SUBMERGED CULTURE OF MAMMALIAN CELLS: THE FIVE LITER FERMENTOR[1, 2]

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Received for publication June 24, 1957

The design and application of a system permitting the active proliferation of mammalian cells, as individual units, in the submerged state, would seem to be of value to individuals interested in investigations relating to the over-all kinetics of the host/cell virus interaction and to studies requiring high concentrations of uniform tissue cells or virus. This report presents data establishing the feasibility of obtaining uniform proliferation of mammalian cells in the submerged state in volumes up to 3 L in a stationary, impeller-agitated fermentor.

Various types of agitated systems have been suggested as satisfactory for permitting growth of tissue cells in the submerged state, i. e., the rotary shaker of Earle *et al.* (1954), 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, Brown and Hardy's (1957) wrist shaker, as well as the glass stirrer of Danes (1957).

Inherent difficulties have been encountered with certain of the submerged culture systems mentioned. Thus, in shake flask systems there is a marked tendency toward the formation of large granules (McLimans *et al.*, 1957). Additionally, proteinaceous precipitates are frequently formed. Certainly, the extrapolation to large vessels, 3 to 16 L, would be difficult in the instance of roller-tube type units or flask systems.

We have reported (McLimans *et al.*, 1957), from our laboratories, the development of a "spinner" culture system in which good growth of various mammalian cell lines has been obtained. Basically, the spinner system is a stationary glass vessel accommodating fluid volumes of 25 to 500 ml agitated by a suspended magnetic bar. The culture medium employed was Eagle's (1955) mixture adjusted in concentrations from 1X to 3X depending on the cell population per unit volume as compared to glass monolayer cultures. Ten per cent horse, calf, or human serum was employed in all instances. It should be noted that the spinner vessel was designed to afford possible extrapolation of data to larger systems of conventional architectural design such as employed in the antibiotic industry, i. e., the New Brunswick fermentor. Sustained logarithmic growth of a variety of cell lines: HeLa (Gey *et al.*, 1952), L (Earle *et al.*, 1951), human conjunctiva (Chang *et al.*, 1954), human amnion (Fogh and Lund, 1957) as discrete units, has been achieved in spinner culture. Additionally, it has been demonstrated by Davis *et al.* (1957), of our laboratories, that the submerged culture cell lines will readily support the proliferation of certain viral agents as judged by the results of the poliovirus/HeLa and Herpes simplex/L cell interaction, and by Brown and Hardy (1957) using the Venezuelan equine encephalomyelitis/L cell systems.

During the initial phases of the reported investigations, it was necessary to study the reaction of the mammalian cell lines to materials of construction other than glass, as well as the apparent toxicity of available antifoam agents. Giardinello *et al.* (1957) have reported the results of this preliminary study. They observed that the antifoam agents NOPCO LEF, GEAF 60, DEMAL M and AF Extra S were nontoxic in the concentrations usually employed for this purpose. Furthermore, it was demonstrated that stainless steel 316, 347, and 321 were satisfactory

[1] Contribution No. 12 from Microbiology in Medicine, Wistar. The investigations herein reported were conducted under the auspices of a grant (Contract no. DA-18-064-404-CML-74) from the U. S. Army Chemical Corps, Fort Detrick, Maryland.

[2] Presented, in part, at the Society of American Bacteriologists meetings May 2, 1957, in Detroit, Michigan.

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TABLE 1

Experimental conditions

No.	Inoculum Source	Aeration	Buffer System	Impeller Speed	Methocel	Conditioned Medium
		%	*M*	*rpm*	%	%
1	Glass	95 Air + 5 CO_2	0.026 bicarbonate* 0.010 phosphate	200	0	10
2	Spinner	95 Air + 5 CO_2	0.026 bicarbonate 0.010 phosphate	160–200	0.2	0
3	Fermentor	95 Air + 5 CO_2	0.026 bicarbonate 0.010 phosphate	160–200	0.2	0
4	Fermentor	Open tank	0.010 phosphate	160–200	0.2	0

* In each instance, with the exception of No. 4, the nutrient medium used was 90 per cent Eagle's mixture and 10 per cent horse serum with the addition of penicillin, streptomycin and Mycostatine. In the instance of No. 4, the identical medium was used with the elimination of the bicarbonate salts.

nontoxic materials of construction (Giardinello *et al.*, 1957).

