CELL GROWTH AND DIVISION

IV. Determination of Volume Growth Rate and Division Probability

E. C. ANDERSON, G. I. BELL, D. F. PETERSEN, *and* R. A. TOBEY

From the Biomedical Research Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544

ABSTRACT Volume growth rate and division probability functions for mammalian cells have been determined as functions of cell volume with good reproducibility and statistical precision using Coulter volume spectrometry and the equations of the Bell model. Results are compared with independent measurements on synchronous cultures. The slow rate of volume dispersion requires that the growth rate $F(\tau, V)$ be closely proportional to volume for cells of a given age. However, when $F(\tau, V)$ is averaged over the age distribution of a population in balanced exponential growth to give the growth rate function $f(V)$, the latter may rise more steeply than V.

INTRODUCTION

In previous papers (Bell and Anderson, 1967; Anderson and Petersen, 1967; Bell, 1968) we have developed and made preliminary application of a generalized model for cell growth and division which relates the differential age-volume distribution spectrum $N(t, \tau, V)$ (the number of cells in a population which at time t have age τ and volume V) to the volume growth rate and the probabilities of division and death. The latter were assumed to be unspecified, time-invariant functions of age and volume: $F(\tau, V)$, $P(\tau, V)$, and $D(\tau, V)$, respectively. The model rests on the assumption that age and volume are adequate and useful parameters by which to characterize the individual cells of a population. The special case of balanced, exponential growth was considered (Bell and Anderson, 1967), and attempts were made to deduce possible growth $f(V)$ and division $p(V)$ functions from the time-invariant volume distribution spectrum $n(V)$ of such a culture. A rigorous solution was not possible when this spectrum was the only experimentally measured function. In the present paper we report the direct calculation of $f(V)$ and $p(V)$ based on the additional determination of the volume distribution spectrum $m(V)$ of dividing cells. The principle of the method has been given previously (Collins and Richmond, 1962), but experimental data have been lacking for a direct solution, and the necessity of

using both age and volume as parameters has not been appreciated. All the data given here were obtained from Chinese hamster cells; similar but less extensive results have been found for HeLa cells. Experimentally, a culture is required which is monodisperse in suspension and which has the proper degree of adherence to glass to permit the separation of mitotic cells by gentle shaking. A preliminary report of these results has previously been given (Anderson et al., 1968).

THEORY

The rigorous derivation of the general equations of the Bell model can be found in the first paper of this series (Bell and Anderson, 1967). We will here be concerned primarily with a single-parameter form of the equations applied to cultures in balanced, exponential growth. For such cultures, time dependence is separable and exponential,

$$N(t, \tau, V) = e^{\alpha t} N(\tau, V), \tag{1}$$

and the experimental volume distribution spectra are measured as summed over the prevailing age distribution:

$$n(V) = \int_0^\infty N(\tau, V)\, d\tau. \tag{2}$$

The corresponding single-parameter forms of the volume growth rate and division probability functions are weighted averages over the age distribution,

$$f(V) = \int_0^\infty F(\tau, V)N(\tau, V)\, d\tau/n(V) \tag{3}$$

$$p(V) = \int_0^\infty P(\tau, V)N(\tau, V)\, d\tau/n(V). \tag{4}$$

When n, f, and p are studied as functions of volume only, some simplifications of measurement and analysis result. These are useful functions for characterizing populations in exponential growth, but we will show that proper understanding of the dynamics of the population requires the use of the corresponding two-parameter functions.

Assuming that cells divide exactly in half[1] (a very good assumption, as will be shown below), a simple conservation equation can be written for the rate of change with time of the number of cells of volume V. (This equation has been derived previously by Collins and Richmond [1962], by Harvey et al. [1967] also by Bell and Anderson [1967].)

