A SIMPLIFIED CHEMOSTAT FOR THE GROWTH OF MAMMALIAN CELLS: CHARACTERISTICS OF CELL GROWTH IN CONTINUOUS CULTURE

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(Received for publication, October 4, 1960)

The continuous culture of bacteria has been described by Monod (1), Novick and Szilard (2), and others (3–7). Sustained growth in a constant environment is achieved by the continuous introduction of fresh medium at a fixed rate, and the simultaneous removal of spent medium and cells. Apparatus has similarly been designed for the continuous cultivation of mammalian cells (8, 9). The present paper describes a simplified procedure, and the characteristics of cell growth under these conditions.

Methods

Apparatus.—As shown in Figs. 1 and 2, the apparatus consists basically of 3 parts:—

1. A reservoir, from which fresh medium is slowly introduced into the culture vessel. The dilution rate (fraction of the culture volume replaced daily) can be controlled by either of two methods: (*a*) a mechanical pump (the Sigma peristaltic pump was used in most of the experiments to be here described), or (*b*) a constant head of gravity between the reservoir (bottom of air vent) and the culture (tip of capillary inlet tube). The former method provided dilution rates reproducible to 1 to 3 per cent; the latter was at best reliable to 10 per cent.

2. The culture vessel. Complete and rapid mixing of the fresh medium with the culture is effected by a magnetic stirrer, which also serves to maintain the cells in suspension.

3. An overflow container. As fresh medium is introduced, an equal volume is removed from the culture through the hollow overflow tube shown in the diagram. The cells collected in the overflow vessel grow normally when maintained in suspension.

Medium and Cultures.—The medium used in these experiments was a minimal growth medium, embodying only the 27 factors demonstrably essential for growth (10), supplemented with 5 per cent dialyzed human or horse serum. Growth supplements, such as the nutritionally non-essential amino acids or pyruvate, may be added; and for routine cultivation, whole serum may be substituted for the dialyzed preparation.

All the experiments here described were carried out with the HeLa-S3 line, using both horse and human serum. Qualitatively similar results have however been obtained with strain KB and human "liver" cultures.

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Assembly and Operation.—The entire apparatus can be autoclaved and made operational in a matter of a few hours. No further care is required other than to refill the medium reservoir and to empty the overflow container. The actual assembly, shown in Fig. 2 may be placed in a warm room; alternatively, the culture vessel alone may be immersed in a suitable 37° bath. Although no attempt was made to control pH, this was only a minor problem, in that the production of lactic acid by the cells was more or less balanced by the loss of CO_2 through the vent tube shown in Figs. 1 and 2. In consequence, the pH varied by no more than 0.5 pH units over a wide range of population densities. The vent tube, as well as the influx of fresh medium, permitted adequate oxygenation of the culture.

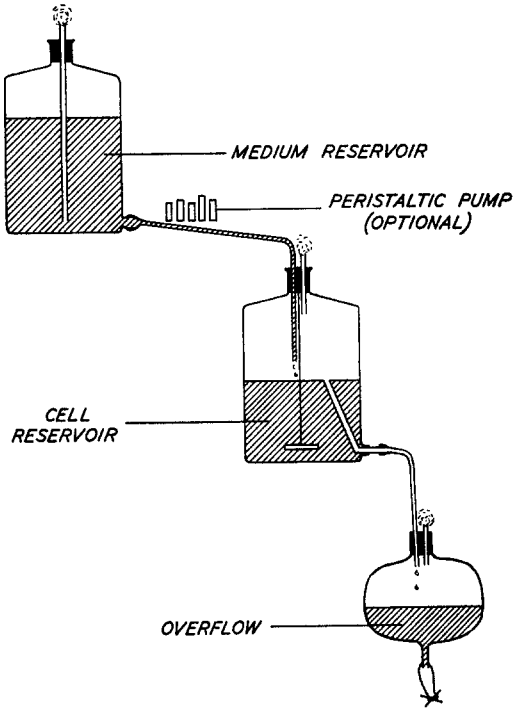

FIG. 1. A mammalian cell chemostat (diagrammatic). Medium is introduced into the cell reservoir either by gravity or a mechanical pump. Spent medium and cell overflow through the tube which projects into the culture, the volume of culture remaining constant.

