A Precise Method for Replicating Suspension Cultures of Mammalian Cells

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A simple, readily assembled shaker-culture system for the cultivation of mammalian cells is described. No specialized glassware and equipment were used in this system, which consists of an Erlenmeyer flask fitted with a breather-sampling assembly. This unit was employed to quantitate the effects of several variables, including medium ingredients, serial transfers, and freezing and storage on two variants of the L-cell line. This system is reproducible and precise and allows for growth of cells in suspension for extended periods of time. Large numbers of cells can be mass-produced. Many replicates can be run simultaneously to yield data for statistical analysis.

To evaluate quantitatively the effect of a variable at several levels or the interaction among a large number of variables in the cultivation of mammalian cells, a procedure is necessary that allows the use of a large number of observations with a small statistical variance among replicates. The recent work of Ham (7) indicates that the optimal concentrations of different medium constituents depend upon the concentration and kind of other ingredients. Ideally, a system involving the use of suspension cell culture (2-6, 8, 9, 12, 14), incubated in Erlenmeyer flasks and agitated by rotary or reciprocal motion, would allow the study of multiple variables in factorial experiments.

This report describes a shaker-culture system for the cultivation of mammalian cells which allows for quantitating the effects of several variables. The variables used to test the system were (i) medium ingredients, (ii) serial transfers, and (iii) the effect of freezing and storage.

Materials and Methods

Cell lines. Two variants of Earle's mouse fibroblast L-cell line (1) were used. One, designated LDR, was obtained from W. F. Daniels, Fort Detrick. The genetic characteristics of the second variant, designated L-MS, have been described by Merchant, Walker, and Parker (11). The LDR variant required 10% bovine serum in the medium for growth; the L-MS variant had been adapted to grow in serum-free medium for a number of years.

Medium. Medium 199 with 0.5% peptone (Difco) and 0.12% Methocel (15 centipoises) was used. The pH of the medium was adjusted to 7.2 at the initiation of each experiment with 6% sodium bicarbonate.

Shaker-flask culture system. Erlenmeyer flasks were fitted with breather-sampling assemblies (Fig. 1). The assembly consisted of a solid rubber stopper pierced by a 15-gauge Luer-Lok needle with the hub sealed to the stopper with Silastic 731 RTV silicon rubber (Dow Corning Corp., Midland, Mich.). The end of the needle protruding into the flask was cut flush with the stopper. A 2.5-ml glass syringe barrel, plugged both with a nonabsorbent cotton plug in the barrel of the syringe and capped with a small rubber stopper, was inserted into the Luer-Lok fitting. Flasks were Siliclad-lined to minimize adherence of cells to the walls. Rubber stoppers were boiled in NaOH solution, thoroughly rinsed with water, and finally rinsed with triple-distilled water to remove inhibitory substances. Assemblies were autoclaved separately from the flask and held sterile until after medium addition. Flasks were plugged with nonabsorbent cotton, covered with aluminum foil, and sterilized by autoclaving. Assemblies were secured with plastic tape. Flasks were shaken on a reciprocal shaker at 100 oscillations per minute through 3-inch (7.6 cm) strokes. The shaker had a capacity of 120 Erlenmeyer flasks (250 ml). Optimal volume of culture medium was 40% of flask capacity. Incubation was at 36 C. The stoppers inserted into the syringe barrels were removed after 24 hr of incubation.

Experimental procedures. Shake flasks were sampled at 1 hr postinoculation and at 24-hr intervals by removing the syringe barrel and aseptically attaching a sterile Luer-Lok syringe, inverting the flask, and withdrawing a 2-ml sample for determination of viable-cell counts, percentage cell viability, and pH. Viable-cell counts and viability determinations were

1 Presented in part at the 67th Annual Meeting of the American Society for Microbiology, New York, N.Y., 1 May 1967.
made with a hemocytometer, using the trypan blue dye exclusion method (10, 13).

