# MODES OF GROWTH IN MAMMALIAN CELLS

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ABSTRACT The increase of cell volume as a function of time was studied throughout the generation cycle in synchronous cultures of Chinese hamster cells using a Coulter aperture and a multichannel analyzer calibrated against known cell volumes. The experimental results were compared to a mathematical model of cell volume increase which considered the effect of the distribution of individual cell generation times on the progress of the population. Several modes of volume increase, including linear and exponential, were considered. The mean volume vs. time curve was rounded at the ends of the cycle even when linear growth was assumed. The experimental results show that cell volume increased in a smooth fashion as a function of time, with no discontinuities in rate detectable at periods when cells may have been undergoing metabolic shifts as, for example, through the phases associated with DNA synthesis, $G_1$, S, $G_2$. A statistical test on the comparison of the modal cell volume vs. time data to the predictions of linear and exponential growth models accepted both hypotheses within the resolution of these experiments. However, exponential growth was favored over linear growth in one cell line. Volume dispersion was almost constant with time in both sublines which is also consistent with exponential growth. Limitations of the electronic technique of volume measurement and indications for future experiments are discussed.

## INTRODUCTION

A number of investigations recently have dealt with the mode of cell growth during the generation cycle in single cells of various species (1–4). In bacteria, exponential increase in accumulation of macromolecules (DNA, RNA, and protein) has been observed (1) while in another study, volume increase appeared to follow a linear mode (4). More complicated growth patterns may exist in cells in which differences in rates of macromolecular syntheses occur during the cell cycle. These apparently occur in bacteria (5) but the times involved are so short that the detection of volume changes concurrent with metabolic shifts, if these occur, would be extremely difficult. In mammalian cells (and in some other species also, e.g. [6]) changes occur especially in DNA synthesis (7–9) which may be sufficiently discrete in time to allow detection of concurrent volume changes. In some instances complex patterns of volume increase have been observed (7), (9), and (10). However, in another study (11), in mouse L cells, dry mass was observed to increase regularly in a manner

indistinguishable from linear or exponential and this increase correlated with RNA content in the cell.

In a preliminary report (12) from this laboratory several years ago, it was shown that the average volume of a synchronous population of Chinese hamster cells, grown in vitro, increased steadily with time during the cell generation cycle. A comparatively simple mode of growth exhibiting no discontinuities related to metabolic changes in the cell (such as onset or cessation of DNA synthesis) was indicated.

Koch (13) has discussed the theoretical limitations of considering modal volume as a function of time and he suggests that the rate of volume increase, $dV/dt$, is a more sensitive test of growth laws. Bell (14) and Bell and Anderson (2) have formulated a model of cell populations which uses rate of volume increase as a population parameter. The merits of this approach have been discussed recently by Anderson et al. (15). However, this model, which has yielded much useful information concerning the interrelationship between the volume spectrum and growth parameters has not revealed definitive information on the nature of growth laws when applied to the asynchronous volume spectrum in particular cases (3, 15). One objection to this method is that the rate of volume increase is not a directly measureable quantity and must be calculated from higher order moments of the volume spectrum and from other experimental data. On the other hand, average or modal volume and volume distribution width during the cell cycle are directly observable quantities not dependent on a subsequent analysis of cell population characteristics. However, the growth parameter of principal interest $\frac{d\bar{V}}{dt}$ must still be derived from the average volume. At the present state of our knowledge presumably both approaches can be exploited to yield useful information.

The present paper discusses the principal features of cell growth as observed in synchronous cultures of two sublines of Chinese hamster cells. An apparatus involving a Coulter counter aperture (Coulter Electronics, Hialeah, Fla.) and a 400 channel analyzer, capable of much higher resolution than our former equipment, was used to measure the peak (modal) volume and the volume distribution half-width at half-maximum height of the volume spectrum as a function of time in the cell cycle. The resulting experimental data was compared with a mathematical model of cell volume increase which predicts the time development of the mean volume in a synchronous population with a small spread in cell doubling times. The conclusion is reached that the sensitivity of methods of studying modes of volume increase is limited by experimental techniques rather than by the sensitivity of the methods of analyzing the data.

## MATHEMATICAL THEORY OF CELL GROWTH AND DIVISION IN SYNCHRONOUS POPULATIONS

A single cell increases its volume within a generation time until the initial volume is approximately doubled. After division its daughters repeat this cycle. A syn-

chronous population of cells exhibits the same behavior in terms of the mean cell volume and the mean generation time. As synchrony decays, due to the dispersion of generation times within a population of cells, the fluctuation of the mean volume becomes damped to an average value and the population approaches an exponential increase in cell number characteristic of log phase, steady-state growth. Our aim here is to provide a model which will predict the time development of cell number and volume of a synchronous population. The model presented is the simplest form that the authors have found convenient for comparison with experiment. The expression to be derived here for the increase of cell number with time can also be derived from more general expressions of Harris (16) and equation 21 of Rubinow (17), which describe the number of cells in a given generation and then sum over all generations present in the population. The method employed in this text is quite applicable to situations in which the coefficient of variation of the distribution of generation times in the population is small and attention is confined to the first few generations so that all cell division takes place between the limits given by equation 1 below. If this condition does not apply (e.g. in later generations) the more general equations are to be preferred.