[1] Equation 5 can also be written in a somewhat more complex form without this assumption (see Harvey et al., 1967). Since the asymmetry of cell division appears small compared with experimental errors, we prefer to use the simpler form.

$$\alpha n(V) = -\frac{d}{dV}[f(V)n(V)] - p(V)n(V) + 4p(2V)n(2V). \quad (5)$$

Here α is the exponential (number) rate constant, $f(V)$ is the time rate of change of cell volume, and $p(V)$ is the probability of cell division per unit time (both averaged over age as indicated in equations 3 and 4). The product $f(V)n(V)$ is the number of cells growing past volume V per unit time, and hence the derivative of this quantity is the net change per unit time in the number of cells per unit volume at V. This term is negative since, if the product fn increases with volume, there will be a net loss of cells at V as a result of volume growth. The second term on the right is the loss of cells at V due to division, and the last term is the gain of cells by birth from dividing cells of volume $2V$. (The coefficient is the product of a factor of 2 for the number of daughters per division and another factor of 2 because $d(2V) = 2\,dV$.) A term could have been included to allow for losses of cells from the population ("death"), but data discussed below indicate this term to be negligible.

Solution of this equation is not possible if only $n(V)$ is known since there are two other unknown functions. However, the division function $p(V)$ is related to the spectrum of dividing cells and, as noted by Collins and Richmond (1962), knowing the latter would permit solution for $f(V)$. Thus, the number of cells $m(V)$ which divide per unit time at volume V is just the probability of division $p(V)$ times the number of cells $n(V)$ which have that volume:

$$m(V) = p(V)n(V). \quad (6)$$

In the absence of experimental data, Collins and Richmond (1962) and Harvey et al. (1967) were forced to assume a probable form for $m(V)$. However, in the case of some mammalian cells, a direct measurement of $m(V)$ is possible since one can separate mitotic from interphase cells. Knowing both $n(V)$ and $m(V)$, one can calculate $p(V)$ from equation 6 and can integrate equation 5 between 0 and V to give:

$$f(V)n(V) - f(0)n(0) = -\int_0^V m(V)\,dV + 2\int_0^V m(2V)\,d(2V) - \alpha\int_0^V n(V)\,dV. \quad (7)$$

The term $f(0)n(0)$ is negligible since both $f(V)$ and $n(V)$ tend to zero with decreasing V. If the unit of time is the e-folding time of cell number (i.e. the mean life T_e of a cell) then $\alpha = 1$. Letting $M(V)$ and $N(V)$ represent the integral spectra (i.e. the number of cells having volumes less than V) equation 7 can be written:

$$f(V) = [-M(V) + 2M(2V) - N(V)]/n(V). \quad (8)$$

The entire right side of the equation is experimentally accessible and one can, therefore, calculate the value of $f(V)$ for every value of V.

There is one normalization problem in the computation: that of adjusting the relative amplitudes of the spectra. This is accomplished by assuming that $f(V)n(V) \rightarrow 0$ as $V \rightarrow \infty$, in which case equation 8 gives

$$N(V) = 2M(2V) - M(V) = M(V) \tag{9}$$

for very large V where $M(2V)$ and $M(V)$ approach the same limit. Both $n(V)$ and $m(V)$ fall very rapidly at large V (about as V^{-6}) and are negligible for volumes greater than 3 or 4 times the modal volume of $n(V)$. There is no experimental problem, therefore, in estimating the limiting values of $M(V)$ and $N(V)$. Physically, equation 9 requires that the total number of cells present be equal to the number of cells dividing per generation.

It is worth noting that equation 8 provides a very powerful method of evaluating the growth rate function, since $f(V)$ is calculated from integrals of the primary experimental data. Thus, one obtains considerable numerical leverage compared with the calculation of this function by differentiating a volume vs. time curve measured for individual cells or for synchronized cultures. The differential volume spectra $n(V)$ and $m(V)$ can be directly measured with high precision and resolution using a Coulter volume sensor and a multichannel pulse-height analyzer.

EXPERIMENTAL METHODS

The cells used in these experiments were local subclones of Chinese hamster (CHO) cells (Tjio and Puck, 1958) grown as previously described (Tobey et al., 1966). Mitotic cells were separated from monolayer by the mechanical selection principle of Terasima and Tolmach (1963) using our modification (Tobey et al., 1967; Petersen et al., 1968) of the technique of Robbins and Marcus (1964). Tightly synchronized cultures were prepared by placing the selected mitotic cells in suspension in spinner flasks (Petersen et al., 1968). Differential cell volume spectra were determined by the Coulter principle according to the detailed procedure previously described (Anderson and Petersen, 1967). Using a 100-channel pulse-height analyzer, approximately 10^5 cells were measured for each spectrum, giving an average amplitude of about 5000 cells in the modal channel corresponding to a statistical coefficient of variation of 1.4%. Channel width was in the range of 3–5% of the modal volume. It is believed that there was no instrumental distortion of the spectra (Harvey and Marr, 1966; Anderson and Petersen, 1967); but, since no other method provides comparable precision and resolution, direct independent proof is not possible. Alternatively, one can test the results indirectly by comparing the growth rates and division probabilities deduced from the spectral distributions with those determined more directly, as will be done here. In addition, there are potential biological sources of error, including unseparated pairs of sister cells (Anderson et al., 1967). Monolayer cultures were released from glass by trypsinization at 37°C for 10–20 min.