**Results and Discussion**

To demonstrate the reproducibility of replicate flasks, four lots of bovine serum were evaluated with LDR cells. The results of growth in triplicate flasks and growth curves plotted from average cell counts for 5 days are shown in Fig. 2. Zero-hour cell concentrations were approximately $2 \times 10^8$ viable cells per ml. Use of bovine serum lot 9 resulted in a lower peak population and longer doubling time compared to the control. The data were analyzed by use of analysis of variance (AOV) on the log transformation of the data. The standard deviation ($\sigma$) of 0.07 log, indicating that the differences among triplicate flasks for each of the four lots of serum tested, was very small. Figure 2 is the only one presenting the three individual flask values, but it is typical for all tests.

The culture system was tested for maintenance of cell growth through several serial transfers. Two 500-ml Erlenmeyer flasks, containing 200 ml of 199 peptone medium without antibiotics and $65 \times 10^4$ viable L-MS cells per ml, were shaken for 4 days, and 50/50 splits were made to four 500-ml Erlenmeyer flasks; these were grown for 3 days and again split. The cultures were carried through five serial transfers. Average values for multiple shake-flask cultures are shown in Fig. 3. The growth curves of each serial transfer were remarkably similar; also it was demonstrated that this growth system has the ability to maintain growth for extended periods of time.

The level of inoculum required to initiate growth before and after freezing (1 C per min) in liquid nitrogen was investigated to demonstrate the sensitivity of the system. Six ampoule lots of LDR cells contained between $2 \times 10^6$ and $8 \times 10^7$ viable cells per ml. Growth was initiated with a minimal count of $21 \times 10^6$ unfrozen viable cells per ml (Fig. 4), whereas $175 \times 10^6$ viable cells per ml were required to initiate growth of stored frozen cells (Fig. 5). The data indicate that the growth potential was impaired by the freezing process. The effect of freezing mammalian cells at these high concentrations and inoculating directly in suspension systems has been further investigated by us and will be reported later.

Mass cultivation of cells was accomplished by seeding 100 ml of 199 peptone medium in a 250-ml Erlenmeyer flask with 1 ml of frozen concentrate cell suspension containing $2 \times 10^7$ viable cells per ml. After 13 days of growth by successive transfer through 500-ml and 1-liter Erlenmeyer flasks, 400 ml of the cell culture containing $2 \times 10^8$ viable cells per ml were obtained. This was a 40-fold increase of the inoculum.

In summary, a method of growing mammalian cells in Erlenmeyer shake flasks that is sensitive and reproducible and that allows for growth of cells in suspension over an extended period of

![Erlenmeyer flask (1,000 ml) fitted with breather-sampling assembly. The same assembly was used also with smaller Erlenmeyer flasks.](image-url)
FIG. 2. Comparison of four lots of bovine serum in 250-ml Erlenmeyer flasks. Cell line: LDR. Medium: 199 peptone containing 50 units of penicillin and 50 μg of streptomycin per ml of completed medium. Viable-cell counts (VCC) of triplicate flasks, ○, ●, and △.

FIG. 3. Effect of five serial transfers of fresh L-MS cells in 500-ml Erlenmeyer flasks containing 199 peptone medium without serum or antibiotics. VCC refers to viable-cell count.

FIG. 4. Growth test of LDR cells, before freezing, in 250-ml Erlenmeyer flasks containing 199 peptone medium supplemented with 10% bovine serum, 50 units of penicillin, and 50 μg of streptomycin per ml of completed medium. VCC refers to viable-cell count.
time has been described. Some of the main advantages of this system are as follows: (i) cells are not gassed, and there are no medium changes between transfers, (ii) specialized glassware and equipment are not needed, (iii) operation is simple, (iv) the system can be set up rapidly, (v) large quantities of cells for various types of studies can be mass-produced, and (vi) many replicates can be run simultaneously for statistical analysis.

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FIG. 5. Growth test of LDR cells, after freezing, in 250-ml Erlenmeyer flasks containing 199 peptone medium supplemented with 10% bovine serum, 50 units of penicillin, and 50 μg of streptomycin per ml of completed medium. VCC refers to viable-cell count.

LITERATURE CITED