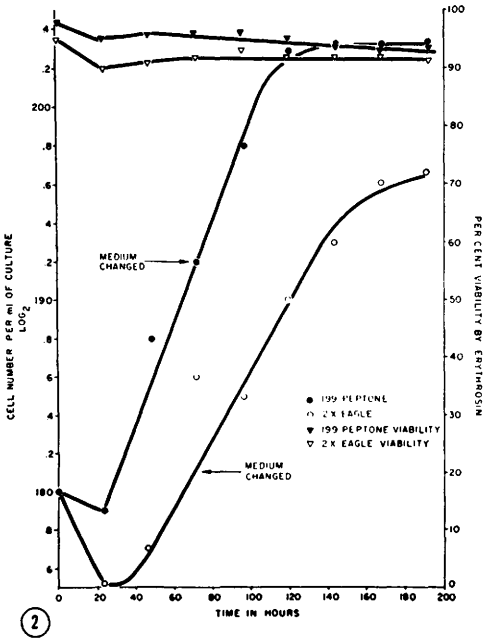
As illustrated in Fig. 2, monolayer cultures of L-M strain cells in 199 peptone exhibit a mean generation time of 40 hours while cells growing in Eagle medium exhibit a mean generation time of 56 hours. From an initial inoculum of $2.5 \times 10^5$ cells per ml (18.0 $\log_2$ units) a final population density of $1.2 \times 10^6$ cells per ml (20.3 $\log_2$ units) was achieved in 199 peptone and $8 \times 10^5$ cells per ml (19.6 $\log_2$ units) in Eagle medium after 7 days. An additional medium change at 6 to 7 days yielded final population densities of 1.2 to 1.6 $\times 10^6$ cells per ml in the Eagle medium.

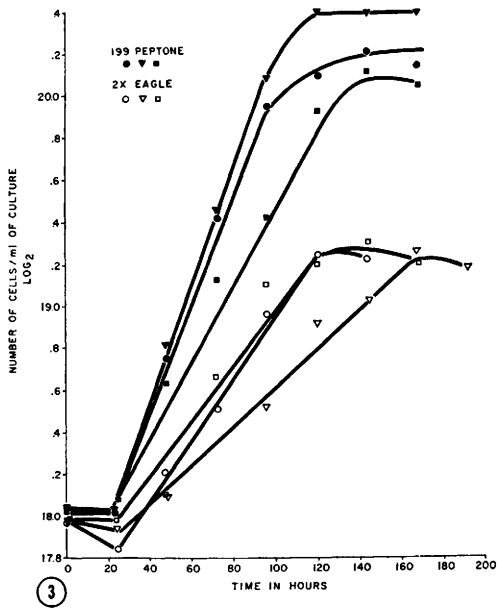
A drop in cell number during the lag phase has been observed both in 199 peptone and in Eagle medium when cells are grown as monolayer cultures. However, the drop observed in Eagle medium is quite pronounced and as yet cannot be completely explained. Viability determinations do not fully account for this, since only a slight decrease from 95% to 90% is observed during the first 24 hours (Fig. 2).

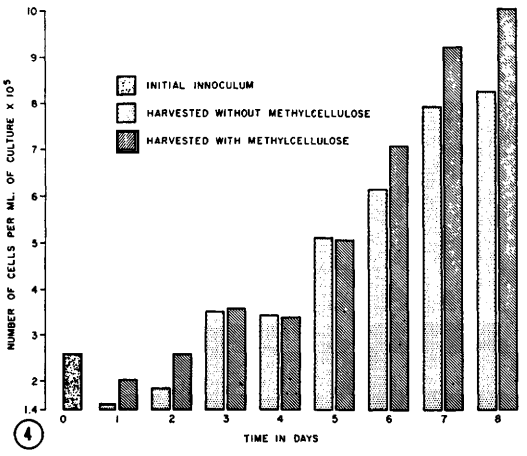
As seen in Fig. 3, it was of interest to note that suspension cultures did not exhibit such a marked drop in cell number during the lag phase. Furthermore, these growth curves illustrate the range in generation time of cultures grown in 199 peptone and in Eagle medium; the mean generation time of cells in 199 peptone being 43 hours as compared with 73 hours in Eagle medium. The generation time of suspension cultures in Eagle medium may be shortened by a complete medium change on the third day as is done with monolayer cultures.