*Cell Number*

Assume that at time $t = 0$, we have a population of cells all of the same size $V_o$, and all of the same age (just divided). For a time no cells will divide, but as the average generation time of the population $T_o$ is approached, a fraction of the cells will divide within any given time interval. Define the $n^{\text{th}}$ interval of division as

$$(n - \tfrac{1}{2})T_o \leq t \leq (n + \tfrac{1}{2})T_o . \tag{1}$$

This is a time period which extends from the middle of the $n^{\text{th}}$ generation cycle to the middle of the $(n + 1)^{\text{th}}$ generation cycle. Then the number of cells that will be found in the $n^{\text{th}}$ interval of division is

$$N_n(t) = N_n^o \left[ 2 \int_o^t f_n(T)\, dT + \int_t^\infty f_n(T)\, dT \right] \tag{2}$$

where $N_n^o$ is the number of cells at the start of the interval.

$$N_n^o = 2^{(n-1)} N_o .$$

$N_o$ is the number of cells at time $t = 0$ and $f_n(T)$ is a distribution function of cell generation times $T$, about a mean generation time $T_o$, given explicitly in equation 3.

Equation 2 has been derived directly from considerations of a synchronous population and its performance in subsequent generations. A different form of this ex-

pression, viz

$$N_n(t) = N_n^o \left[ 1 + \int_o^t f_n(T)\, dT \right]$$

is more often used for populations in general when the number of cells present is obtained by summing over all generations.

Equation 2 is valid only if there is no overlap of generations between $(n - \frac{1}{2})T_o$ and $(n + \frac{1}{2})T_o$. This is the case in the experimental situations examined in this paper.

The distribution function $f_n(T)$ describes the distribution of cells dividing with time during the $n^{\text{th}}$ generation. [In the first generation this corresponds to the distribution of generation times.]

$$f_n(T) = \left[ \frac{2}{\pi n \sigma_1^2} \right]^{1/2} \exp \left[ -\frac{1}{2} \left( \frac{T - nT_o}{n^{1/2}\sigma_1} \right)^2 \right]. \tag{3}$$

We have assumed that the distribution of generation times is normal about a mean value, $T_o$, and that the distribution width increases in a well known manner, viz $\sigma_n$ (for the $n^{\text{th}}$ generation) $= n^{1/2}\sigma_1$ if there is no correlation between successive divisions. Thus, $f_n(T)$ describes the frequency of cells dividing in any given time interval for an initially synchronous population. As $n$ becomes large, the number of cells dividing in any time period approaches a constant times the number of cells present and the increase in cell number becomes exponential with time.

There is some difference of opinion as to whether the distribution of generation times $f(T)$ or generation rates $f(1/T)$ is gaussian in an asynchronous population (18, 19). Assuming the generation times to be distributed normally implies that the distribution of generation rates is skewed towards slower rates and similarly assuming the rates to be gaussian means that the distribution of generation times is skewed towards longer times. This effect was investigated in the function $f_n(T)$ and for an initial coefficient of variation $(CV) = \sigma_1/T_o = 10\,\%$, the amount of skewness of either distribution has less than a 1 % effect on the predictions of this theory.

The initial behavior of the synchronous population is incorporated into the parameters $T_o$, the average generation time, and $\sigma_1$ the initial distribution width of $f_n(T)$ for $n = 1$. The effective distribution width in the $n^{\text{th}}$ cycle, $\sigma_n = n^{1/2}\sigma_1$ describes the decay of synchrony.

*Mean Cell Volume*

If the volume of a single cell can be written as a function of time $t$, and its generation time $T$, we can calculate the mean volume of the population by averaging over the distribution of interdivision times $f_n(T)$.

$$V_n(t) = \frac{2^{n-1}N_o}{N_n(t)} \left[ 2 \int_o^t f_n(T) V_n(t, T)\, dT + \int_t^\infty f_n(T) V_n(t, T)\, dT \right]. \tag{4}$$

The factor of two in front of the first integral is due to the doubling of all cells with intervals of division $T \leq t$. The normalization factor is just the number of cells in the $n^{th}$ interval as a function of time.