MATERIALS AND METHODS

Cell line. The studies herein reported were conducted with the strain "L" cell (Earle *et al.*, 1951). The tissue cells were routinely propagated in Blake bottles using a nutritive medium composed of 90 per cent Eagle's (1955) constituents and 10 per cent horse serum. The medium also contained penicillin (100 units/ml), streptomycin (50 μg/ml), and Mycostatin (15 units/ml) (McLimans *et al.*, 1956). The cells were harvested by either scraping from the glass or by exposure to 0.25 per cent trypsin (Difco) for 5 min. The culture medium employed in the fermentors was as indicated in table 1. In some instances the cell inoculum was obtained from a suspended cell system, namely the spinner cultures or another New Brunswick fermentor.

The fermentor. The fermentor unit, as commercially available,[4] consists of three 5-liter fermentors which are individually connected, through flexible rubber couplings, to a common variable speed drive whose operating range is 150 to 750 rpm. The fermentors are partially submerged in a thermostatically controlled water bath. The gas supply for each fermentor is metered through individual Fischer-Porter flowraters which are mounted on the control panel. Individual gas filters are also supplied with the unit.

[4] The fermentors used were obtained from the New Brunswick Scientific Company of New Brunswick, New Jersey.

The main body of the fermentor consists of a standard 6″ by 12″ five-L pyrex jar (figure 1). There are 4 baffles, a gas sparger, a sample line, and an impeller suspended from the stainless steel head. All of the metallic constituents which come into contact with the medium are constructed of 316 stainless steel. The gas sparger consists of a single orifice immediately below the flat blade, turbine-type impeller. The introduction of gas through the sparger may result in severe foaming, a condition which can be readily controlled by the addition of General Electric Antifoam 60. However, it is interesting to note that to date we have obtained as good growth of tissue cells using a gas overlay as when sparging. The overlay gassing system is preferred because of its simplicity and the elimination of need for antifoam agents. Those fermentor ports which are not used for the continuous transfer of gas are sealed with rubber vaccine stoppers.

The built-in sample line is not used for sampling since frequent flushing of the line would be required to obtain a representative sample and would thus appreciably reduce the operating volume over an extended period of time. The fermentors were sampled at least once daily by passing a 10-in 18-gauge needle through the vaccine stoppered port.

The reaction of submerged culture cells to trypan blue serves as a useful guide to follow the state of the culture. As a result of permeability changes in degenerating cells they readily stain with trypan blue. For this purpose 0.5 ml of 0.5 per cent water solution of trypan blue (Coleman and Bell, Norwood, O.) is mixed with a 1.0 ml of the cell suspension, placed in a hemocytometer