RESULTS

Applicability of the Equations

Use of equations 6–8 for the calculation of division and growth functions is justified only if the experimental cultures fulfill the conditions required by equation 5. These

conditions are for the exponential culture:

1. The entire population is growing at a constant exponential rate.
2. Loss of cells is negligible.
3. For the dividing cells, the selected mitotic subpopulation provides an unbiased measure of $m(V)$.

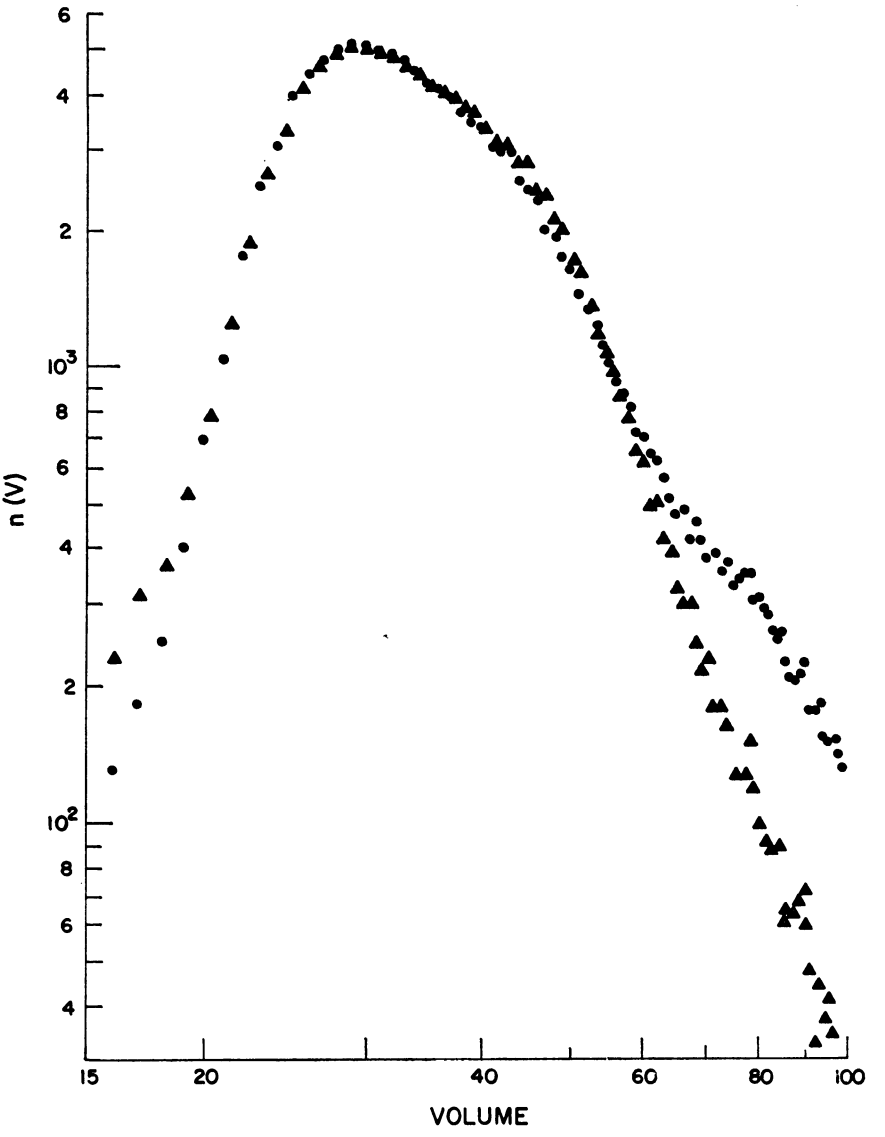

FIGURE 1 Volume spectra of exponentially-growing suspension (●) and monolayer (▲) cultures of CHO cells. (The volume scale is the channel number of the multichannel pulse-height analyzer.)

