ibid., 1949, v177, 793.

3. Anson, M. L., *J. Gen. Physiol.*, 1938, v22, 79.

4. Hussey, R. G., and Northrop, J. H., *J. Gen. Physiol.*, 1922, v5, 335.

5. Duthie, E. S., and Lorenz, L., *Biochem. J.*, 1949, v44, 167.

6. Shulman, N. R., *J. Exp. Med.*, 1952, v95, 571.

7. McCann, S. F., and Laskowski, M., *J. Biol. Chem.*, 1953, v204, 147.

8. Innerfield, I., Angrist, A., and Schwarz, A., *J. A. M. A.*, 1953, v152, 597.

9. Kleinfeld, G., and Habif, D. V., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 585.

10. Beilar, J. M., Brendel, R., and Martin, G. I., *ibid.*, 1955, v89, 274.

11. Miechowski, W. L., and Ercoli, N., *J. Pharm. Exp. Therap.*, 1956, v116, 43.

Received May 8, 1956. P.S.E.B.M., 1956, v92.

Propagation of Strain L (Earle) Cells in Agitated Fluid Suspension Cultures.* (22620)

ROBERT J. KUCHLER AND DONALD J. MERCHANT. (Introduced by W. J. Nungester.)

Department of Bacteriology, University of Michigan Medical School.

Several workers, using various technics for measuring increases in cell populations, have shown that a characteristic growth curve exists for mammalian tissue cells cultivated *in vitro*(1,2,3,4). As is true with microbial populations, this growth cycle can be separated into 4 major phases: 1) lag phase, 2) logarithmic growth phase, 3) stationary phase, and 4) logarithmic death phase(2,5). The study reported here made use of the rate of change of growing cell populations to demonstrate the effects of inoculum size, pH and serum concentration on strain L mouse fibroblasts in agitated fluid suspension cultures. It was hoped that an analysis of the growth curve would make it possible to establish a more efficient system for cultivation of these cells.

Materials and methods. Strain L mouse fibroblasts(8) were used in this study. The stock strain was grown in stationary cultures on the surface of bottles with a capacity of 250 ml and a surface area of approximately 56 cm^2. Each culture contained 10 ml of a nutrient medium composed of 20% horse serum, 60% modified synthetic solution 199† (9), 20% Hanks balanced salt solution, and 100 units per ml each of penicillin and streptomycin. The fluid suspension culture method (6,7) was used in these studies. In this type of culture, 80 ml of medium of the same composition as previously described, and containing the desired number of cells, was placed in a 250 ml Erlenmeyer flask stoppered with a #6 rubber stopper. During incubation such cultures were placed on a New Brunswick rotary shaker, modified to give a speed of 100 rpm. Daily or twice daily, counts were made from a small sample using a hemocytometer. Since the cells in such cultures remain in a homogenous suspension, no treatment was required to disperse the cells prior to making the counts. The pH of samples taken for counting was determined with a Beckman Model G pH meter. For other cultures used as controls, cells were harvested from a stock culture by removing the growth from the wall of the bottle with a rubber scraper. These cells were evenly suspended in the medium by repeated pipetting. A series of replicate cultures was set up with an inoculum of approximately 200,000 cells per ml by pipetting 10 ml of the cell suspension into each of 10-15 bottles, as described for stock cultures. The initial pH was adjusted to approximately 7.4. Two or 3 of these cultures were harvested each day, and cell counts were made using a hemocytometer.

Results. When the logarithm, to the base 2, of the cell number was plotted against time in

* This work was supported by USPHS Grant No. C2539 (C) from the National Cancer Institute.

† Purines and pyrimidines omitted and medium sterilized by autoclaving.

days, typical growth curves resulted in both stationary and in fluid suspension cultures. It could be observed that after a lag period of about 2 to 3 days the cells began to divide at a logarithmic rate. During the lag period pH of the medium dropped from 7.4 to approximately 7.0 in the fluid suspension culture. A value for the rate of change could be calculated in the logarithmic growth phase, using the formula: $R = \frac{\log Nx2 - \log Nx1}{t_2 - t_1}$ (10), where Nx2 is the number of cells at time t_2 and Nx1 the number of cells at time t_1. This value for strain L mouse fibroblasts for both stationary and agitated cultures was shown to be approximately 2.5×10^{-2}. This calculation was based on population change per hour and would correspond to a generation time, for these cells, of about 40 hours. Two generations and sometimes 3 could be observed in the logarithmic growth phase. The apparent maximum cell density for the system described was about 1.5×10^6 to 2×10^6 cells per ml. When cells were removed from a culture in the logarithmic growth phase and inoculated into fresh medium at a pH of 7.4 the culture exhibited a shortened lag period (Fig. 1). Other growth characteristics were not altered. In these experiments, the logarithmic growth phase began when the pH of the medium had fallen to approximately 7. The rate of growth, generation time and maximum cell density appeared to exhibit the same characteristics as previously described. Since pH 7 seemed to be optimal for growth in this system, medium was adjusted to pH 7 by equilibrating with CO_2 prior to the inoculation of cells. When cells growing in the logarithmic phase were inoculated into this medium, no lag was observed, the cells remaining in the logarithmic phase of growth with no further pH adjustment (Fig. 2).