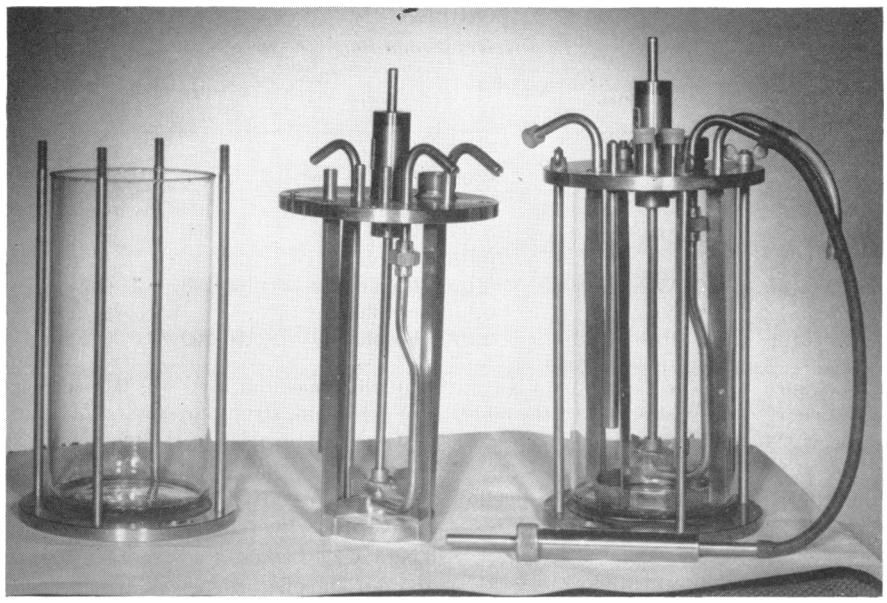

Figure 1. Construction details of the 5-L New Brunswick fermentor

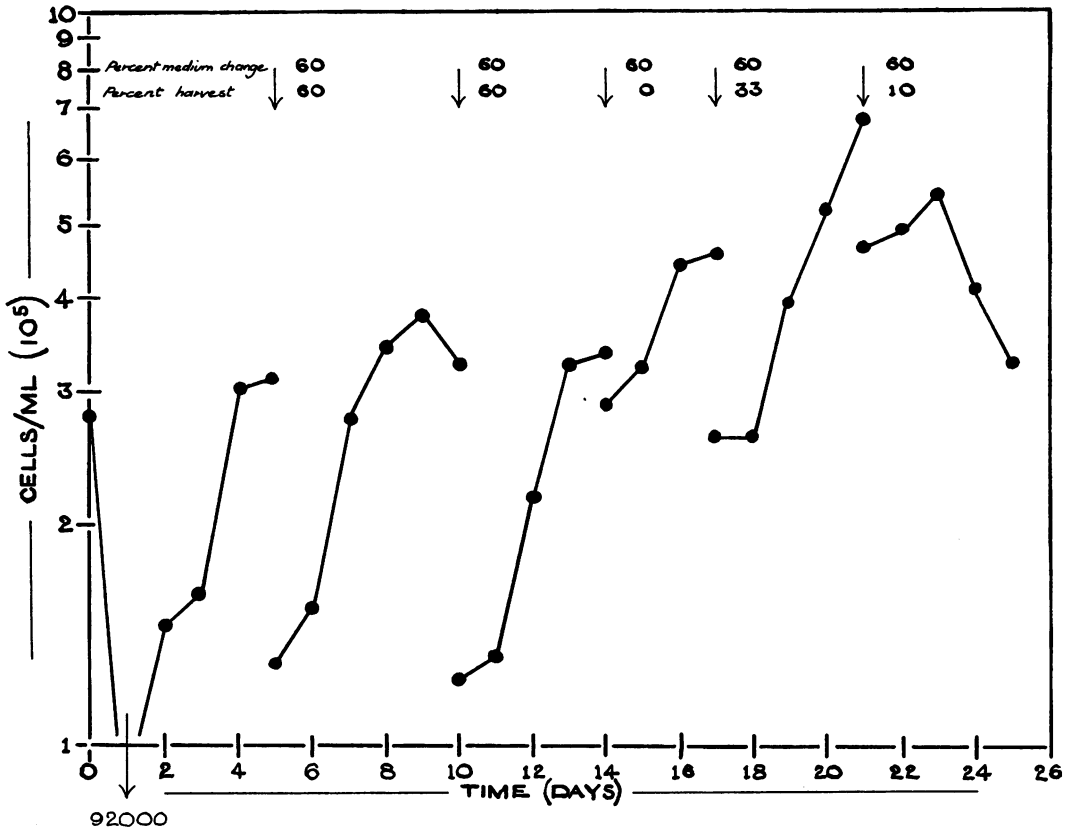

Figure 2. Continuous growth of L cell in the New Brunswick fermentor (vol, 3 L) over a period of 22 days. Drop in cell count following 22nd day was a result of bacterial contamination. Reduced cell count at 5, 10, and 17th day was result of harvesting cells for other investigations.

and a direct cell count made of both total and viable (nonstained) cells. The viability of the culture, as defined, appears to satisfactorily reflect the general state of the fermentor culture. It is usual to record 95 per cent or better viability. A decrease to less than 90 per cent appears to be associated with an unsatisfactory substrate depletion or accumulation of acid or other metabolic end products, viral action, etc.