The longest period of continuous operation has been 11 weeks, at which time the culture was discontinued. The most common source of difficulty was contamination incident to the removal of samples from the culture vessel.

RESULTS

The kinetics of cell growth in the continuous flow apparatus here described generally paralleled those of bacteria grown under similar conditions.

Population Density.—The cell count per milliliter varied inversely with the rate of input of fresh medium. When that dilution rate was decreased, the

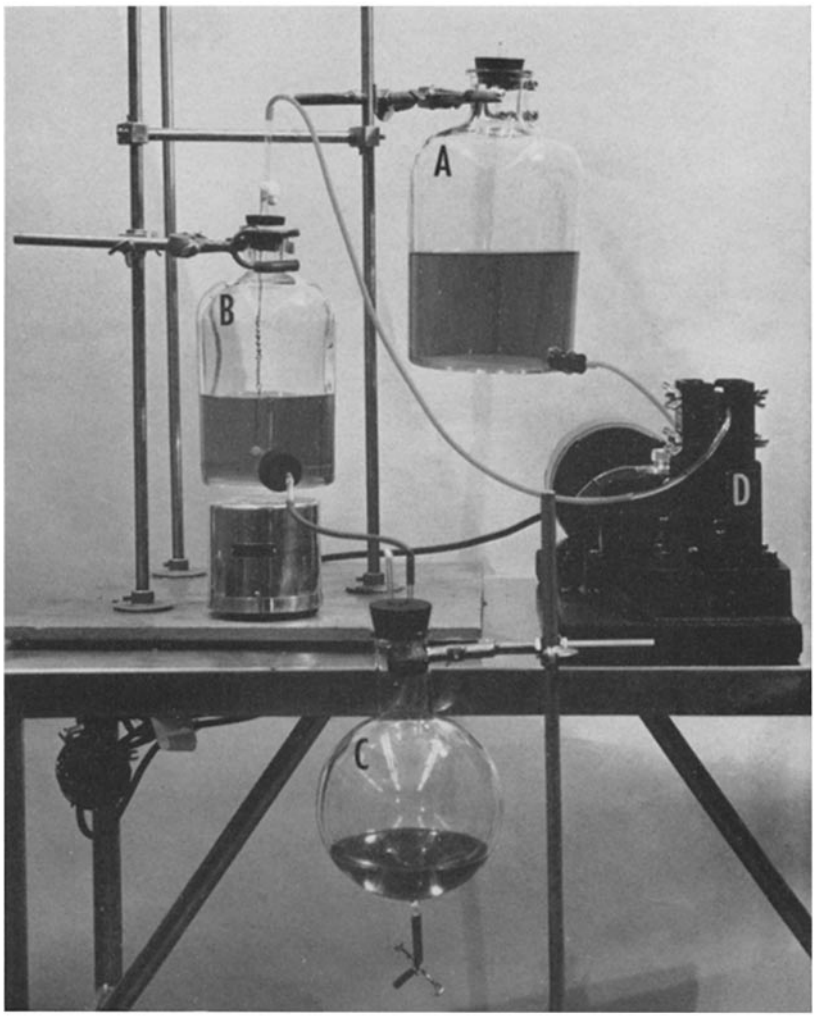

FIG. 2. Photograph of the mammalian cell chemostat with a Sigma peristaltic pump in place.

A, the medium reservoir; *B*, the cell reservoir; *C*, the overflow; *D*, the peristaltic pump.