Point 1 implies that a time-invariant steady state exists in which all intensive properties of the population are constant and all extensive properties increase exponentially with the same rate constant. In particular, the age distribution is time-invariant, evidence of which is the constancy of the rate of logarithmic number increase of the entire population, $d \log n/dt$. Also, the shape of the volume spectrum is time-invariant (i.e., for all V, $d \log n(V)/dt$ is the same). Both of these requirements were confirmed experimentally for the populations studied. Further evidence of the stability of the growth pattern is provided by the continued invariance of these criteria as the population was shifted from suspension to monolayer culture and back again. Doubling times remained the same and the volume spectrum was essentially constant under such transfer, as shown in the example in Fig. 1, in which the circles represent the spectrum of the suspension stock culture of CHO cells and the triangles the spectrum one generation after transfer to monolayer. The only difference between the two spectra occurs at large volumes where, above channel 60, the monolayer spectrum continues to decline smoothly while the suspension spectrum shows a pronounced shoulder. The fraction of cells contained in this shoulder is small (a few per cent), and the explanation appears to be that these are pairs of daughter cells whose separation from one another has been delayed. The absence of such doublets in the monolayer culture is probably due to their separation by the trypsinization necessary to remove the cells from monolayer.

That few cells are being lost from the culture (by death, attachment to the walls of the spinner flask, etc.) is shown by the similarity of the number doubling time of the exponential culture (T_A, the apparent generation time) with the time between successive division waves in synchronized culture (T_G). Thus, for three representative experiments, T_A/T_G was 14.0/13.6, 19.0/19.2, and 19.0/18.7 hr.

Turning now to a consideration of the selection of dividing cells, we present evidence that requirement 3 is fulfilled. We have recently published (Petersen et al., 1968) an analysis of the mitotic selection method in terms of the point in the life cycle at which the cells are released from monolayer and the point at which they reattach. It was shown that such parameters as the mitotic fraction and the age width of the separated population change with the time between successive separations as predicted, and that the yield of cells per separation and the increase in this yield with continued sequential processing of a given monolayer are consistent with continued unperturbed entry of cells into mitosis and their quantitative removal. There remains, of course, the possibility—which is difficult to disprove—that some discrimination with respect to volume may exist in the selection process. However, the near identity of the volume spectra obtained by 25 or more successive extractions of the same monolayer (extending in time over one-quarter of a generation) suggests that such discrimination may be small.

The age distribution of a typical mitotic population prepared by the selection method is indicated by the data of Fig. 2. in which the lower curve is a plot of the mitotic fraction as a function of time after the population was separated and

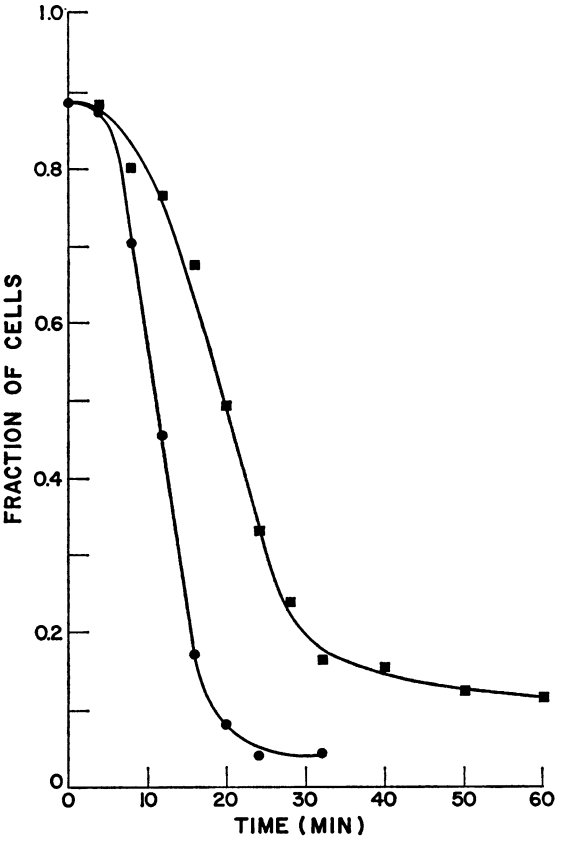

FIGURE 2 Fraction of cells in mitosis (●) and fraction which has not yet separated (■) for a suspension culture as a function of time after synchronization by mitotic selection.

