

The Propagation of Mammalian Cells in a 20-Liter Stainless Steel Fermentor^{1, 2}

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The propagation of mammalian cells in stainless steel fermentors of a type used in antibiotic fermentations represents a marked departure from the techniques employed in classical tissue culture studies. Since the pioneer work of Harrison (1907), in which he demonstrated the outgrowth of cells from nerve tissue maintained *in vitro*, many techniques for the *in vitro* culture of cells have been developed.

An older method of cell culture, which is still used, consists of imbedding small fragments of excised tissue in a drop of nutrient medium on the glass surface of a test tube or flask. The medium generally includes embryo extracts and blood plasma which form a coagulum about the tissue mass. The imbedded tissue may be bathed in a nutrient solution, composed of serum, carbohydrate, amino acids, or protein hydrolyzates, and salts. The small amount of tissue produced as well as the nature of environmental conditions make application of this system to viral, nutritional, and physiological studies most difficult (Parker, 1950).

Another technique, the cultivation of cells as a monolayer on glass surfaces, was described by Carrel and Ebeling (1922). Today the culture of cells as monolayers in flasks and bottles is the most widely used method (Hanks *et al.*, 1955). The preparation of cells for monolayer culture requires their dispersion from clumps and tissue masses by mechanical means or by the use of agents such as trypsin and Versene. The dispersed cells are suspended in a nutrient medium and allowed to settle onto the bottom of the glass vessel where they adhere to the glass surface and multiply. This method makes possible investigations concerned with the nutrition of the cells as well as the kinetics of virus-host cell reactions. However, the amount of cells which can be produced is necessarily limited and quantitation of cell responses to imposed conditions lacks precision.

Recent investigations have established the feasi-

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bility of propagating several mammalian cell lines as discrete units in agitated fluid suspension (submerged culture). A variety of techniques and environmental conditions have been proposed for the cultivation of tissue cells in the submerged state. These include the rotary shaker of Earle *et al.* (1956), the tumble tube of Owens *et al.* (1953), the roller tube of Graham and Siminovitch (1955), the suspended stirrer in Erlenmeyer flasks of Cherry and Hull (1956), Powell's (1954) hexagonal roller tube, Hardy and Brown's (1957) wrist shaker, and the glass stirrer of Danes (1957). Investigations conducted in our laboratories led to the development of a culture system designated by us as the spinner culture (McLimans *et al.*, 1957a). Using the spinner culture system, the following stable cell lines were propagated successfully in submerged culture as single discrete cells; HeLa (Gey *et al.*, 1952), L cell (Earle *et al.*, 1943), human conjunctiva (Chang, 1954), and human amnion (Fogh *et al.*, 1957).

The spinner culture consists of a stationary flask in which a Teflon-covered magnet is suspended by a swivel. Agitation of cell suspensions is obtained by placing the flask with the suspended Teflon magnet in the field of a magnetic stirrer. This system permits the cells to proliferate, in most instances, as suspensions of single discrete cells, without clumping or sticking to the walls of the flask. The spinner culture has served as the prototype for scale-up of the submerged culture system to successful propagation of several cell lines in a conventional 5-L New Brunswick Fermentor⁴ (McLimans *et al.*, 1957b).

The successful adaptation of these techniques to larger size equipment is the subject of this paper. The propagation of mammalian cells, by the methods described, permits one to contemplate the production of viral vaccines, hormones, and other physiological agents by methods analogous to techniques employed in microbiological fermentations.

MATERIALS AND METHODS

Twenty-L Stainless Steel Fermentor. A diagram of the 20-L stainless steel fermentor⁵ is given in figure 1.

⁴ New Brunswick Scientific Co., New Brunswick, New Jersey.

⁵ Twenty L Stainless Steel Fermentor fabricated by Stainless & Steel Products Co., St. Paul, Minnesota.

The fermentor shell of this equipment was fabricated from type 316 stainless steel alloy, an alloy known to be nontoxic to certain mammalian cells cultured *in vitro* (Giardinello *et al.*, 1958). All lines and service connections which are in direct contact with the culture chamber are constructed of stainless steel of a suitable alloy. Valves, including those in the air lines, steam lines, and drain lines, are diaphragm-type valves with stainless steel bodies. Neoprene⁶ diaphragms have been found to be satisfactory in the operation of these valves. However, direct toxicity tests of the Neoprene diaphragm material have not been carried out.