We now require an expression for $V_n(t, T)$: for linear volume increase,

$$\begin{aligned}
V_1(t, T) &= V_o(1 + t/T_o) && o < t < T \\
V_2(t, T) &= 1/2V_o(1 + T/T_o) + V_o\left(\frac{t - T}{T_o}\right) && T < t < 2T \\
V_3(t, T) &= 1/4V_o(1 + T/T_o) + 1/2V_o(T/T_o) + V_o\left(\frac{t - 2T}{T_o}\right) && 2T < t < 3T \\
&\vdots && (5)
\end{aligned}$$

For exponential volume increase,

$$\begin{aligned}
V_1(t, T) &= V_o \exp((\ln 2)t/T_o) && o < t < T \\
V_2(t, T) &= 1/2V_o \exp((\ln 2)T/T_o) \exp\left((\ln 2)\frac{t - T}{T_o}\right) && T < t < 2T \\
V_3(t, T) &= 1/4V_o \exp((\ln 2)2T/T_o) \exp\left((\ln 2)\frac{t - 2T}{T_o}\right) && 2T < t < 3T \\
&\vdots && (6)
\end{aligned}$$

These sets of equations, 5 and 6, are merely cyclic ramps with either a linear or an exponential slope. We shall assume: (*a*) that different cells grow to different maximum volumes depending on their generation time $T$, i.e. control of division is independent of volume, and (*b*) that parent cells divide into two daughters each with one-half the parent cell volume (cf. reference 15). Then, the initial volume of the cell after division is treated as a function of the generation time within that cycle only. Biologically, one may expect that the volume of a cell is related to more of its past history, including all of its past generation times; however, it was not necessary to include these other variables in this model to obtain an accurate prediction of population behavior.

A more complete description of the volume increase of a population of cells would include, in addition to the distribution of generation times, the possibility of a distribution of rates, rather than the constant rate $1/T_o$. The authors have performed calculations including these other factors, but have found that more detailed theory does not greatly add to the analysis of results in the present investigation.

When the analytic expressions for cell growth (equations 5 and 6) are evaluated in the averaging formulas of equations 2 and 4, the parameters of cell number and mean cell volume can be plotted as continuous functions of time. The calculation requires numerical integration, which was performed on a digital computer. Fig. 1

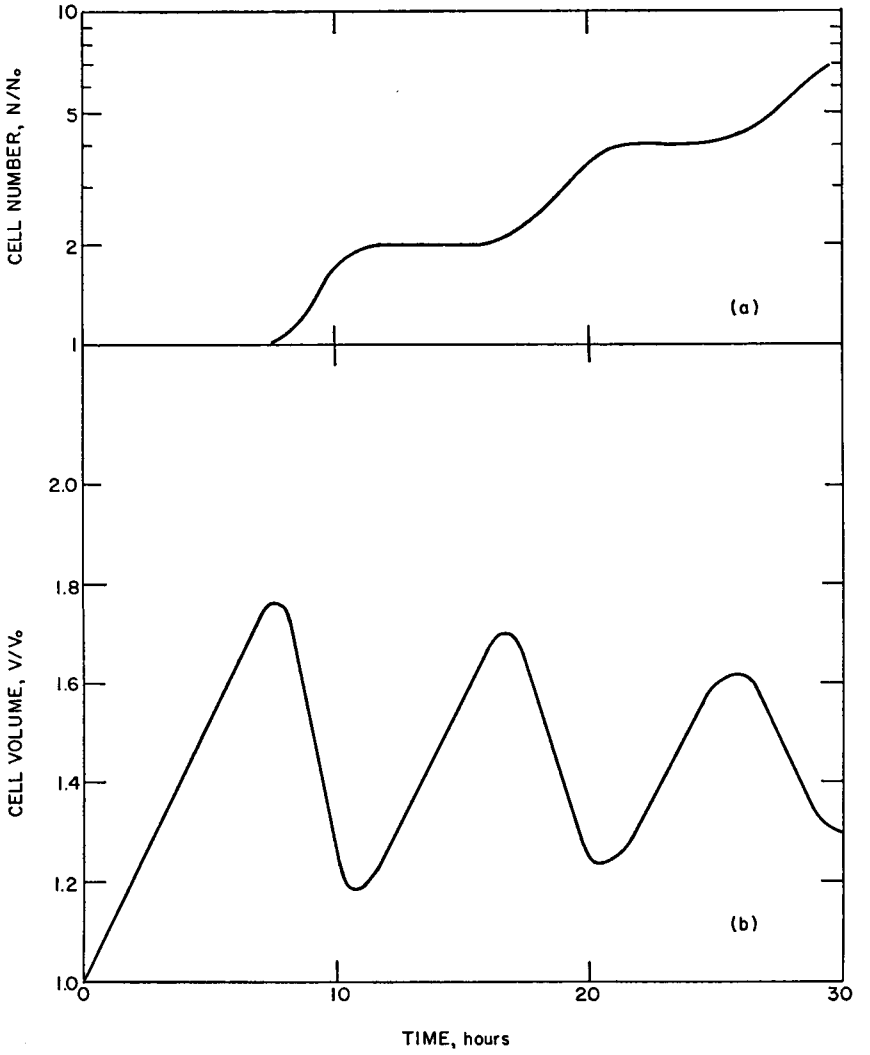


FIGURE 1 Population parameters as a function of time for an initially synchronized population: 2½ cycles of a cell line with an average generation time $T_o$ = 9.5 hr. (*a*) Number of cells vs. time and (*b*) cell volume vs. time assuming linear growth. Note, increasing "rounded" effect due to decay of synchrony.