placed in spinner culture. (The upper curve is discussed below.) At the time of separation the mitotic fraction was 0.89 and it remained constant at this level for about 6 min and then dropped precipitously to 0.05 by about 18 min. 84% of the population was, therefore, within an age span of 12 min, or about 1% of a mean time. This span corresponds well with the 10 min time interval between successive separations which could be expected to determine the range in age of the cells selected if the latter are those and only those which grow into the loosely-bound state between separations.

Except as evidence of the quality of the separation process, the age range of the mitotic cell population is not of primary importance in the calculation of $f(V)$ and $p(V)$, provided that it lies close enough to the age of physical separation (see below) and is narrow enough so that no significant volume changes result. The volume change resulting from a given age change, of course, depends on $f(V)$, but an approximate estimate (probably good to a factor of 2 or so for the probable set of growth laws) can be made by assuming volume growth to be exponential, $f(V) = V$.

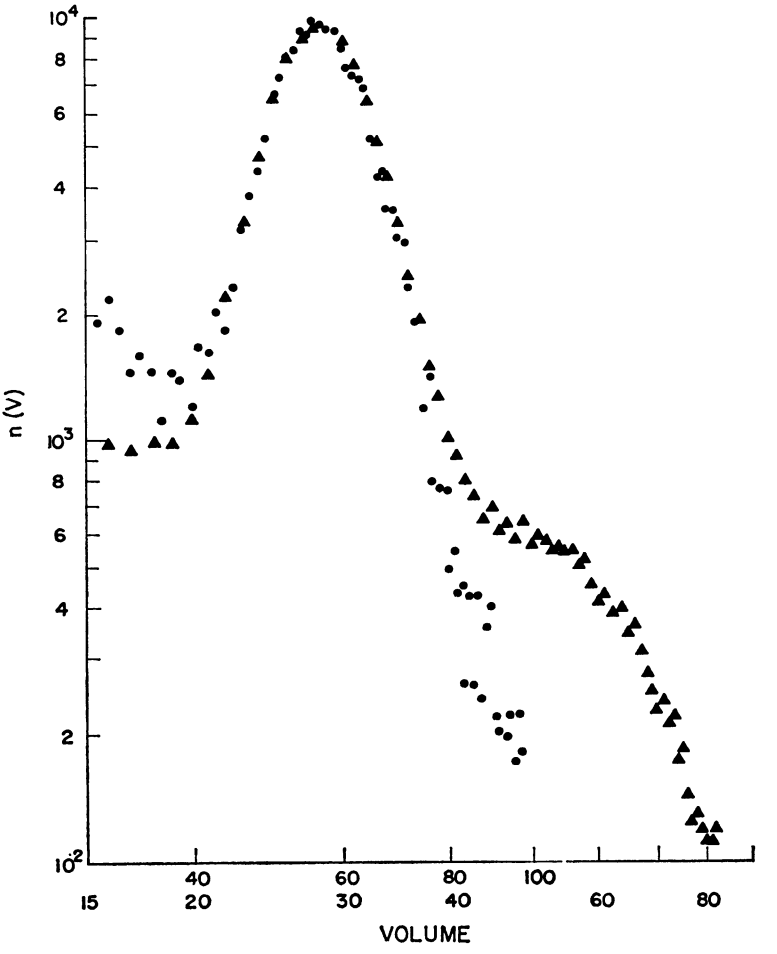

FIGURE 3 Volume spectra of mitotic cells (●, upper volume scale) and of the same population after division (▲, lower volume scale).

In this simple case, the fractional volume change in a given short time is equal to the time interval expressed as a fraction of the mean time, ($d \ln V = 1/T_e\, dt$). The age width of about 1 % of T_e noted above corresponds, therefore, to a volume increment which is small compared with the coefficient of variation of $m(V)$, which is about 18 %.