To avoid gradual reduction of the operating volume as a result of evaporation, the gaseous feed to each tank was saturated by passing through a gas washing bottle filled with water. Both the inlet and outlet gases are filtered through glass wool. The fermentor, along with its auxiliary gas filter, are autoclaved at 121 C for 40 min. It appears essential to add about 20 ml of distilled water to the fermentor prior to autoclaving. The culture harvests are made by attaching a rubber transfer line to the sample line of the tank and then pressuring out the desired tank contents.

Certain qualitative factors influenced the selection of the mild agitation conditions. First, the mammalian cells do not appear to require high oxygen tensions and, accordingly, it was not deemed necessary to increase the impeller speed to obtain more efficient aeration. Additionally, greater impeller speeds would tend to increase the cellular trauma. Therefore, agitation condi-

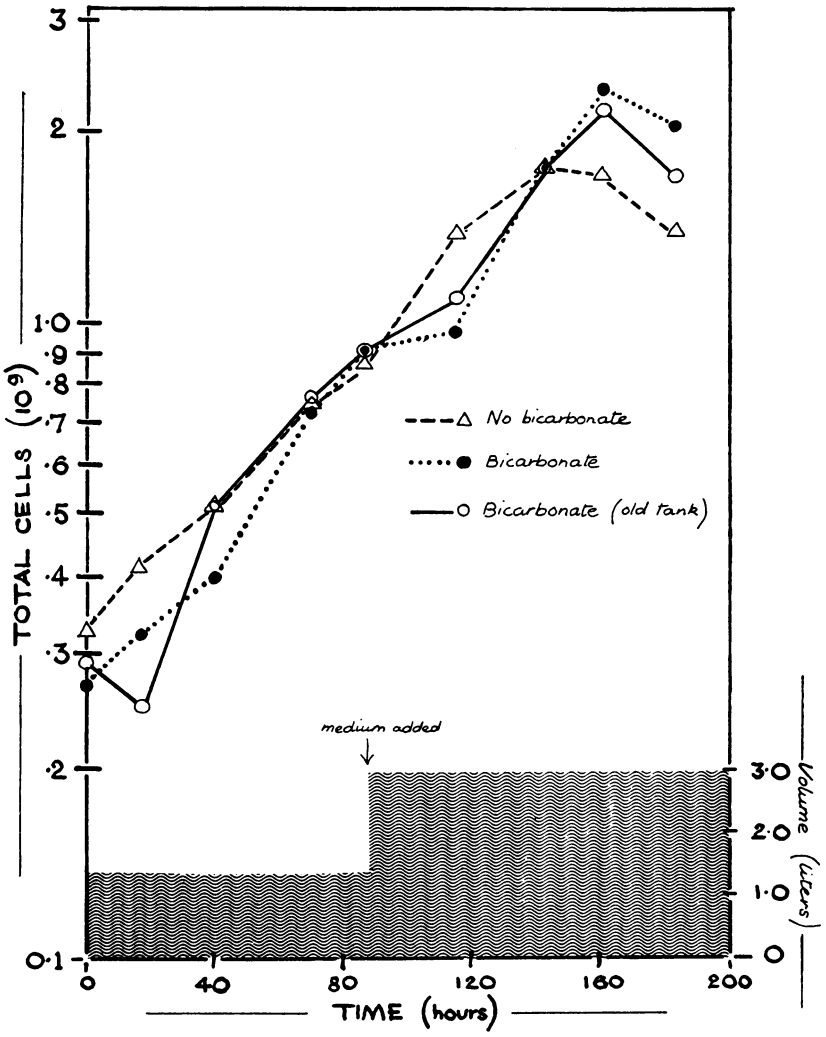

Figure 3. Cell proliferation in 5 L fermentor

tions as gentle as possible were selected, with consideration given to obtaining impeller speeds just sufficient to maintain the suspended state of the cells.