is a plot of these functions for a cell line with a generation time of 9.5 hr. Fig. 1 *a* shows the increase in cell number which, as Engelberg (20) has shown, increases in a step wise fashion but with longer periods of time during which cells divide in successive cycles as synchrony in the population decays. In Fig. 1 *b* is shown a plot of mean volume as a function of time assuming linear growth. The maximum mean volume does not reach 2 $V_o$ and the new minimum mean volume after division

is greater than $V_o$. This "rounding" of the mean cell volume vs. time curve is inherent in equation 4 and gets progressively worse in successive cycles. It shows clearly that even if growth is truly linear, "rounding" occurs because of the spread of generation times and this reduces the length of the linear portion of growth in the next cycle. With data of poor resolution this effect may even give the appearance of other modes of growth (e.g. exponential). This fact has been used to support a hypothesis of linear growth in *Escherichia coli.* (4).

## MATERIALS AND METHODS

### *Cell Lines and Growth Conditions*

The cells used were sublines of the V79 line of Chinese hamster cells which was originally grown from female lung tissue by Ford and Yerganian (21). One subline, designated V79-S171, had a generation time of about 9.5 hr, subdivided into a pre-DNA synthetic period, $G_1$, of ~1.5 hr, a DNA synthetic period, S, of ~6 hr, a post-DNA synthetic period, $G_2$, of ~1.5 hr, and mitosis lasted 0.5–1 hr. The other subline, designated V79-325, had a longer $G_1$, ~3.5 hr and S ~9 hr, while $G_2$ was about 1.5 hr. Mitosis presumably lasted about 0.5–1 hr also. Cells were grown in monolayer cultures attached to the surface of plastic dishes at 37° C in EM-15 medium (22) in a humid atmosphere of 2% $CO_2$ and air.

### *Synchronous Cultures*

Synchronous cells were obtained from asynchronous cultures in log phase by selection for poorly attached dividing cells using a standard shake-off procedure (23, 24). Aliquots of the resulting suspension of synchronous cells were inoculated into a series of dishes and incubated at 37°C. This temperature was maintained constant in the dishes to ±0.1° C throughout the experiment. At half-hour intervals a dish was removed and cells were detached from the surface by treatment with trypsin (0.03%). Cells of this suspension were counted and their volume distribution was determined by passing them through a 100 × 100 $\mu$ Coulter counter aperture, the signals from which were fed through amplification stages to a 400 channel analyzer. An X-Y plotter was used to obtain immediate visual results of the volume distribution. A further set of culture dishes was used at less frequent intervals to establish, by labeling the cells with tritiated thymidine ($^3$HTdR) and subsequent autoradiography, the subdivisions of the cell generation cycle (21). Mitotic index, Blumenthal-Zaler index, applied to the first half-cycle (25), and L index (24) were used to measure the degree of synchrony in the population, as a check on the quality of the experiment. Typical values of these parameters for V79-325 shown in Fig. 5, were 77%, 95%, and 82%, respectively.

### *Electronic Volume Measurements*

Two methods were used to calibrate the electronic system used for volume measurement. A photographic study of cell size was performed on 700 cells of an asynchronous population by measuring diameters of trypsinized cells under an optical magnification of 1800. This is compared in Fig. 2 to a volume distribution of 500,000 cells from the same population sized electronically. The high degree of correlation achieved (Chi-squared test shows that, to a greater than 95% confidence level, they are identical) provides a means of relating channel