In addition to the age range of the mitotic population, one must also be concerned with the average age. Thus, the experimental $m(V)$ is the spectrum of the separated mitotic cells, whereas the $m(V)$ required by theory is the spectrum of dividing cells. If there were a significant time delay between these stages and during this time the cells changed in volume, then the calculated values of $f(V)$ and $p(V)$ would be in error. Measurements of separation times show that, for many cultures, the time lag between completion of mitosis and separation of sister cells is short. This is shown

in Fig. 2 in which the lower curve (as discussed above) is the mitotic fraction of the population and the upper curve is the fraction of the population which has not yet separated as deduced from total cell counts.[2] At the midpoint of the declining portions of the curves, the time difference between end of mitosis and cell pair separation is about 10 min.

A more direct and convincing proof of the relevance of the measured $m(V)$, however, is the measurement of the volume spectrum of the same population both during mitosis and again after separation of sister cells has occurred. Such a pair of volume spectra is shown in Fig. 3 in which the logarithm of $m(V)$ is plotted against the logarithm of volume. The circles are the spectrum of the parent cells immediately after separation while the mitotic fraction was 0.95; the triangles are the spectrum of daughter cells when the mitotic fraction had fallen below 0.05. The volume scale for the latter spectrum is twice that of the former, and the nearly exact superposition indicates both that no detectable volume change has occurred and that the cells have divided almost exactly in half. Therefore, either separation follows promptly after mitosis or there is no detectable volume growth during the time delay. In either case, the measured spectrum of mitotic cells cannot be significantly different from the required spectrum of dividing cells and the former is, therefore, appropriate for use in the equations.

The small differences between the two spectra are explicable as due to the presence of a few per cent of nonmitotic cells or of unseparated pairs in the respective cases. Test calculations have shown that the extremities of the spectra have little effect upon the results for $f(V)$ and $p(V)$ in the regions of interest, and the spectra will, therefore, be smoothed by extrapolating a straight line (on a log-log plot) from the linear portions of the measurements. Failure to observe a significant broadening after division indicates that the distribution of relative volumes of sister cells is narrow compared with that of the total mitotic population.

Calculation of Growth and Division Rates

The results of using equation 8 to calculate cell growth rates, $f(V)$, of CHO cells are shown in Fig. 4 which illustrates the degree of reproducibility of the results as well as the type of divergence observed. Fig. 4 *B* displays $f(V)$ as a function of V and, to provide a scale against which to judge the significance of the structure of $f(V)$, Fig. 4 *A* shows a representative total monolayer spectrum, $n(V)$, and the corresponding spectrum of mitotic cells, $m(V)$. Curve 1 in Fig. 4 *B* is derived from the pair of spectra in Fig. 4 *A*. Curve 2 was obtained from the same experiment using the spectrum of the corresponding suspension stock culture instead of that of the monolayer culture. Curves 3 and 4 are from an independent experiment with the same clone, the total spectrum used being that of a monolayer for curve 3 and of the suspension

[2] The unseparated fraction is given by $[N_0(1 + MF_0) - N_t]/N_t$, where N_0 and N_t are the number of cells present at times zero and t, and MF_0 is the mitotic fraction at time zero.