RESULTS

To date, there have been 10 cultures initiated in the New Brunswick fermentor under a variety of conditions; a cell population increase was experienced in every case. The viability, as determined on the basis of failure to stain with trypan blue (Pappenheimer, 1917), generally was in excess of 95 per cent. The various experimental test conditions are presented in table 1. In the early experiments medium changes were made at varying intervals of time (figure 2). Approximately 50 per cent or more of the cell suspension was pressured out of the fermentor. The culture could then be restored to the original volume by the addition of fresh medium. Generally, the cells harvested at the time of medium change were used for other purposes. On a few occasions, however, i. e., day 14 of figure 2, the cells removed were centrifuged and returned to the fermentor with the fresh medium.

The first modifications in the fermentor system were made on the basis of experience obtained in spinner culture. Original observations (Earle *et al.*, 1954) that the incorporation of a small amount of Dow Methocel (4,000 cps) into

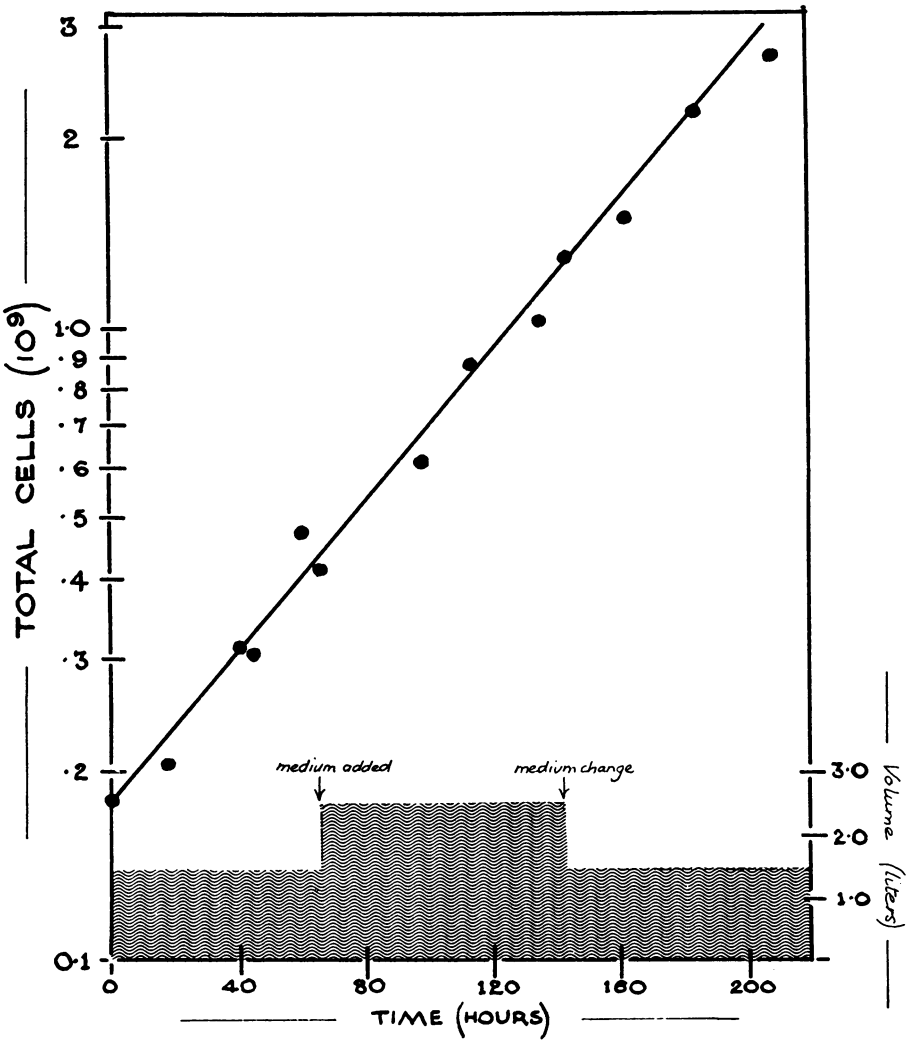

Figure 4. Cell proliferation in 5 L fermentor

the medium enhanced growth and promoted more uniform cell suspensions were confirmed. Silver (1957, *personal communication*) further observed that the sodium bicarbonate/CO_2 buffer systems could be eliminated and replaced with a phosphate buffer system. Thus, cultures could be maintained without CO_2 gassing. These changes were incorporated into the fermentor system. An experiment was performed in which a comparison was made between the phosphate buffer system without gassing and the sodium bicarbonate/5 per cent CO_2 in air gas overlay system. There appeared to be no significant difference between the rates of growth of the cell suspensions (figure 3). Morphologically the cells were identical. The pH range observed with the bicarbonate system was 7.2 to 7.5. The phosphate buffer system occasionally required addition of alkali to maintain the pH above 7.0.

DISCUSSION

To date, no attempt has been made to determine the maximum attainable cell concentration for this type of vessel. The maximum concentration achieved, to this point, in the fermentor system is 2.2×10^6 cells per ml (figure 4). This level appears to be consistent with the 2×10^6 cells per ml reported by Earle *et al.* (1954) and Kuchler and Merchant (1956) for L cell suspension (shake flask) systems.