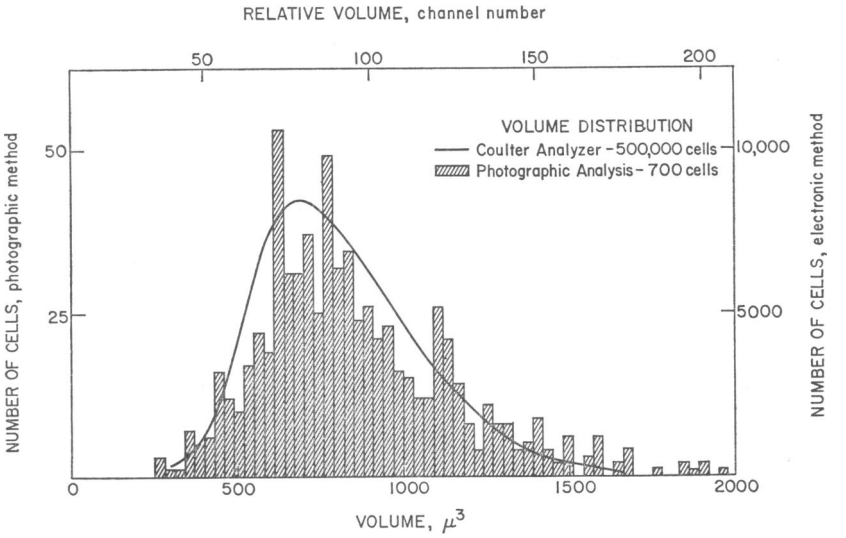


FIGURE 2 Comparison of experimental volume distributions for Chinese hamster cells V79-S171, asynchronous population. Histogram is a photographic analysis of 700 cells. Smooth curve is electronic measurement of volume by Coulter aperture and multichannel analyzer; 500,000 cells.

number to absolute volume in cubic microns. Also a reference distribution of microscopic latex spheres in the same range of volumes as the cells (7–14 $\mu$ in diameter) was checked every few hours to monitor the stability of the system.

The possible effect of the size of the aperture on the volume distribution was checked at the suggestion of H. E. Kubitschek. In our experiments an aperture 100 $\mu$ in diameter and 100 $\mu$ long was used. Similar results for the peak of both the reference distributions and asynchronous distributions of cells were observed with an aperture of 85 $\times$ 185 $\mu$, lent to us by Kubitschek. This longer aperture permits greater transit times, which however appeared to be unnecessary in this instance because our analyzer was adjusted to obtain faster electronic response times. Coincidence counts (two cells simultaneously in the aperture) were nearly eliminated by the speed of the sizing system. A delay-monostable gate was set at 10 $\mu$s to hold the flow of information through the system open for only that length of time necessary for the full volume of the cell to be recorded.

The contents of the 400-word memory were presented to an X-Y plotter which had a null-balance feature to assure accurate plotting. Six typical distributions obtained from a synchronous population during the first two cycles of division are shown in Fig. 3. Arrows above the volume spectra indicate the modal volume of the population. Three of the frames (*a*), (*d*), and (*f*) show spectra at times when the population was undergoing cell division. For these distributions it was possible to separate the population into two subgroups; those cells about to divide and those cells which have recently divided. The arrows over the spectra in these frames indicate the modal volumes of each of these two subgroups and also of the population taken as a whole. The modal volume for the whole population is the weighted mean of the bimodal volume, with the weight factor being the fractional number of cells in each mode. Note: the model actually predicts the *mean* volume of the whole population rather than the peak or modal value. However, the mean and the mode were nearly identical for

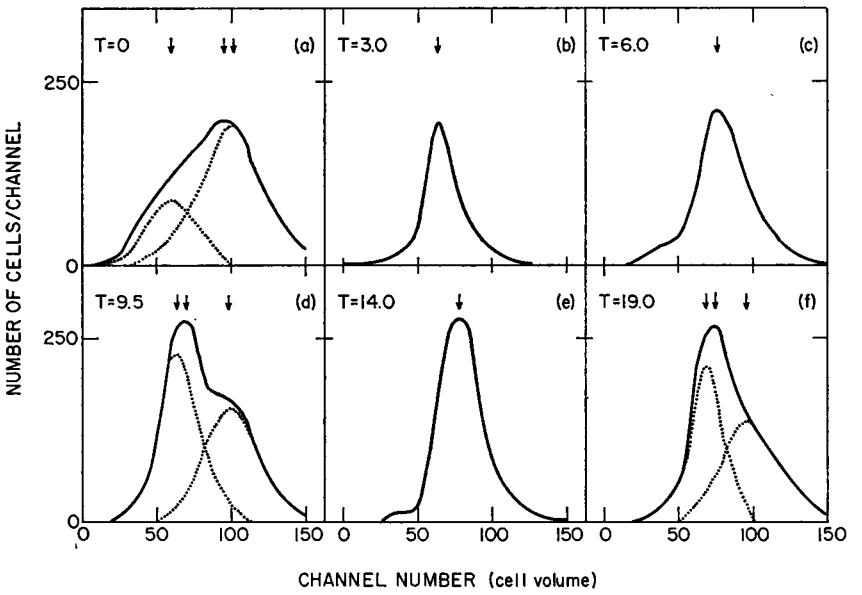


FIGURE 3 Typical volume distributions for synchronous Chinese hamster cells V79-S171; six distributions cover two cycles of division ($T_o$ = 9.5 hr). Experimental values of the peak volume of the distribution are denoted by arrows; dotted curves are separation of distribution into parent and daughter cell subgroups at times when whole population is dividing. Channel numbers correspond linearly to single cell volume.

these distributions and the peak volume was considered to be the better parameter since it is less sensitive to noise and shape factors.