A 6-bladed, 4-in. impeller driven by a Reeves Vari-Speed Motodrive⁷ is used to agitate the cell suspensions. Cell culture in this equipment has been carried out with agitation speeds varying from 140 rpm to 190 rpm. The speed of agitation of the culture has not appeared to be a critical factor as evidenced by the similar rates of growth observed under different rates of agitation. Generally, the speed of agitation was adjusted to a point at which vigorous movement of the suspension was attained without foaming. Volumes of 6 L, the smallest volume which can be handled in this equipment, were agitated at 140 to 160 rpm. Volumes of 12 L were agitated at 170 to 190 rpm. Under these conditions, there was no evidence that the cells settled out or adhered to the walls of the fermentation vessel.

Sterilization of all components of the fermentor was accomplished by in-place steam sterilization. A steriliza-

⁶ Grinnell Company, Inc., Providence, Rhode Island.

⁷ Reeves Pulley Co., Columbus, Indiana.

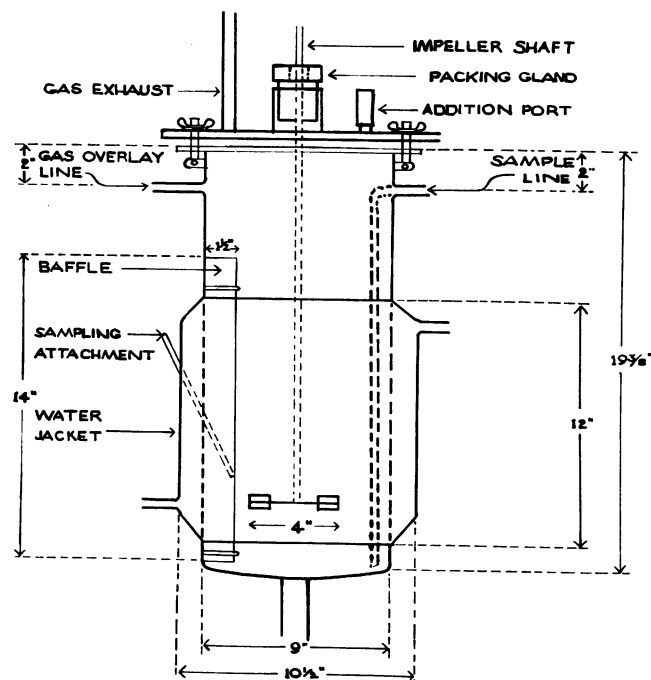


Figure 1. Twenty L stainless steel fermentor

tion period of 2 hr at 18 lb per in.² with steam being bled through the filters and outlets has given completely satisfactory results, both in actual operation and in sterility tests.

Aeration of submerged tissue cultures by sparging gas into the bottom of the fermentation vessels has been found unnecessary. Hence, tank cultures were maintained with a gas overlay. Further simplification of the aeration system was afforded by the use in the growth medium of an increased phosphate buffer concentration, which eliminated the necessity of the use of the conventional bicarbonate-gaseous carbon dioxide buffer. Since the need for carbon dioxide gassing was circumvented, the submerged cultures were aerated only with air from a small air compressor at a flow-rate of 0.5 to 2.0 L per min. The air was sterilized by passage through a glass wool-packed column. A positive pressure of 5 to 8 lb per in.² within the fermentors appeared to aid in the exclusion of contaminants from the tissue culture system.

An incubation temperature of 36 to 37 C was maintained by the circulation of water from a constant temperature water bath through the water jacket of the fermentor.

