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Chemostatic Concentrated Cultures of Heteroploid Mammalian Cell Suspensions in Dialyzing Fermentors

I. Experimental Evidence and Theoretical Considerations

GIO B. GORI

Microbiological Associates, Inc., Bethesda, Maryland

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GORI, GIO B. (Microbiological Associates, Inc., Bethesda, Md.). Chemostatic concentrated cultures of heteroploid mammalian cell suspensions in dialyzing fermentors. I. Experimental evidence and theoretical considerations. Appl. Microbiol. **13**:93–98. 1965.—Concentrated chemostatic cultures of HeLa S3-1, KB, and HEp #2 cells have been grown in a dialysis fermentor. Stationary cell concentrations of approximately 1.2×10^6 cells per ml have been produced at rates of 15×10^{-3} to 20×10^{-3} cells per hour for as long as 40 days. The dialysis fermentor appears to be useful in controlling the effects of nutrients on the growth rate of the cultures. Theoretical considerations are offered.

Sustained growth of concentrated animal cell suspensions is limited by accumulation of toxic catabolites, and depletion of nutrilites in the culture fluids (Earle, 1958; McLimans et al., 1958; Cohen and Eagle, 1961).

Graff and McCarty (1957) devised a perfusion device to overcome these limitations, but the complications of their apparatus discouraged further attempts. A simple perfusion apparatus was developed in our laboratory, but proved to be impractical for continued operations; medium-porosity sintered-glass filters clogged in a few days at flow rates of 0.001 ml per cm^2 per min. Moreover, at these low medium velocities, the cells probably utilized the filter surface as a stationary growth matrix, which also contributed to a shorter service life.

To avoid these filtration difficulties, it was decided to dialyze the cell culture continuously against a large amount of growth medium. The dialysis technique was utilized in bacteriology as early as 1896 (Metchnikoff, Roux, and Salimbeni, 1896), was used for nutritional studies in tissue culture by Eagle (1960), and has been recently reviewed and applied to pilot fermentors for concentrated growth of microorganisms by Gallup and Gerhardt (1963).

MATERIALS AND METHODS

Theoretical analysis. The diagram of the apparatus for dialyzed chemostatic cultures is indicated in Fig. 1. For extensive mathematical analysis of the growth and fluid kinetics in this type of fermentations, we refer to the original works of Monod (1950), Novick and Szilard (1950), and the excellent elaboration of Herbert, Ellsworth, and Telling (1956). A simplified version of this theoretical analysis, and its extension to dialyzed or perfused cultures in ideally mixed fermentors, is presented as follows. The growth dynamics are regulated by the equation:

$$\frac{dN}{dt} = aN \tag{1}$$

where N is the cell concentration, a is the growth rate, and t is the time. Theoretically, the growth rate a is related to the concentration of nutrients in the medium by the following relation:

$$a = A \frac{c}{k + c} \tag{2}$$

where A is the maximum value of a, c is the concentration of nutrients in the fermentor, and k is a constant numerically equal to the value of c when $a = \frac{1}{2} A$. The dilution rate D is defined as:

$$D = W/V \tag{3}$$

where W is the flow rate of medium through the fermentor, and V is the volume of the culture in the fermentor itself. Therefore, the variability of the cell concentration N in a chemostatic system

is expressed as:

$$\frac{dN}{dt} = aN - DN \tag{4}$$

Similarly, the concentration c of nutrients in the fermentor follows this relation:

$$\frac{dc}{dt} = D(C - c) - uaN \tag{5}$$

where D is the dilution rate, C is the concentration of nutrients in the medium entering the fermentor, c is the concentration of nutrients in the fermentor, u is the utilization coefficient representing the moles of nutrients utilized by a single cell, a is the growth rate, and N is the cell concentration.

Stationary conditions of the culture are verified by $dN/dt = 0$ and $dc/dt = 0$. Therefore, substituting equation 2 in equation 4, and solving equation 4 for $dN/dt = 0$, the value of the stationary concentration c of nutrients in the fermentor is:

$$c_s = \frac{Dk}{A - D} \tag{6}$$

Similarly, substituting equation 2 and 6 in equation 5, and solving equation 5 for $dc/dt = 0$, the stationary cell concentration N is:

$$N_s = \frac{1}{u}\left(C - \frac{Dk}{A - D}\right) \tag{7}$$

Equations 6 and 7 predict all stationary states possible in a given chemostatic system, if the values of A, k, and u are known.