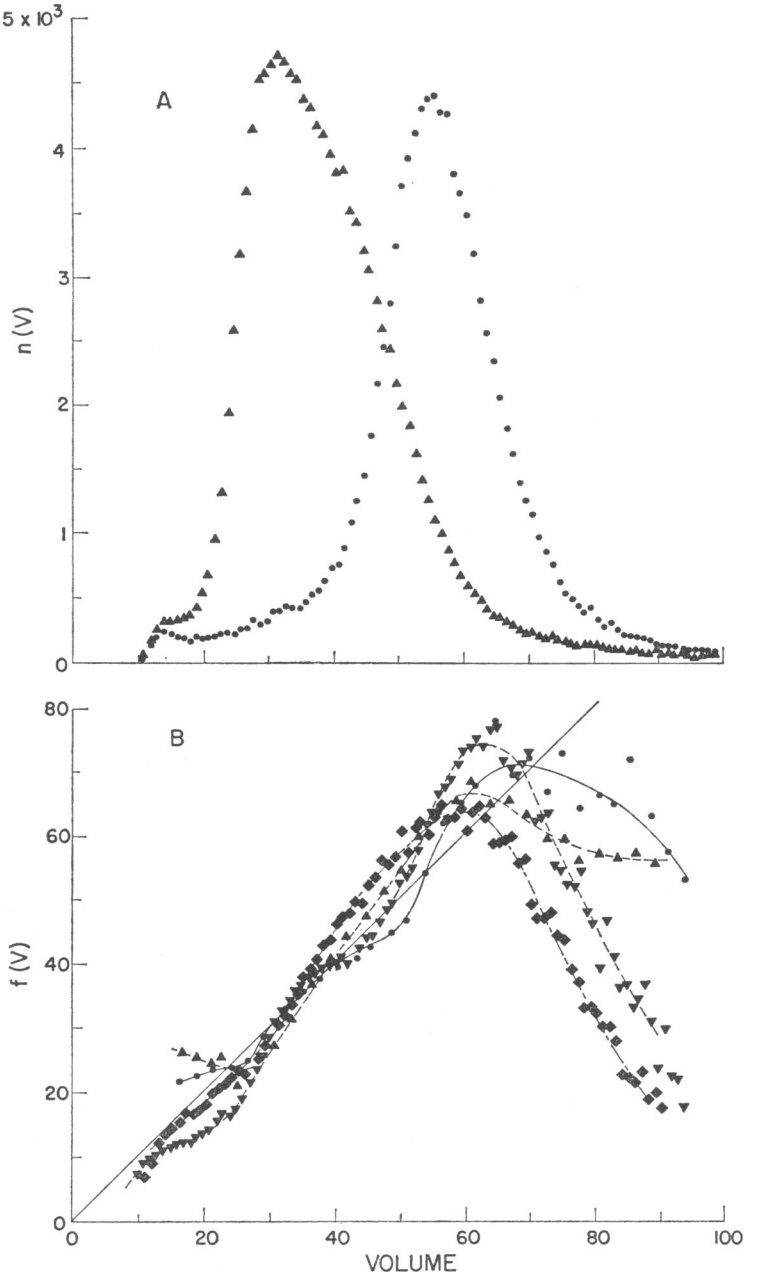

FIGURE 4 (*A*) Volume spectra of exponentially-growing, total monolayer population (▲) and of the corresponding selected mitotic population (●). (*B*) Results of four separate measurements of the rate of volume increase per generation, $f(V)$, as a function of volume: (▼) (curve 1) from monolayer and mitotic populations of Fig. 4 *A*; (◆) (curve 2) from suspension and mitotic populations (same experiment); (●) (curve 3) from monolayer and mitotic populations (second experiment); and (▲) (curve 4) from suspension and mitotic populations (second experiment).

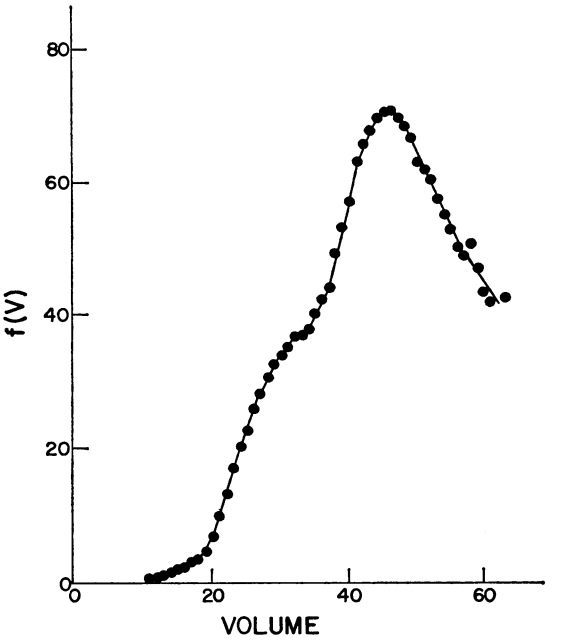

FIGURE 5 Rate of volume increase per mean time (T_e) as a function of volume showing a case of apparently nonexponential growth.