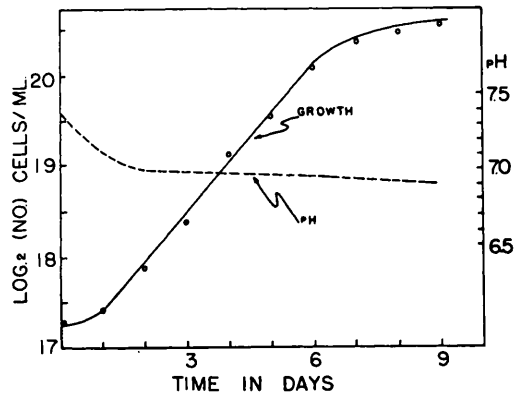

FIG. 1. Growth curve of L strain fibroblasts in shake culture, using cells growing at logarithmic rate as inoculum.

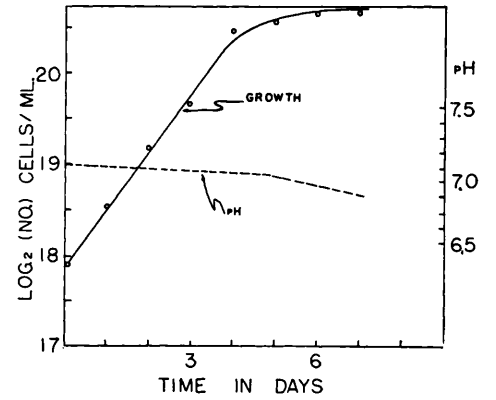

FIG. 2. Growth curve of L strain mouse fibroblasts in shake culture, using cells growing at logarithmic rate as inoculum, and starting at pH 7.

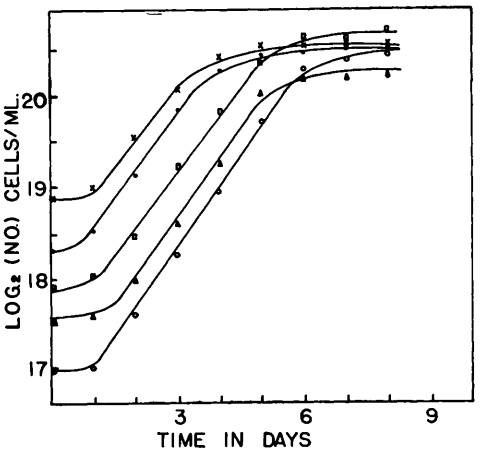

FIG. 3. Growth curves of L strain mouse fibroblasts varying the initial cell inoculum.

With starting inoculums ranging in size from 100,000 to 500,000 cells per ml, rate of growth in the logarithmic phase was independent of the starting inoculum (Fig. 3). An inoculum of 50,000 cells per ml gave the same type of curve. Maximum cell density appeared to be approximately the same for all inoculums tested. It was not possible to es-

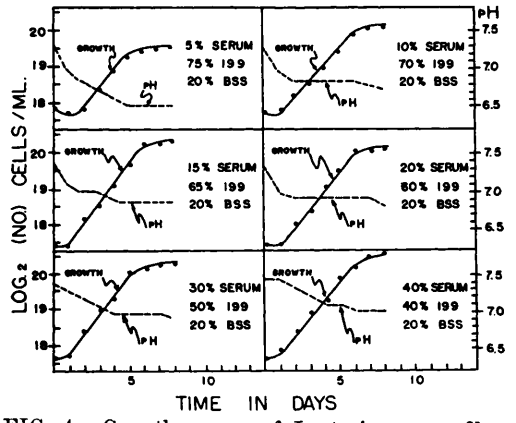

FIG. 4. Growth curves of L strain mouse fibroblasts varying ratio of serum to synthetic medium.

tablish a significant relationship between inoculum size and the length of the lag phase.

With variations of serum concentrations over the range of 5 to 40%, in a complete growth medium, it could be seen (Fig. 4) that rate of growth in the logarithmic phase was essentially independent of the serum concentration. The inoculums for this experiment were taken from a culture growing in a 20% serum medium and in the logarithmic phase of growth. There appeared to be a lower maximum cell density in the cultures containing less serum. It was noted that the apparent optimum pH for growth during the logarithmic phase varied with the concentration of serum in the medium.

Finally, by starting with cells in the logarithmic growth phase and adjusting the transfer interval to a 4 day cycle, conditions of steady state growth were approached. The cells were continually transferred when they were in the logarithmic phase so that cells were available at all times for other studies. When cultures were handled in this manner in 20% serum, no shift in pH below 7 occurred.

These experiments have been repeated, using LLCMl strain mouse fibroblasts(11), with essentially the same results.