The water bath, which surrounds culture vessel *B*, and which includes a heater, thermostat, and circulator, is not shown. The rubber stopper at the bottom of the culture vessel projects through a hole cut into the wall of the water bath.

population density increased over a period of several days to reach a new plateau, at which it usually remained until dilution rate was again changed (Fig. 3). At low dilution rates the cell count did not remain stable indefinitely, but sometimes varied in a cyclical pattern illustrated in Fig. 4. A similar observation has been made by Cooper (8), and the basis of that fluctuation is under

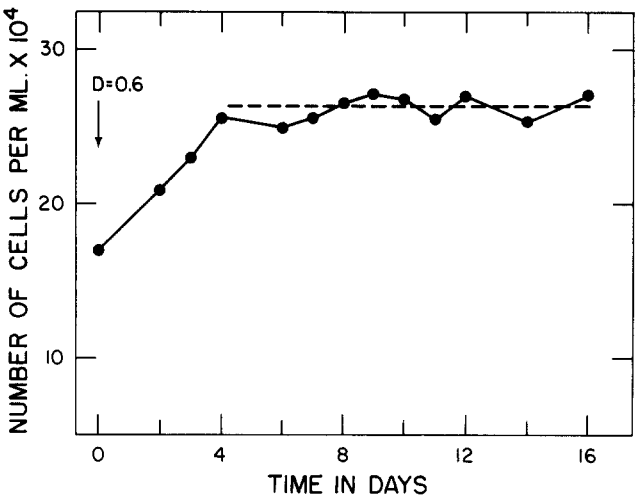

FIG. 3. Illustrating the stabilized cell count at a constant rate of medium input (dilution rate of 0.6 volumes per day).

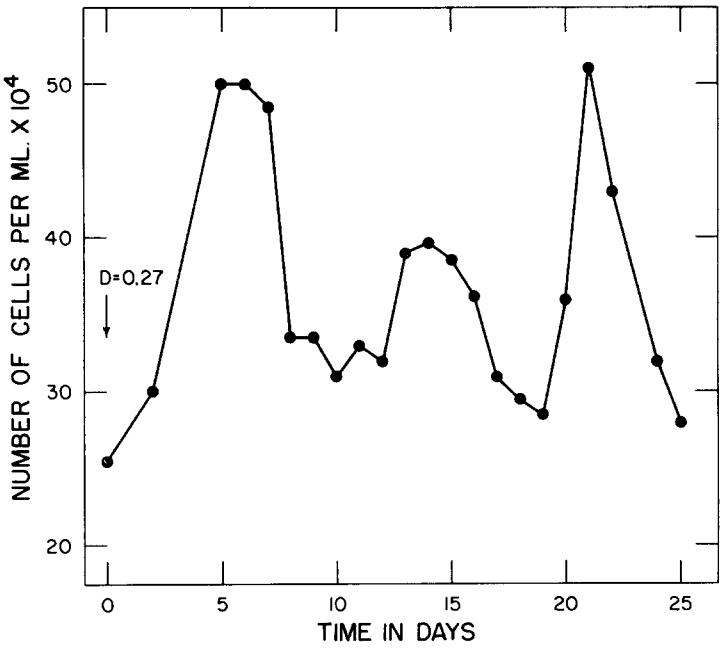

FIG. 4. Cyclical variation in cell population at a low input rate of fresh medium.

present study. The correlation between the dilution rate and the stabilized cell count is shown for a single experiment in the left-hand portion of Fig. 5; qualitatively and quantitatively similar results have been obtained in a large number of experiments. Not shown in the figure is the fact that with excessively