The resolution of the volume measurement performed by this electronic technique was about 1% (±1 channel number). In terms of absolute units, a measurement of an average cell of volume 710 $\mu^3$ had an uncertainty of ±25 $\mu^3$, including the uncertainty in calibration. A method of increasing the precision would be, in theory, to spread the volume distribution over more channels of the analyzer, narrowing the width of the window on any one channel. This procedure also serves in our apparatus to introduce more statistical noise in the number of counts in any one channel for the number of cells available (about 5000 cells/spectrum) and there is no net gain in accuracy. In fact 200 channels was found to be an optimal spread of the volume distribution for this population of cell sizes. The electronic sizing technique was limited to a certain resolution, probably about 1%, by the nature of the conversion of cell sizes to current pulses inside the Coulter aperture. Any attempt at achieving greater precision in the analyzer would have been fictitious since the conversion of cell volumes to current pulses was limited to about 1% in precision. The system would not respond in a reproducible way to cell size fluctuations less than this amount.

A feature of this method of volume spectroscopy is the necessity of obtaining cells in suspension in the rounded up form. In these experiments cells were treated with trypsin in order to detach the cells from the surface of the dish. Thus, although the condition of the cells at assay is constant this condition may not be the same as undisturbed cells growing on the dish. In this respect cells growing in monolayer are somewhat less suitable for such experiments than suspension cultures if cells in the latter can be kept single.

## RESULTS: COMPARISON OF EXPERIMENT AND THEORY

The results of an experiment performed throughout two generation cycles of the V79-S171 subline are shown in Fig. 4, and for one generation cycle of the V79-325 line in Fig. 5. These figures show the development of the peak volume as a function of position in the cell cycle. Points in Fig. 4 where three separate modal volumes occur at the same time were obtained by separating the volume spectra into its parent and daughter cell components as discussed earlier. The volume scale is shown in units of $V/V_o$, where $V_o$ is the mean size of daughter cells. The conversion of the volume scale from channel number to $V/V_o$ was performed by requiring the experimental data to be symmetric about 1.47 $V_o$ ($(1.50 + 1.44)V_o/2$) in the first cycle. The experimental curves can then be extrapolated to 1.0 $V_o$ and 2.0 $V_o$ at the beginning and end of the cell cycle. Several additional experiments were performed with each cell line with results very similar to those shown in Figs. 4 and 5. In both cell lines the mode of increase of cell volume with time is relatively simple

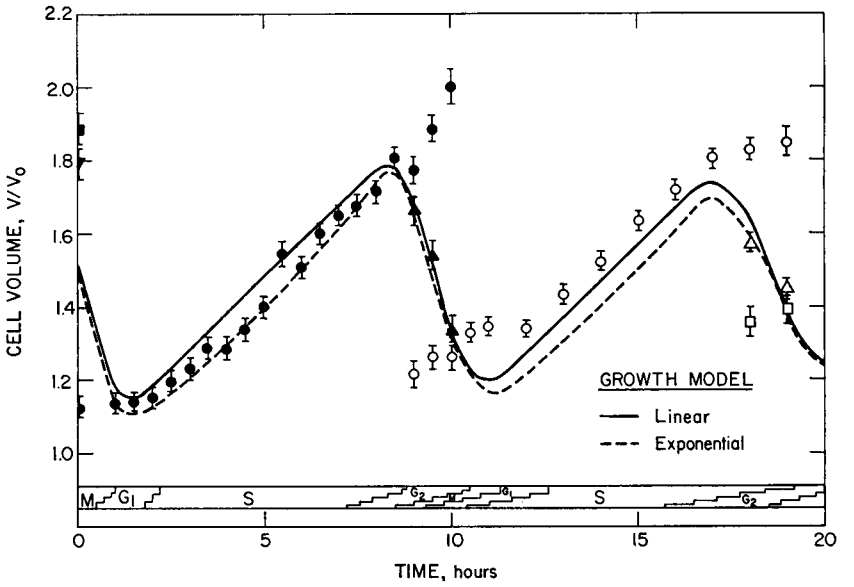


FIGURE 4 Cell volume (peaks) vs. time for synchronous cultures of Chinese hamster cells V79-S171. The smooth curves are theoretical computations for linear and exponential growth models, equations 5 and 6, choosing, as indicated by the experimental data, an average generation time $T_o$ = 9.5 hr and $CV$ = 9.5% on the first cycle, and $T_o$ = 9.0 hr (due to a shortening of $G_1$) in the second cycle. Approximate subdivisions of the cell cycle as defined by DNA synthesis are shown above the time axis. ▼, starting population, mean size; ■, starting population, mean size of undivided cells; ●, mean size of cells during first cycle after synchronization; ○, mean size of cells during second cycle after synchronization; ▲, mean size of both divided and undivided cells during division at the end of the first full cycle after synchronization; □, mean size of cells during the third cycle after synchronization; and △, mean size of divided and undivided cells at the end of the second full cycle after synchronization.