Discussion. The development of a growing population of mammalian tissue cells inoculated into an adequate nutrient medium can be divided into a number of successive growth phases that are analogous to microbiological culture cycles and can be described with the nomenclature of Buchanan(5) and Monod(10). Fluid supension cultures of L strain mouse fibroblasts, used in this study, showed growth characteristics analogous to those cited by Rinaldini(2) for chick embryo cells and Graham and Siminovitch(1) for monkey kidney cells. At least 2 distinct advantages of fluid suspension cultures for the study of growth are apparent. When such a culture is established in the logarithmic phase of growth, the cells grow as a uniform, single cell suspension and can be sampled at intervals over a period of time thus eliminating the variation which might exist between replicate cultures. Moreover, cells making up a population in the logarithmic growth phase are more uniform physiologically than cells from other phases of the growth cycle. The complete elimination of the lag period by careful equilibration of pH at the time new medium is added was demonstrated to be possible but not too practical under the conditions of the experiments. A serum concentration of 20% was found to be optimal for L strain cells under the conditions of these experiments. While cultures containing lower serum concentrations showed the same rate of population change during the logarithmic growth phase, the maximal cell density was significantly lower at 5% serum levels and it was more difficult to maintain cultures in the lower concentrations over an extended period of time. Since growth rate and maximal cell density were not appreciably increased at higher serum levels, it was felt that 20% serum represented the most efficient level in terms of utilization of available nutrient materials.

The role of pH in the experiments reported here appeared to be complex. It had been noted in earlier experiments that pH 7.4 was required to assure sticking of L strain cells to a glass substrate. When the pH was allowed to fluctuate significantly above or below this value, many cells failed to attach and remained floating in the medium. Low pH levels, however, while they inhibited cell attachment, did not interfere with the growth of previously attached cells. This fact was not at first appreciated and it was assumed that pH 7.4 was optimal for growth of these cells. When attempts were made to establish fluid

suspension cultures at pH 7.4, it was found that the cells had to adjust the medium to a lower pH value before they could begin logarithmic growth. For medium containing 20% serum, this level was pH 7. The pH then remained rather constant during the remainder of the logarithmic growth phase and dropped more rapidly as the cells entered the stationary phase. This phenomenon of adjustment of environmental pH by bacterial populations has been adequately described by Gale(12). The relationship of this adjustment to duration of the lag period was illustrated by the fact that no lag occurred in cultures started at pH 7. It was further noted that one of the possible roles of serum in the medium was to act as a buffering agent. In this regard it was of interest to note that the level at which the pH stabilized during logarithmic growth was directly proportional to the serum concentration.

Summary. The agitated culture has been used in studying the growth cycle of strain L (Earle) mouse fibroblasts. From data available, it is seen that mammalian cells growing *in vitro* display a growth pattern quite similar to that of other microbiological populations. Some points which have been studied and can now be evaluated are the following: 1) the lag period for these cells appeared to be eliminated completely when log-phase cells were used as inoculum in media equilibrated to pH 7, 2) growth rate appeared to be independent of the inoculum size and also independent of serum concentration, 3) generation time for these connective tissue cells, under conditions studied, was approximately 40 hours; 2 generations could usually be observed in these cultures and saturation concentration in some instances reached 2,000,000 cells per ml, 4) steady-state cultivation of tissue cells has been approached. The results indicated that populations could be maintained in such a manner, and this problem will be investigated.

1. Graham, A. F., and Siminovitch, L., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 326.
2. Rinaldini, L. M., *Nature,* 1954, v173, 1134.
3. Eagle, H., *J. Biol. Chem.,* 1955, v214, 839.
4. Puck, T. T., Marcus, P. I., and Cieciura, J. J., *J. Exp. Med.,* 1956, v103, 273.
5. Buchanan, R. L., *J. Infect. Dis.,* 1918, v23, 109.
6. Owen, O. von H., Gey, G. O., and Gey, M. L., *Proc. Am. Assn. Cancer Research,* 1953, v1, 41.
7. Earle, W. R., Schilling, E. L., Bryant, J. C., and Evans, V. J., *J. Nat. Cancer Inst.,* 1954, v14, 1159.
8. Sanford, K. K., Earle, W. R., and Likely, G. D., *ibid.,* v9, 229.
9. Morgan, J. F., Morton, H. J., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.
10. Monod, J., *Ann. Rev. Microbiol.,* 1949, v3, 371.
11. Hull, R. N., *Science,* 1953, v117, 223.
12. Gale, E. F., The Chemical Activities of Bacteria, Academic Press, New York, Ed. 2, 1948, 60.

Received May 17, 1956. P.S.E.B.M., 1956, v92.

Equivalence between Vaccinia Particles Counted by Electron Microscopy and Infectious Units of the Virus. (22621)

JOHN R. OVERMAN AND IGOR TAMM.
Rockefeller Institute for Medical Research, New York City.

In earlier studies on the relation of the number of vaccinia virus particles to the infectious unit variable results were obtained (1-3). The lowest average ratio of particles to infectious units heretofore reported is 4.2:1(3).

Three features of experiments relating number of virus particles to the infectious unit are most important. Clearly, the method of virus titration to obtain the number of infectious units must be highly sensitive and reproducible. Burnet showed that infectivity of vaccinia virus suspensions could be determined by counting the pocks produced by the virus on the chorioallantoic membrane of the embryonated egg(4). Such titrations have the advantage of expressing infectivity in terms of pock-forming units rather than re-