From equation 4 it is evident that, in order to maintain sustained growth, the dilution rate D must not exceed the growth rate a; otherwise dN/dt becomes negative, and the culture is progressively diluted to extinction. Theoretically, the maximal dilution rate corresponds to the highest possible growth rate:

$$D_{max} = A\frac{C}{k + C} \tag{8}$$

where C is the concentration of nutrients in the medium entering the fermentor. Each dilution rate below D_{max} determines a particular stationary state of the culture.

The yield DN of a chemostat is the product of the dilution rate and the cell concentration, and substituting equation 7 it is:

$$\text{Yield} = D\frac{1}{u}\left(C - \frac{Dk}{A - D}\right) \tag{9}$$

The optimal dilution rate which gives the maximal yield is defined by differentiating equation 9 with respect to D and equating to zero:

$$D_{optimum} = A\left(1 - \sqrt{\frac{k}{k + C}}\right) \tag{10}$$

Therefore, substituting equation 10 in equation 7, the optimal stationary concentration is:

$$N_{s_{optimum}} = \frac{1}{u}(C + k - \sqrt{k^2 + kC}) \tag{11}$$

Theoretically, the value of the cell concentration in the fermentor is directly related to the volume of dialyzing medium. In other terms, if a nondialyzed culture has a volume V and a maximal concentration N_{max} compatible with optimal growth, then the same culture dialyzed against a volume Vd of optimal medium could reach the following maximal concentration compatible with optimal growth:

$$Nd_{max} = N_{max}\left(e\frac{Vd}{V} + 1\right) \tag{12}$$

where e (min = 0, max = 1) is a coefficient of dialysis efficiency, depending on the surface and porosity of the dialyzing membrane and on the rate of dialysis.

When the cell concentration in the fermentor from a value N_o reaches the value Nd_{max}, the metabolite-catabolite balance of the system be-

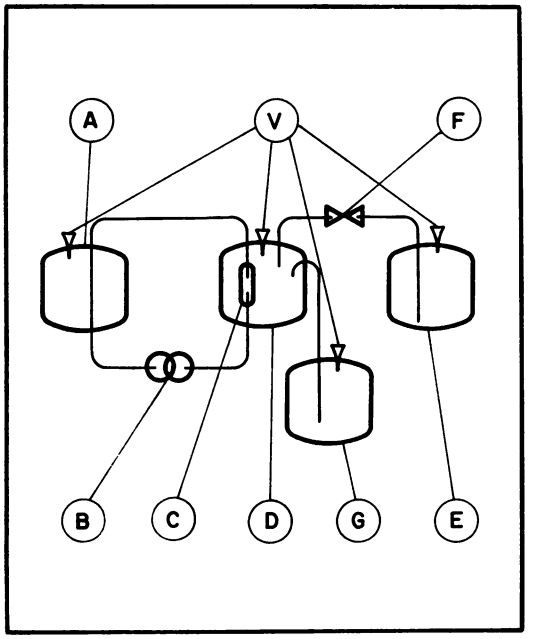

FIG. 1. *Chemostatic dialysis fermentor. (A) Dialyzing medium reservoir. (B) Pump. (C) Dialysis membrane. (D) Fermentor. (E) Eluting medium reservoir. (F) Eluting medium pump. (G) Cell collecting reservoir.*

comes critical, and the growth rate a decreases eventually to self-extinction. The time T required to reach this condition is directly related to the growth rate a, and can be generally expressed as:

$$T = \frac{1}{a} \ln \frac{Nd_{max}}{N_o} \quad (13)$$

However, if a chemostatic culture is maintained at a stationary concentration Nd lower than the maximal concentration Nd_{max}, the service life of the dialyzing medium will last until the total number of cells, compatible with the nutrient capacity of the dialyzed medium and of the diluting medium, is produced. This total number of cells is approximated as follows:

$$m_{max} = Nd_{max} (V + Vd + Ve) \quad (14)$$

where V is the initial and constant volume of the cell culture in the fermentor, Vd is the volume of the dialyzing medium, and Ve is the volume of medium eluted through the fermentor.