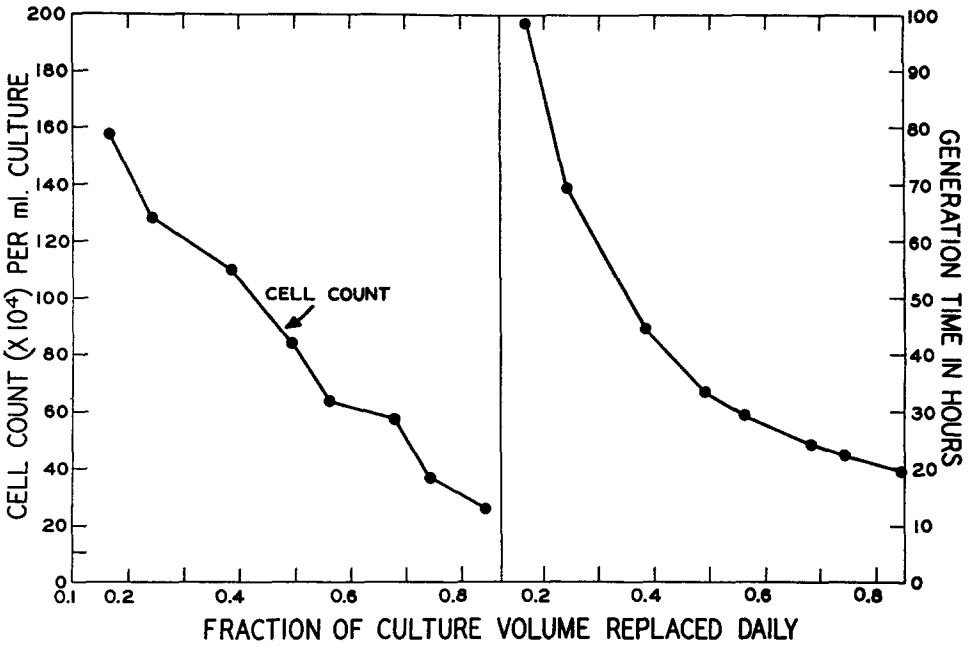

FIG. 5. A single experiment illustrating the relation between dilution rate of the culture with fresh medium, cell population density, and generation time. Each point in the figure is the average of 3 successive days during which the cell population level remained essentially constant (*cf.* Fig. 3). The generation (doubling) time in hours is calculated as $\frac{24 \times 0.693}{\text{fraction of culture replaced daily}}$ (1, 4).

high dilution rates, the cells were washed out of the culture faster than they could multiply, and the count fell progressively without ever reaching a stabilized level.

Generation Time.—At a stabilized cell count, the generation time was obviously an inverse function of the dilution rate (*cf.* Fig. 5). In that experiment, the generation time increased from 20 to 99 hours as the dilution rate was decreased from 0.85 to 0.15. The shortest generation time has usually been approximately 16 hours; in one experiment it was, however, as little as 11.5 hours.

In all these experiments, the cellular protein N per unit culture volume

paralleled the cell number, indicating that the protein content per cell did not vary significantly over widely varying growth rates and population densities (Table I).

The possibility was considered that at low dilution rates, many of the microscopically visible cells might have been non-viable, and the apparently increased generation time an artefact. This was excluded by the finding that the cloning

TABLE I

The Constant Cellular Protein Content and Volume in Cultures Growing at Widely Varying Rates

Dilution rate	Stabilized cell population per ml.	Packed cell volume/liter culture	Packed cells		Packed cells
			Cold TCA residue	Hot TCA residue	
		ml.	*Mg. N/ml.*	*Mg. N/ml.*	*Cell No./ml.*
0.73	13×10^4	0.58	14.8		2.18×10^8
0.48	30	1.16	13.8		2.58
0.29	67	2.4		11.0	2.73
0.27	55	2.2	11.9		2.50
0.22	54	2.0	12.2	10.1	2.71

TABLE II

Showing That the Slow Rate of Cellular Growth at Low Impact Rates of Medium Is Not Due to the Death of a Large Portion of the Cells Counted

Day No.	Dilution rate	Cell count $\times 10^4$/ml.	Cells/clump	No. of clumps cultured	No. of clones after 8 days
1	0.2	75	1.4	100	60, 59, 52
3	0.2	80	1.2	100	78, 73, 68
6	0.18	75	1.2	100	70, 69, 66
8	0.18	103	1.5	100	74, 59, 56
10	0.18	100	1.25	100	63, 61, 57
13	0.19	95	1.6	100	54, 52, 43

efficiency, *i.e.* the proportion of cells capable of adhering to glass and growing out to form a visible colony, was essentially independent of the dilution rate (Table II). This renders unlikely (but does not rigorously exclude) the possibility that at low input rates, a small proportion of the microscopically visible cells was multiplying at a normal rate.