Medium. The culture medium employed in these studies was composed of amino acids, glutamine, and vitamins in concentrations recommended by Eagle (1955) and a modification of the balanced salts-buffer solution devised by Earle *et al.* (1956). It should be noted that an increased phosphate concentration has permitted the omission of sodium bicarbonate. Comparative studies with several cell lines have demon-

TABLE 1

Modified Eagle's medium employed for submerged culture studies

L Amino Acids (g/L)		Vitamins (mg/L)	
Arginine·HCl	0.021	Biotin	1.0
Cystine	0.012	Choline	1.0
Histidine·HCl	0.008	Folic acid	1.0
Isoleucine	0.026	Nicotinamide	1.0
Leucine	0.026	Pantothenic acid	1.0
Lysine·HCl	0.026	Pyridoxal	1.0
Methionine	0.008	Thiamin	1.0
Phenylalanine	0.016	Riboflavin	0.1
Threonine	0.024		
Tryptophane	0.004	Glucose	2.5 g/ml
Tyrosine	0.018	Phenol red	0.01 g/L
Valine	0.024	Methocel* (methylcellulose-4000 CPS)	1 g/L
Glutamine	0.300	Serum (heat inactivated: 56 C, 10 min)	10%
		Penicillin	100 units/ml
		Streptomycin	50 µg/ml
		Mycostatin	30 units/ml
Salts (g/L)			
NaCl	7.0		
KCl	0.4		
MgSO ₄ ·7H ₂ O	0.2		
Na ₂ HPO ₄	1.44		

* Dow Chemical Co., Midland, Michigan.

strated that medium buffered with M/100 phosphate permits cell proliferation equal to the growth obtained in a CO₂: bicarbonate buffered medium. The complete ingredients of the medium are listed in table 1.

The necessity for complete medium changes was eliminated by the periodic addition of the amino acid, arginine (Thomas *et al.*, 1958), to the actively proliferating cell suspension. The use of this technique, together with the addition of fresh medium, allowed the cultures to be maintained for periods of 10 to 20 days without medium changes.

Cell lines. Propagation of three cell lines was studied in the 20-L fermentation equipment. These are strain L, a mouse fibroblast; strain HeLa, an epithelial-type cell isolated from a human cervical carcinoma; and strain KD of the ERK cell line (Westwood *et al.*, 1957), isolated from embryonic rabbit kidney. The latter cell line is of special interest because it supports the proliferation of the virus of poliomyelitis (Sheffield and Churcher, 1957).

Cell stocks of each of these cell lines were maintained routinely both in spinner culture (suspended agitated culture) and in bottle culture (stationary culture). Inoculum for the initiation of the stainless steel fermentors was produced in 3 L volumes in New Brunswick fermentors.

Inocula for cultures initiated in the New Brunswick fermentors were obtained either from a spinner culture or from glass-grown cells. Either source of inoculum has proved to be satisfactory. Tissue cultures in the New Brunswick equipment were started with 1.5 L of cell suspension containing 1.5 to 3.0 × 10⁵ cells per ml. As cell multiplication was noted, fresh medium was added until a total volume of 3 L of cell suspension was obtained. The maximum cell population usually attained was 1.0 to 1.5 × 10⁶ cells per ml. The detailed operation of tissue culture in this equipment has been reported elsewhere (McLimans *et al.*, 1957b). A culture was initiated in the larger 20 L fermentor by introduction of 2 to 3 L of cell suspension inoculum plus sufficient fresh medium to give a total volume of 6 L, with a resultant cell population of 1.7 to 3.0 × 10⁵ cells per ml.

Sampling and cell counting. Cell multiplication was determined by counting the cells in a hemocytometer. The general state of a culture was reflected by the viability of the cells which was determined by a staining method employing trypan blue (Pappenheimer, 1917; Hewitt, 1953). It has been observed that degenerating cells have an affinity for the stain, trypan blue, whereas actively proliferating cells are not stained. The viabilities of cell cultures were determined by making differential counts of stained and unstained cells. The publication of McLimans *et al.* (1957a), describing the development of the spinner culture system, includes a description of the trypan blue method. Additionally,

Gwatkin *et al.* (1957) have demonstrated a good correlation between the total cell count as obtained by a direct counting procedure and actual isolated cell culture using a feeder layer (Puck and Fisher, 1956).