The time for exhaustion of the dialyzing medium is now:

$$T = \frac{1}{a} \left(\ln \frac{Nd_{max}}{N_o} + \ln 2 \left(\frac{m_{max}}{V Nd_{max}} - 1 \right) \right) \quad (15)$$

Apparatus. The apparatus is illustrated in Fig. 1 and 2. The medium reservoir A is a 25-liter flask immersed in a water bath cooled at 4 C. A tubulation passing through the peristaltic pump B (Sygmamotor, Inc., New York, N.Y.) circulates the medium through the dialyzing tube C (regenerated cellulose; 48 Armstrong A.P.D.; Arthur H. Thomas, Philadelphia, Pa.; 4-cm diameter, 30-cm length) at approximately 200 ml/min, and with a positive pressure of approximately 0.01 kg/cm² inside the dialyzing tube. The fermentor B is a 2-liter flask with an overflow at the 1,800-ml level. Elution medium is added to the fermentor at a constant rate, but a syphon outlet provides for an intermittent discharge of the cell suspension between 2,000 and 1,800 ml, thus avoiding the accumulation of cell sediments in the tubes. The fermentor is immersed in a water bath at 37 C, and the culture is stirred by a suspended magnetic stirrer (5-cm bar) at 200 rev/min. The eluting flow-metering pump F is a piston pump (Beckman, solution-metering pump, model 746) regulable from 0 to 20 ml/min. The collecting reservoir G is a 2-liter flask. Media containers and fermentor are usually shielded from light to prevent degradation. All tubing is of surgical latex boiled in 5% sodium bicarbonate for 30 min and rinsed in distilled water. Vents are provided with cotton air filters. The entire unit is autoclaved at 120 C for 15 min.

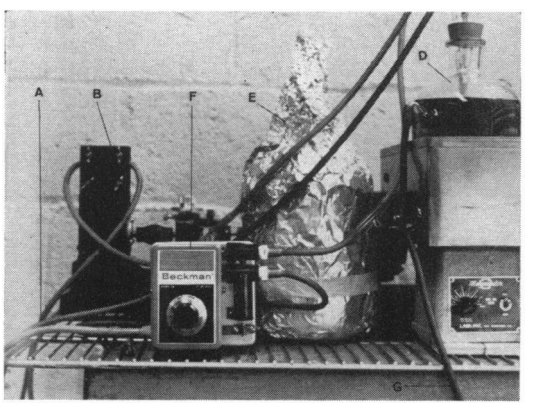

FIG. 2. *Chemostatic dialysis fermentor. (A) Pipelines to dialyzing medium reservoir. (B) Dialyzing medium pump. (D) Fermentor. (E) Eluting medium reservoir. (F) Eluting medium pump. (G) Pipeline to cell-collecting reservoir.*

Media. Minimal essential medium for suspended cultures (SMEM), described by Eagle (1959), was used in all experiments. This medium was supplemented with 10% inactivated calf serum (SMEM, 90-CS, 10).

In several instances, it has been found that cultures seriously contaminated by *Mycoplasma* can not endure sustained growth in chemostatic systems at high cell concentration. The addition of chlortetracycline, 5 μg/ml, has been most helpful in containing the effect of contaminating *Mycoplasma*. Aeration has been achieved by partial change of the air present in the reactor if the culture was too acid.

Medium toxicity has been found to be a major cause of failures, and it is imperative to test all medium batches for cloning efficiency prior to use. No methylcellulose or other compounds have been used to increase medium viscosity.

Cell cultures. HeLa, Clone S3-1, was obtained from N. Saltzman of the National Institutes of Health, Bethesda, Md. This culture, originally grown in SMEM-horse serum 5%, has been adapted to grow in SMEM, 90-CS, 10. Slight *Mycoplasma* contamination was present, approximately one colony per 1 million cells. KB (Eagle) and HEp #2 (Toolan) suspension cultures were obtained from production stocks of Microbiological Associates, Inc., Bethesda, Md. These lines are *Mycoplasma*-free and are usually propagated in SMEM, 90-CS, 10. Cell counts of viable cells have been performed in duplicate at the hemocytometer, after mixing in equal parts with a 1% trypan blue solution in isotonic saline.

RESULTS

Effect of dialysis. The growth curves of HeLa S3-1 cultures started from the same cell suspension are indicated in Fig. 3. All were 2,000-ml undiluted and unfed batch cultures; one was not dialyzed, and the others were continuously dialyzed against varying amounts of growth medium SMEM, 90-CS, 10. The maximal cell concentration reached by each culture was proportional to the volume of dialysis medium, approximately as predicted by equation 12, and assuming a 50% efficiency of dialysis ($e = 0.50$).