The effectiveness of methylcellulose in permitting growth of L-M cells as suspension cultures in a defined medium suggested that this compound might act to protect the cell surface. To determine its effectiveness in protecting cells grown in monolayer during transfer, cultures of a replicate series were harvested, with and without addition of methylcellulose, at 24-hour intervals for 8 days. When methylcellulose was used, the old medium was discarded and fresh medium containing 0.12% methylcellulose was added and left in contact with the culture for 2 to 5 minutes. The cultures were then harvested by scraping and cell counts were made. Control cultures were handled in a similar manner except that methylcellulose was omitted

from the fresh medium added to the cells. As illustrated in Fig. 4, an appreciable difference in cell number is noted during the lag phase and again in the plateau phase, cultures harvested with methylcellulose having a cell count ranging from 70,000 to 200,000 cells per ml greater than the controls. There is no appreciable difference in cell yield during the



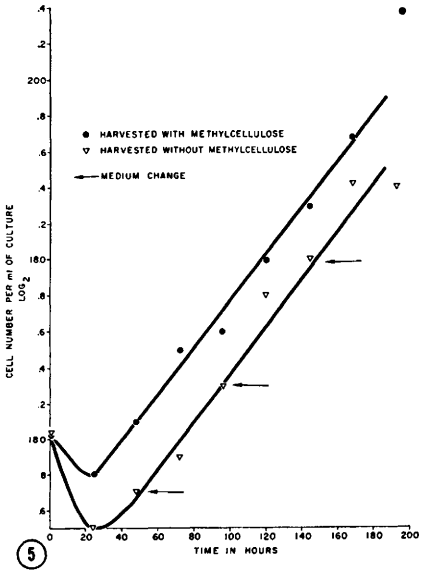




FIG. 2. Comparative growth curve of L cells in monolayer culture using 199 peptone and 2× Eagle basal medium.

FIG. 3. Comparative growth curves of L cells in suspension culture using 199 peptone and 2× Eagle basal medium.

FIG. 4. Comparative yields of L cells from 2× Eagle harvested with and without methylcellulose.

FIG. 5. Comparative yields of L cells grown in 2× Eagle harvested with and without methylcellulose: complete medium change every 48 hr.

log phase. However, as can be observed in Fig. 5, repeated medium changes at 48-hour intervals had a marked effect on the cell yields obtained when harvesting with and without methylcellulose. Under these conditions a consistent difference in cell yield was noted throughout the growth cycle.

Addition of peptone to 1× or 2× Eagle basal medium failed to provide a suitable growth medium for L-M cells. Moreover, we were unable to adapt the cells to grow in either 1× or 2× medium 199 without peptone. This was true even for cells maintained through many generations in 2× Eagle medium.

*Discussion.* The results of this study confirm the findings of Eagle regarding the essential nutrients required for growth and maintenance of L strain cells *in vitro*(16) though it was found that a 2-fold increase in amino acid, vitamin, and glutamine concentrations was necessary for rapid cell multiplication in absence of added protein or peptides. A total replenishment of medium at 3 days was also necessary to achieve maximum cell growth.

These results are in agreement with those of McLimans(21) who found that even in the presence of added protein, in the form of serum, a modification of Eagle basal medium and repeated medium changes were necessary for attainment of adequate cell yields of L or HeLa strains in suspension cultures. Subsequent to McLimans' studies Eagle reported a "minimal essential medium" (22) which is similar in amino acid composition to the 2× Eagle basal medium used in the present studies.

Added protein or peptides are required for serial propagation of most mammalian cells. The L strain, clone 929, was grown for several years in this laboratory in a medium composed of 60% 199, 20% horse serum, 20% BSS. The serum content gradually was reduced and finally was replaced by 0.5% peptone. This is thought to have resulted in a selection of the population for ability to grow in a simplified medium(17) since it is now possible to grow these cells in 199 peptone or in 2× Eagle medium interchangeably. The resulting cell strain is designated L-M. Similar morphology, chromosome complement and enzyme patterns as well as the ease of interchange between media suggest that the basic selection occurred during adaptation to 199 peptone and that no marked additional selection occurred upon growth in 2× Eagle medium.

The function of protein or peptides in the growth of cells *in vitro* has not yet been determined fully. Aside from a nutritional role it has been suggested that they function in attachment of cells to glass or to other cells (23,24,25). Eagle has suggested a carrier function for proteins(26) and Evans postulated their role as reservoirs of nutritional components(27). Results of the present study strongly suggest that in addition to these roles proteins or peptides may act as physical protective agents since they can be replaced, in certain situations, by methylcellulose which is inert nutritionally. Further data relating to this point and to the use of methylcellulose as a protective agent will be published elsewhere.

With adjustments in concentration of components, relatively frequent medium change and the use of a macromolecular substance as a stabilizing agent it is possible to attain the continued rapid growth of the L-M strain of cells either in monolayer or in suspension in this simple, chemically defined medium. Population densities up to $2 \times 10^6$ cells/ml have been obtained in monolayer cultures. The extreme simplicity of the medium, the characteristics of the cells grown under these conditions and the degree of quantitation attainable make this system ideally suited for a wide variety of investigations ranging from biochemical analysis and metabolic studies to host cell-parasite interactions.