It seems possible that concentrations considerably in excess of this will be achieved with this cell line when the appropriate nutritional and physical conditions are more clearly delineated. Through the expediency of making complete medium changes every 24 hr, Graham and Siminovitch (1955) have achieved a concentration of 10^7 cells per ml with a strain of monkey kidney cells grown in a suspension system. In a spinner system, Dr. Silver, in our laboratories, has achieved a cell concentration of 3.5×10^6 employing the L cell.

Cultures were frequently initiated at reduced volumes and increased at intervals of time via addition of nutrient medium. Figure 4 depicts a growth curve incorporating these changes. From our experience it would appear that glass grown cell inoculum routinely exhibits an initial drop in cell population or at least an increased lag period while submerged culture cells do not.

Although we have established that the L cell can be grown in this type of system, it is obvious from examination of the varying generation times, 24 to 60 hr, that we do not as yet have precise control of all variables. From a cytologic viewpoint, the L cell as it proliferates in the fermentor is indistinguishable from that previously described using a spinner culture (McLimans *et al.*, 1957) (figure 5).

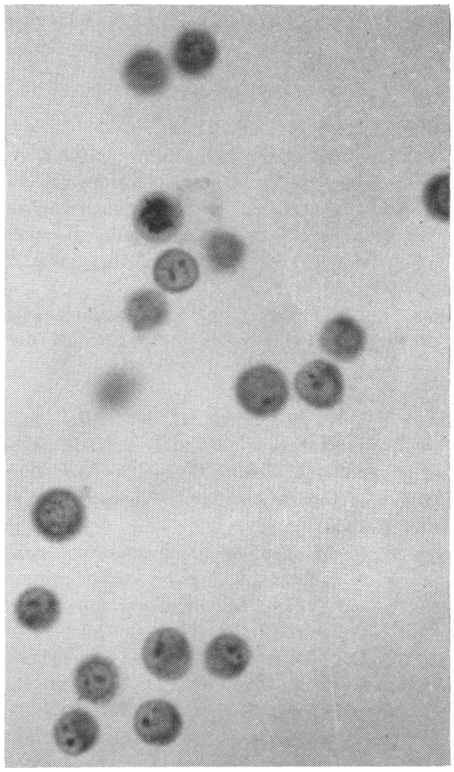

Figure 5. L cell (Earle) grown in a 5-L vol New Brunswick fermentor. Giemsa stain, × 960.

ACKNOWLEDGMENTS

We wish to gratefully acknowledge the invaluable assistance rendered in this investigation by Mr. Fletcher Glover, Mr. Thomas Orr, Mr. Elmer Bennett and to thank the Fort Detrick personnel for their assistance during many conferences in the prosecution of this investigation.

SUMMARY

A variety of conditions is described under which the growth of "L" cells has been attained in a 5-L fermentor which is a prototype of the antibiotic fermentors. A thorough evaluation of these conditions is contemplated. The growth exhibited in this type of equipment establishes the feasi-

bility of growing mammalian cells on a large scale in submerged culture.

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