Dialyzed chemostatic cultures. In Fig. 4, the growth curve of a dialyzed chemostatic culture of HeLa S3-1 is indicated. The culture was continuously dialyzed against 10 times its volume of SMEM, 90-CS,10. The growth rate a was calculated by the following formula:

$$a = \frac{\ln 2}{td} = \frac{0.693}{td} \qquad (16)$$

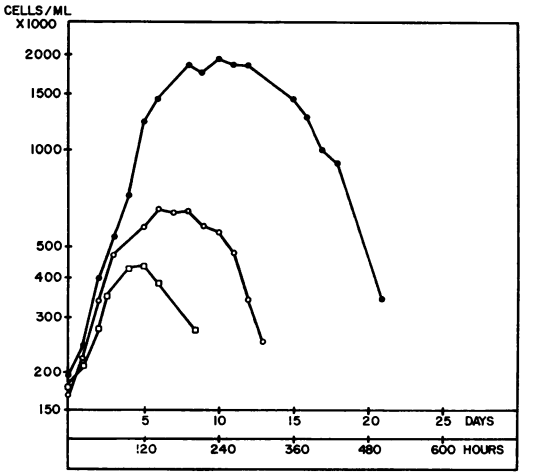

FIG. 3. *Effect of dialyzing medium volume on cell concentration. Symbols:* ●, *culture dialyzed against 10× its volume;* ○, *culture dialyzed against 5× its volume;* □, *culture not dialyzed.*

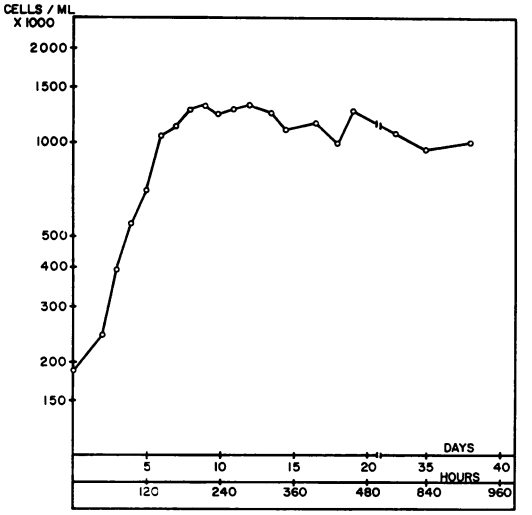

FIG. 4. *Dialyzed chemostatic culture of HeLa-S3-1 cells. Growth rate,* $a = 16 \times 10^{-3}$ *per hour; dilution rate,* $D = 5 \times 10^{-3}$ *per hour started at 156 hr. Dialyzing medium was changed at 432 hr.*

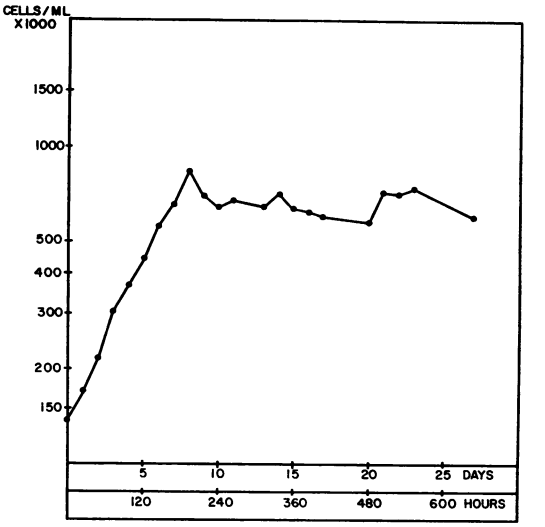

FIG. 5. *Dialyzed chemostatic culture of KB cells. Growth rate,* $a = 11 \times 10^{-3}$ *per hour; dilution rate,* $D = 5 \times 10^{-3}$ *per hour started at 192 hr. Dialyzing medium changed at 480 hr.*

where the value of the duplication time td was extrapolated from the logarithmic growth curve. The calculated growth rate was approximately 16×10^{-3} per hour. At 144 hr, before toxic cell concentrations could be reached, the culture was diluted at a dilution rate $D = 5 \times 10^{-3}$ per hour, thus establishing a chemostatic culture. The cell concentration in this chemostatic culture was not stationary, because the dialysis medium was being recycled and therefore accumulated toxic materials. This increasing toxicity of the medium caused a progressive decrease of the growth rate and of the stationary cell concentration, until the dialyzing medium was substituted with fresh medium at approximately 432 hr. At this point, the growth rate and the stationary cell concentration increased again. As a consequence, the growth curve has a "saw tooth" behavior.

Similar curves are shown in Fig. 5 and 6 for cultures of KB and HEp #2.