*Summary.* Using the criteria of generation time, final population density, and chromosome analysis, it has been demonstrated that Eagle basal medium is adequate for continued propagation of L-M strain mouse cells both as monolayers and in suspension. Three distinct advantages to this system are: (1) the relative simplicity of the medium in terms of number of components as compared to other defined media currently available, (2) the 3- to 5-fold increase in cell popula-

tion achieved in 7 to 10 days, in contrast with results using most defined media, (3) the growth of these cells in suspension culture, which is an ideal system for both nutritional and parasite-host cell relationship studies. The effectiveness of methylcellulose as a physical protective agent for cells grown in a simple, chemically defined medium has been demonstrated.

1. Waymouth, C., *Texas Rep. Biol. and Med.*, 1955, v13, 522.
2. White, P. R., *J. Nat. Cancer Inst.*, 1955, v16, 769.
3. Evans, V. J., Bryant, J. C., Fioramonti, M. C., McQuilkin, W. T., Sanford, K. K., Earle, W. R., *Cancer Res.*, 1956, v16, 77.
4. Evans, V. J., Bryant, J. C., McQuilkin, W. T., Fioramonti, M. C., Sanford, K. K., Westfall, B. B., Earle, W. R., *ibid.*, 1956, v16, 87.
5. McQuilkin, W. T., Evans, V. J., Earle, W. R., *J. Nat. Cancer Inst.*, 1957, v19, 885.
6. Healy, G. M., Fisher, D. C., Parker, R. C., *Canad. J. Biochem. and Physiol.*, 1954, v32, 327.
7. Healy, G. M., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 71.
8. Pasieka, A. E., Morton, H. J., Morgan, J. F., *Canad. J. Biochem. and Physiol.*, 1958, v36, 771.
9. Waymouth, C., *J. Nat. Cancer Inst.*, 1959, v22, 1003.
10. White, P. R., *Growth*, 1946, v10, 231.
11. ———, *J. Cell. and Comp. Physiol.*, 1949, v34, 221.
12. Morgan, J. F., Morton, H. J., Parker, R. C., PROC. SOC. EXP. BIOL AND MED., 1950, v73, 1.
13. Briggers, J. C., Webb, M., Parker, R. C., Healy, G. M., *Nature*, 1957, v180, 825.
14. Morgan, J. F., Morton, H. J., *J. Biophys. and Biochem. Cytol.*, 1957, v3, 141.
15. Bakken, P. C., Evans, V. J., Earle, W. R., Stevenson, R. E., *Am. J. Hygiene*, 1961, v73, 96.
16. Eagle, H., *Science*, 1955, v122, 501.
17. Hsu, T. C., Merchant, D. J., *J. Nat. Cancer Inst.*, 1961, v26, 1075.
18. Earle, W. R., *ibid.*, 1943, v3, 555.
19. Sanford, K., Earle, W. R., Likely, G. D., *ibid.*, 1948, v9, 229.
20. Merchant, D. J., Kahn, R., Murphy, W. H., Jr., in *Handbook of Cell and Organ Culture*, 1960, Burgess Publ. Co., Minneapolis.
21. McLimans, W. F., *J. Immunol.*, 1957, v79, 428.
22. Eagle, H., *Science*, 1959, v130, 432.
23. Fisher, H. W., Puck, T. T., Sato, G., *Proc. Nat. Acad. Sci. U.S.*, 1958, v44, 4.
24. Lieberman, I., Ove, P., *J. Biol. Chem.*, 1958, v233, 637.
25. Kuchler, R. J., Marlowe, M. L., Merchant, D. J., *Exp. Cell Research*, 1960, v20, 428.
26. Eagle, H., *Proc. Nat. Acad. Sci. U.S.*, 1960, v46, 427.
27. Evans, V. J., *J. Nat. Cancer Inst.*, 1957, v19, 539.

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## Multiplication and Cytopathology of Coxsackie A-21 Virus in Rotated and Stationary Tissue Culture. (27465)

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Coxsackie A-21 virus (Coe) is an enterovirus which propagates and produces a cytopathic effect (CPE) in certain human continuous and primary embryonic tissue cultures (1,2,3,4). This agent was recently shown to be associated with acute undifferentiated upper respiratory illness in military personnel (5,6). During controlled epidemiologic studies which established the role of Coxsackie A-21 virus in respiratory disease it was observed that production of CPE by this virus was enhanced when inoculated HEp-2 tissue cultures were rotated during incubation. The quantitative aspects of virus replication and cytopathology as effected by rotation of cul-

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