RESULTS AND DISCUSSION

Growth of strain L. The techniques employed in culturing the three cell lines were essentially similar; hence, a detailed description of the procedures used will be presented for the strain L cell only. The inoculum was obtained from a cell suspension produced in a New Brunswick fermentor. Three L of cell suspension containing 4.9 × 10⁵ cells per ml (1.47 × 10⁹ total cells) were transferred to the 20 L fermentor. Three L of fresh medium were added to the fermentor, giving an initial total volume of 6 L with a cell concentration of 2.45 × 10⁵ cells per ml. The results of this cell culture are represented graphically in figure 2. The data reported in this figure show both the actual cell concentration at the various sampling periods as well as the total number of cells produced. The cell concentration is represented by the line curve while vertical bars represent the total number of cells produced.

Frequently it has been observed in our laboratories, as well as in reports from other laboratories, that upon initiation of a cell culture a "lag period" of variable duration was noted. Since the delayed period of growth was not observed consistently in all cultures, it may possibly be interpreted as being caused by the physiologic condition of the cells in the inoculum rather than by conditions imposed by the environment in the newly established culture. The physiological condition of the cells which determines the character of growth

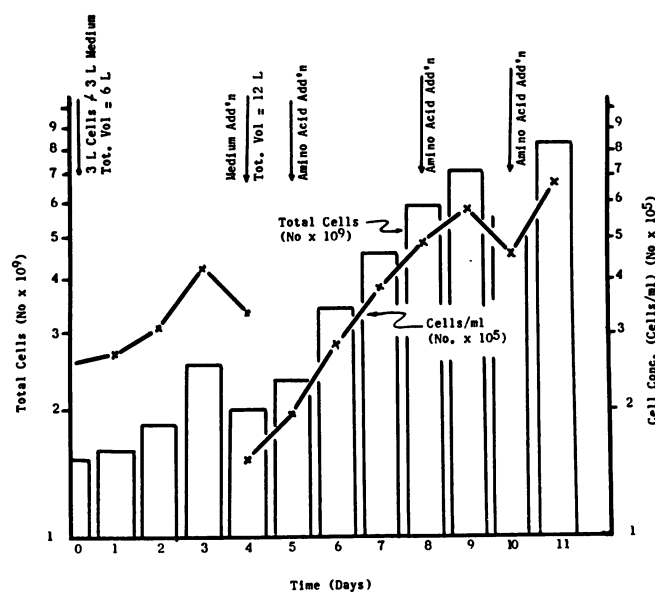


Figure 2. Growth of "L" cells in 20 L fermentor. Tank no. 1. Date initiated 3-8-58. Inoculum: cells from New Brunswick fermentor.

upon subculture may reflect the nutritional state of the cells or the growth phase of the cells at the time of subculture. Accordingly, an inoculum of cells from a culture in a stationary growth phase would produce a "lag" upon subculture, while cells from a culture in log-growth phase would not exhibit an initial "lag period."

The "lag period" shown in figure 2 was minimal and was followed by an increase in the cell population. After 4 days of growth, the cell suspension contained approximately 4.0×10^5 cells per ml and at this point an additional 6 L of medium was added to the tank. A constant increase in the cell population resulted and was maintained over the 7-day period by three additions of Eagle's amino acid concentrates. The amount of each amino acid addition was equal to the original concentration of the amino acid in fresh medium. Work by Thomas *et al.* (1958) has shown that arginine present in the amino acid mixture is the vital factor which may be used to supplement medium additions during the first 10 days in this type of culture. The addition of arginine alone in subsequent cell cultures was equally as effective as the addition of the complete amino acids mixture.

After a growth period of 11 days, the cell concentration reached 6.75×10^5 cells per ml, which represents an increase in total cells from 1.47×10^9 cells to 8.1×10^9 cells in 12 L of suspension. Although complete medium changes with cell suspensions in large volumes are unwieldy and are to be avoided, it was of interest to explore the possibility of successfully performing this operation. Hence, the 12 L of cell suspension were withdrawn from the tank, centrifuged, and the cells resuspended in fresh medium. The cell suspension was equally divided between two 20 L fermentors. The