In general, the time at which the dialyzing medium became toxic and had to be changed was shorter than predicted by equation 15, because the medium change was performed before critical toxicity could develop.

Effect of dilution rate variation. The growth curve of a chemostatic HeLa S3-1 culture, continuously dialyzed against 10 × its volume of SMEM, 90-CS,10, is shown in Fig. 7. The growth rate $a = 18 \times 10^{-3}$ per hour was cal-

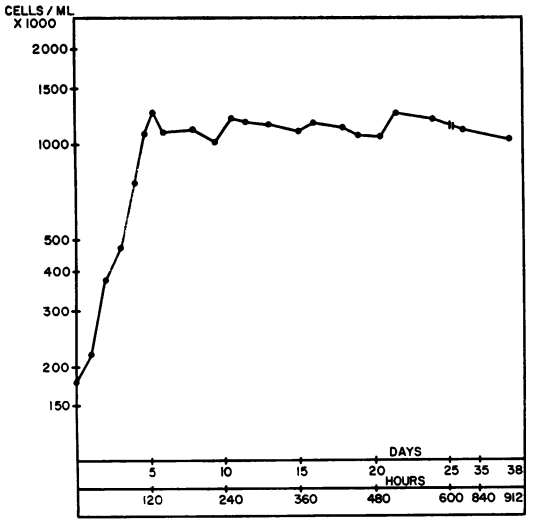

FIG. 6. *Dialyzed chemostatic culture of HEp #2 cells. Growth rate, $a = 17 \times 10^{-3}$ per hour; dilution rate, $D = 5 \times 10^{-3}$ per hour started at 120 hr. Dialyzing medium was changed at 228 and 492 hr.*

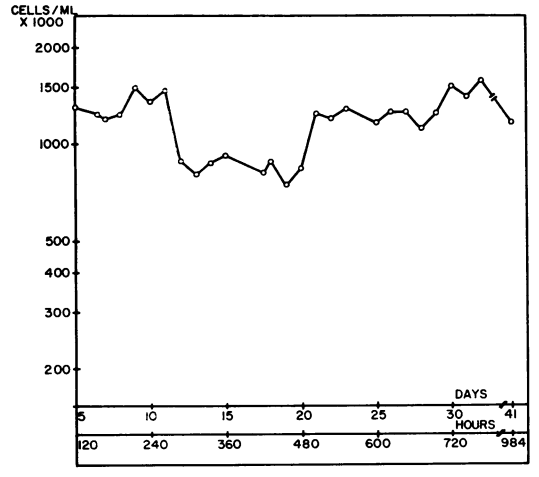

FIG. 7. *Effect of dilution rate variation on cell concentration of a dialyzed chemostatic culture of HeLa-S3-1 cells. Growth rate, $a = 16.5 \times 10^{-3}$ per hour at 48 hr; dilution rate, $D = 5 \times 10^{-3}$ per hour at 120 hr, $D = 10 \times 10^{-3}$ per hour at 264 hr, and $D = 5 \times 10^{-3}$ per hour at 480 hr. Dialyzing medium was changed at 192 and 672 hr.*

culated as described in equation 16. At 120 hr the dilution rate was set at $D = 5 \times 10^{-3}$ per hour, resulting in a stationary concentration around 1.35×10^6 cells per ml. At 192 hr the dialyzing medium was changed, and at 264 hr the dilution rate was adjusted to 10×10^{-3} hr, resulting in a stationary concentration of approximately 0.875×10^6 cells per ml.

At approximately 480 hr, the dilution rate was again set to $D = 5 \times 10^{-3}$ per hour, which increased the stationary concentration to approximately 1.25×10^6 cells per ml. All variations of the stationary concentrations followed the postulations of equation 7.

DISCUSSION

The experimental data indicate the feasibility of concentrated chemostatic cultures of heteroploid mammalian cell suspensions. The cultures must be either dialyzed or perfused to remove toxic catabolites and to provide nutrients. The medium used must permit cloning at a high efficiency, and the dilution rate of the chemostat must be lower than the maximal growth rate of the culture. The growth dynamics of these cultures follow mathematically predictable patterns; yet they have not reached theoretical maximal values in actual experiments. The limiting factors are unknown, but if they are not dialyzable (Cohen and Eagle, 1961), then the best results will probably be attained in dialyzed and perfused fermentors.

Studies are in progress to determine reliable and efficient perfusion methods, the nutritional variables of this type of culture, and their general application to continuous automatic growth of virus suspensions.

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