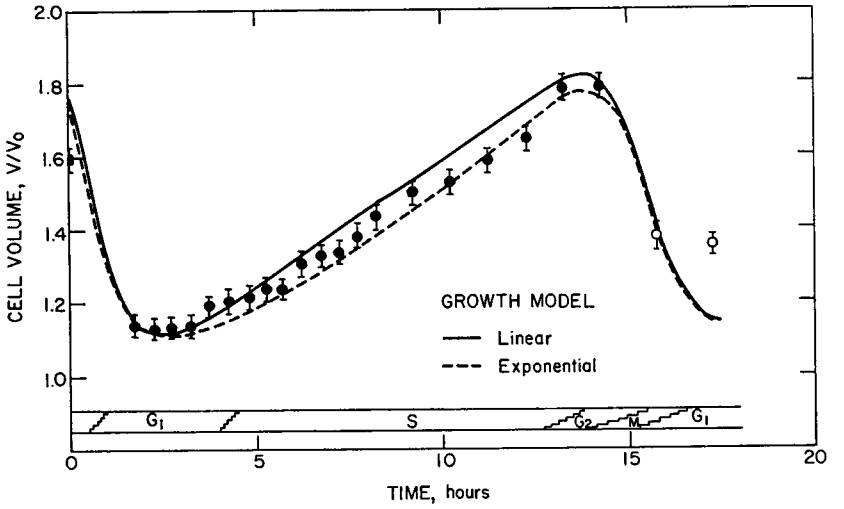


FIGURE 5 Cell volume (peaks) vs. time for synchronous cultures of Chinese hamster cells, V79-325. The smooth curves are based on theoretical calculations for linear and exponential growth, equations 5 and 6, choosing, as indicated by the experimental data, an average generation time $T_o$ = 15.3 hours and $CV$ = 6.5%. Approximate subdivisions of the cell cycle as defined by DNA synthesis are shown above the time axis.

and no obvious discontinuous changes in the rate of volume increase were observed. However, the degree to which this statement is true depends upon the form of the growth law ultimately established. Evidently for linear growth $\frac{d\bar{V}}{dt}$ is constant whereas for exponential growth, $\frac{d\bar{V}}{dt}$ is proportional to $\bar{V}$. Thus, considering the $G_1$-S transition in Fig. 5, the data could be simulated by two different linear rates of growth, one before the transition and one after, differing by as much as 70%. However, if an exponential law is assumed, only a much smaller change (of the order of 20%) can be tolerated by the data. In both cases the limited length of $G_1$ in this cell line and the effects of "rounding" due to late dividing cells, influences this estimate. Within these limitations, changes in the pattern of macromolecular synthesis as cells progress from $G_1$ to S, or S to $G_2$ did not appear to cause changes in rate of cell volume increase. Such changes (had they occurred) were expected to be observed more easily in the V79-325 subline than in V79-S171 because of the longer $G_1$ period in the former.

A Chi-Squared test of fit was performed on the data to determine the degree of correlation with either the linear or exponential models. In the first cycle of Fig. 4, although both linear and exponential hypotheses are accepted with a high degree of confidence, exponential growth is slightly favored. The second generation cycle of Fig. 4 appears to have a linear region. The early part of the cycle shows very clearly the rounding of the growth curve that occurs due to the spread of genera-

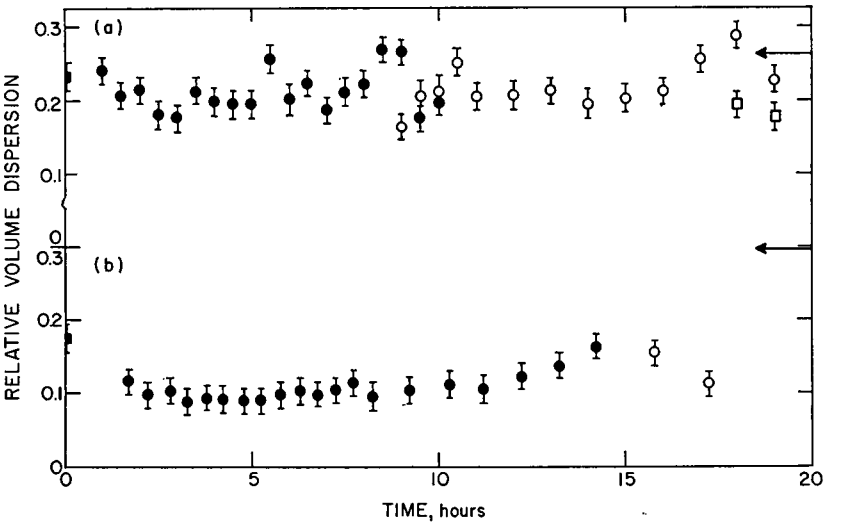


FIGURE 6 The relative volume dispersion (measured by the relative halfwidth of the distribution at halfheight) vs. time for synchronous cultures of Chinese hamster cells; (*a*) V79-S171 and (*b*) V79-325. The symbols for the experimental points change after each cell division. Arrows at upper right indicate values for the asynchronous volume distribution widths of these cell lines. Note: better synchrony is seen in (*b*).