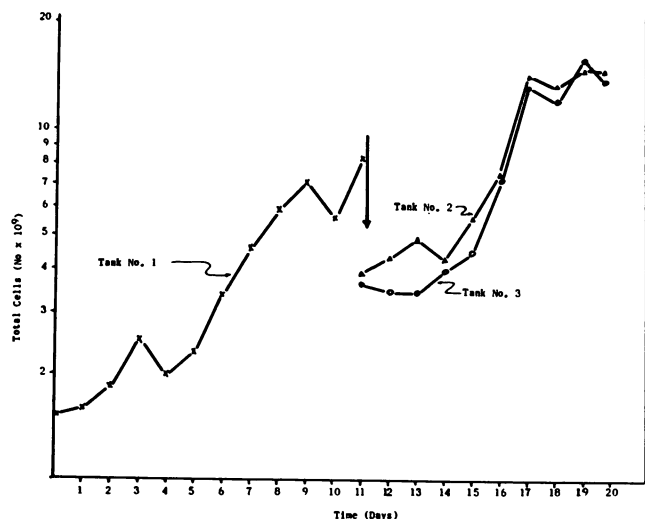


Figure 3. Growth of "L" cells in 20 L fermentors. Cells from tank no. 1 centrifuged and resuspended in fresh medium; cells equally divided between tank no. 2 and tank no. 3.

total volume in each of the two fresh tanks was 6 L at a cell concentration of approximately 6.0×10^5 cells per ml.

After 5 days an additional 6 L of fresh medium were added to each tank. Subsequent to the medium addition, the culture in one tank received periodic additions of complete Eagle's (1955) amino acid concentrate, while the other culture received only additions of the single amino acid, arginine. In each case, the amounts of the complete amino acid mixture or arginine alone were equal to the initial concentration of the respective components in fresh medium. The cell growth in each of these tanks was nearly identical, indicating that addition of arginine alone promoted growth as well as the addition of the complete amino acid mixture.

The total cell growth obtained in the three separate tank cultures is shown in figure 3. A cell build-up from 1.47×10^9 cells contained in 6 L to a total of 30.0×10^9 cells in 24 L of suspension was obtained.

Growth of strain HeLa. Three L of a HeLa cell suspension plus 3 L of fresh medium were added to a 20 L fermentor. The resultant cell density was 4.6×10^5 cells per ml. Although this cell culture was initiated with a relatively high cell concentration, smaller cell inocula in subsequent cell cultures produced satisfactory growth. A somewhat different procedure was used in maintaining the HeLa culture than was employed in the maintenance of the L culture. Three smaller medium additions of 1 and 2 L were made instead of a single 6 L addition such as was carried out with the L cell culture. No advantage was noted in the more frequent additions of smaller volumes of medium.

Two additions of arginine were also employed, which, in conjunction with the medium additions, permitted cell multiplication to be maintained over an 11-day

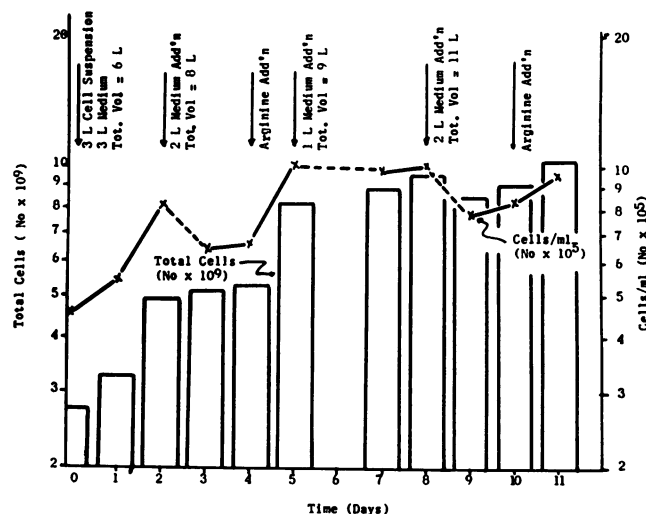


Figure 4. Growth of HeLa cells in 20 L fermentors. Date initiated 4-15-57. Inoculum: cells from New Brunswick fermentor.

period. During this period, the total cell population increased from 2.8×10^9 cells suspended in 6 L to 10.6×10^9 cells contained in 11 L. The results obtained from this cell culture are shown in figure 4.

The possibility of producing large amounts of viral agents in submerged culture-produced cells is indicated by the demonstration that strain HeLa, as propagated in spinner culture, supported the proliferation of all three types of poliovirus (Davis, 1957).