Growth-Limiting Factors in Stabilized Cultures.—As the dilution rate of the culture with fresh medium was progressively decreased, there was necessarily a corresponding decrease in the growth rate of the cells (*cf.* Fig. 5). This strongly suggested that one or more components of the medium were being reduced to growth-limiting levels. However, as seen in Table III, no such deficiency could

be demonstrated. Over a wide range of dilution rates and cell population densities, in which the generation time varied 6-fold, the amino acids and glucose of the medium remained at levels sufficient for rapid growth. Cystine and methionine were present in least concentration; but the addition of excess

TABLE III

Showing That at Slow Dilution Rates, with Prolonged Generation Time, the Medium Had Not Been Depleted of Essential Amino Acids or Glucose

Stabilized dilution rate*	Stabilized cell count per ml.	Concentration (mM) in culture fluid											
		Arg.	Cys.	Glut.	His.	Isol.	Leuc.	Lys.	Meth.	PhAl.	Thr.	Tyr.	Val.	Gluc.
Original medium		0.60	0.10	2.0	0.2	0.4	0.4	0.4	0.1	0.2	0.4	0.2	0.4	5.5
0.85	26 × 10^4	0.39	0.022	>0.3	0.128	0.18	0.15	0.247	0.20(?)	0.14	0.23	0.19	0.20	2.2
0.64	34		0.005	>0.3		0.26	0.24		0.078	0.13	0.25	0.15	0.23	
0.66	56		0.023	>0.3		0.19	0.16		0.016	0.12	0.23	0.14	0.24	0.65
0.55	66		0.018	>0.3		0.16	0.12		0.019	0.097	0.21	0.12	0.16	0.66
0.38	110	0.31	0.011	>0.3	0.13	0.14	0.099	0.17	0.021	0.09	0.20	0.11	0.14	0.99
0.17	158		0.012	0.28		0.11	0.069		0.02	0.084	0.24	0.12	0.11	0.49

* Fraction of culture volume replaced in 24 hours.

TABLE IV

Concentration (mM) in Culture Fluid of Amino Acids Synthesized by the Cells and Released into Medium

Stabilized dilution rate*	Stabilized cell count per ml.	Serine	Proline	Glycine	Alanine
Original medium		0	0	0	0
0.85	26 × 10^4	0.13		0.004	0.137
0.64	34		0.062	0.008	0.149
0.66	56		0.054	0.009	0.279
0.55	66	0.04	0.088	0.14	0.251
0.38	110	0.065	0.067		0.32
0.17	158				0.233

* Fraction of culture volume replaced in 24 hours.

cystine, methionine, and glucose to such slowly growing cultures caused no increase in growth rate. Except for glycine, the concentrations of the nutritionally non-essential amino acids synthesized by the cells and released into the medium similarly did not vary greatly over a wide range of cell populations (Table IV). Bubbling a 5 per cent CO_2-air mixture through the culture at three different population levels also had no significant effect on population density or generation time.

In some experiments at low dilution rates, when the culture fluid was examined for its ability to support growth in freshly planted monolayer cultures, the cells died. The addition of either dialyzed serum, vitamin, or complete amino acid supplements did not reactivate the material. Further, the spent medium inhibited the growth-promoting effect of fresh medium. The growth inhibitory factor(s) was nondialyzable; studies on its characterization are in progress.

SUMMARY

A simplified technique has been described for the continuous growth of mammalian cells in suspension culture. The cell population density increased as the rate of input of fresh medium was decreased, and the average generation time was concomitantly prolonged. At relatively high input rates, the population remained stabilized for an indefinite period, but at low flow rates, there was sometimes a cyclical variation in population density. The factor limiting growth rate at input rates of approximately 0.2 volumes per day was not the exhaustion of the medium; but in some experiments a non-dialyzable material appeared which inhibited cell growth.

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