tion times. The Chi-Squared test however accepts both linear and exponential hypotheses. The growth curve of the longer generation time cell line, V79-325, in Fig. 5 appears visually to match exponential growth. With the stated accuracy of $\pm 1$ channel number in peak volume resolution, the test of fit still accepts both hypotheses with greater than 95% certainty. Only when the degree of accuracy is fictitiously increased to $\pm 0.2$ channel does the Chi-Squared criterion allow for the linear hypothesis to be rejected and exponential volume increase to be accepted, both with greater than 95% confidence. This is taken to favor the exponential hypothesis in this cell line but not to prove it.

In the above comparison between theory and experiment it has been assumed that the population of synchronous cells starts with initial volume $V_o$. If mitotic cells divide over a period of time initially this will have the effect of an initial lag in the volume vs. time curve. Such a lag would further reduce the opportunity to distinguish between different modes of growth in the subsequent portion of the cycle. There may be evidence of such an initial lag in the experimental data of Fig. 4 but it is less apparent in the data of Fig. 5.

Fig. 6 shows a plot of the relative volume dispersion (relative distribution halfwidth at halfheight $= \frac{\Delta V}{V}\Big)$ as a function of cell cycle for the experiments shown in Figs. 4 and 5. The parameter $\frac{\Delta V}{V}$ (which is about 0.83 of the coefficient of variation)

remains nearly constant throughout the cycle. Anderson et al. (15) have pointed out that this is the expected behavior of a population undergoing exponential volume increase. A variety of other growth laws also lead to dispersionless growth, but this does not include a linear growth law. However, the experimental error associated with a determination of distribution width is greater than the 1–2% accuracy achieved in determining the peak volume of the distribution.

## DISCUSSION AND CONCLUSIONS

In the two sublines of Chinese hamster lung cells tested, no relationship was detected between volume increase and changes in patterns of macromolecular synthesis within the limits of accuracy attained. In addition to the discontinuities with regard to DNA synthesis occurring at the beginning and the end of the S period there is also the fact that in these cells RNA and protein synthesis appear to reach a peak about the middle to latter part of the S period (8) and decline thereafter. [This is based on the incorporation into synchronous cells of labeled uridine and labeled leucine respectively, but pool sizes were not specifically measured.] For this reason it would be of interest to undertake a volume increase vs. time experiment in synchronous cells of different lines such as HeLa cells or L cells known to have even longer $G_1$ periods than V79-325 and with different patterns of RNA and protein synthesis (7, 11, 26, 27).

In both sublines of Chinese hamster cells the distinction between linear and exponential modes of volume increase is not definite. However the statistical tests applied appear to favor exponential rather than linear growth in the case of the long $G_1$ cell line V79-325. In addition exponential growth is consistent with the lack of volume dispersion observed in both cell lines, whereas, linear growth is not (15). Further distinctions between modes of growth would appear to depend more upon the availability of appropriate experimental material rather than improvements in the accuracy with which volume distributions can be determined by electronic apparatus.

The mathematical models discussed here indicate that the existence of a distribution of generation times in the population is responsible for the "rounding" of the curve of cell volume vs. time whatever the mode of growth. This fact, together with uncertainties in the assumptions, for example the exact form of the distribution of generation times in the population, and the experimental difficulty of starting with a population of cells all of exactly the same size, means that distinctions between different modes of growth by the direct observation of average volume with time as Koch (13) and Anderson et al. (15) have pointed out, may be expected to be very difficult. However it is clear the direction experiments designed to measure average volume as a function of time must take in order to yield optimum experimental data. For example, in the case of mammalian cells the experimental conditions would be greatly improved if the initial distribution of synchronized cells were only

a fraction (e.g. the largest or the smallest group) of the initial population distribution used here. Selection of appropriate groups of cells may be feasible by electronic methods (28) or by other physical techniques, such as "STAFLO" sedimentation (29).

It should be noted that the method of synchrony employed should not perturb the normal mode of growth of the cell population. In the case of the mitotic selection synchrony used here there is reasonable assurance that this is the case (e.g. 30). In some other methods, biochemical inhibition of DNA synthesis for example, this may not be so (e.g. 31) and indeed, since cells continue to grow during the inhibitory period, normal growth is not to be expected immediately thereafter.

In other systems however, two further choices are possible, first, a longer period of relative growth could be observed in some system in which the material not only doubles but quadruples before fission (e.g. *Chlorella*). Second, the effect of a distribution of generation times could be reduced or eliminated in situations where synchrony is controlled for long periods, either naturally (e.g. sea urchin eggs) or by temperature control (e.g. Tetrahymena [32]).

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