Growth of strain KD. Figure 5 presents data describing the growth obtained with a third cell line, strain ERK/KD cultivated in the stainless steel equipment. The inoculum was a cell suspension produced in a New Brunswick fermentor. The cell inoculum, plus sufficient medium to make a total initial volume of 6 L containing 5.6×10^5 cells per ml, was introduced into a tank. In this culture, as in the other cell cultures, growth was maintained by a medium addition and additions of arginine. On the third day following the initiation of the culture, the addition of fresh medium appeared to cause a slight lag in the rate of cell multiplication. The reason for the "lag" has not been determined, but in this culture and in studies with other cell lines, it seems that the lag may be caused by an endogenous depletion of essential factors in the cells prior to the addition of fresh medium. Although the addition of arginine permits continued growth without medium changes for a period of 10 days, the question relating to the depletion of other metabolites remains to be elucidated.

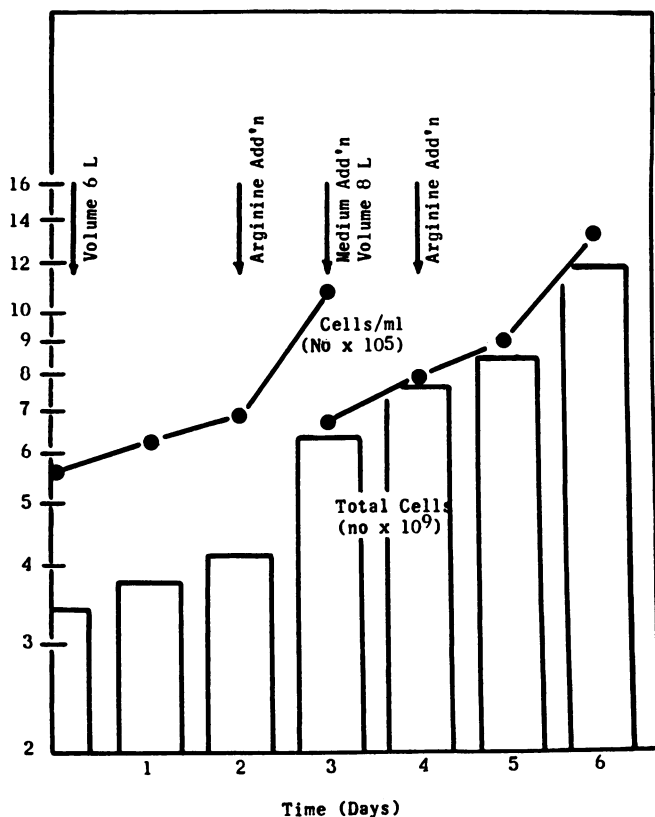


Figure 5. The growth of strain KD cells in the 20 L fermentor

During the 6-day growth period, the use of arginine and fresh medium promoted a continuous increase in the total cell population from 3.4×10^9 cells to 11.1×10^9 cells with a final cell density of 1.3×10^6 cells per ml. Although satisfactory growth of the ERK/KD cell, as well as with strain HeLa and strain L, was obtained with these procedures, future nutritional studies undoubtedly will permit both more rapid rates of multiplication and the attainment of higher cell densities. This particular nonprimate cell line seems to be of interest since it supports the proliferation of poliovirus when cultured in the submerged state (McLimans *et al.*, 1957c) in volumes up to 10 to 15 L.

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SUMMARY

Twenty-liter stainless steel fermentors, designed for use in the fermentation industry, were adapted for use in the propagation of mammalian cells in an agitated fluid suspension system. The medium and cultural conditions employed in the investigations are discussed.

Three unrelated cell lines (strain L, a fibroblastic cell of mouse origin; strain HeLa, an epithelial-type cell isolated from a human cervical carcinoma; and strain ERK/KD, isolated from embryonic rabbit kidney) were successfully cultivated in the large stainless steel equipment. It was possible to obtain 3- to 6-fold increases with each of the cell lines during a 6- to 12-day growth period.

Since cells of three different origins were readily cultured under the conditions described, these results offer promise that many stable cell lines may be propagated in mass in equipment designed for large scale fermentations. The possibility of using submerged culture cells to produce viral vaccines, hormones, and other physiologically active cell products is indicated.

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