stock for curve 4. Over the volume range of 20–60 all four curves are in good agreement with the line of unit slope, which corresponds to $f(V) = V$, or exponential growth. As can be seen by comparison with Fig. 4 *A*, this range covers the greater portion of the total cell spectrum and over half of the spectrum of dividing cells. Below $V = 20$ the curves of $f(V)$ diverge, but there are few cells in this region and the primary spectra may be contaminated with a small background of irrelevant objects. Above $V = 60$ the curves also diverge, in one experiment showing a drastic decline in $f(V)$ and in the other a leveling off at a more or less constant value. However, the range of exponential volume growth (20–60) brackets the volume span of the average cells (modal birth volume 28, modal division volume 56), and over this range there is good agreement among the experiments. The calculated values of $f(V)$ in this range were little affected by various methods of smoothing, truncating, or extrapolating the primary data at either end of the spectra. Truncation of the spectra at $V = 100$ does indeed force $f(100)$ to zero, but this effect does not extend much below $V = 90$ and is not responsible for the apparent deviation from exponential growth above $V = 60$.

We do not always observe exponential volume growth in CHO cell cultures. Fig. 5 is a plot of $f(V)$ vs. V illustrating a case, observed a number of times, in which a population in stable exponential number growth shows a volume growth pattern which is steeper than exponential. This somewhat unexpected result was confirmed

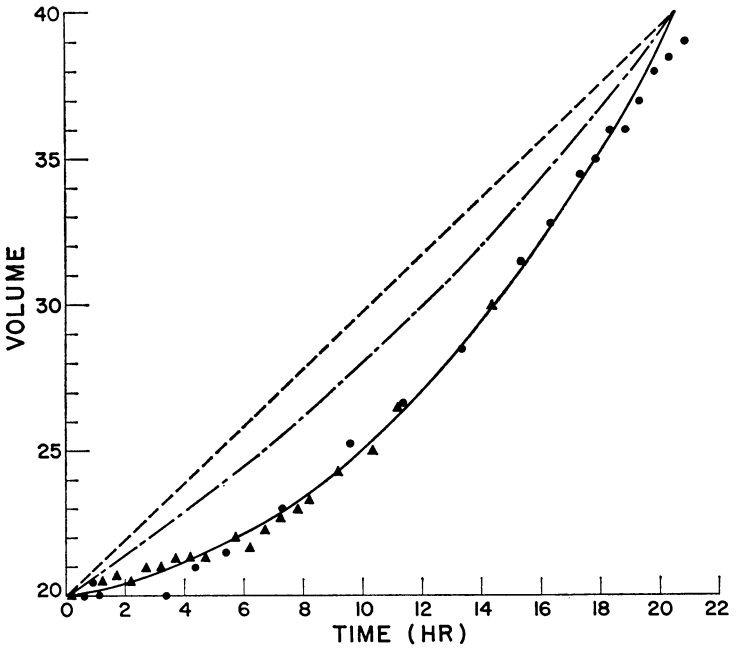

FIGURE 6 Cell volume as a function of time after birth. The solid curve is calculated by integration of $f(V)$ from Fig. 5. The points are experimental results for the modal volume of a synchronous culture: (●) first generation cells, and (▲) second generation cells. The upper and lower broken lines represent linear and exponential growth rates, respectively.

independently by direct observation of the modal volume of a synchronous culture prepared from mechanically-separated mitotic cells. Numerical integration of Fig. 5 gives the volume vs. time curve shown in Fig. 6 as a solid line. (The two broken lines are for linear and exponential volume growth, respectively, for comparison.) The points are experimental results for the modal volume of a synchronous culture over a period of about one and one-half generations. The circles are the data for the first generation cells, and the triangles are for the second generation plotted 17.3 hr earlier than measured, a value taken to be the generation time in the integration of $f(V)$. The synchronous culture data are, roughly speaking, an experimental integration of $F(\tau, V)$ along the trajectory $V_m(t)$, the modal volume of that population, whereas the calculated line is the integral of $f(V)$ for the exponential population. If the spectrum $N(\tau, V)$ of the latter population is rather narrow in age so that most cells of volume V have about the same age, then the difference between $f(V)$ and $F(\tau, V)$ will be small and not apparent in the integrated functions of Fig. 6. We will show later that the differences between $f(V)$ and $F(\tau, V)$ are highly significant with respect to volume dispersion, but in the present context the agreement between the two types of measurements can be taken as a significant confirmation of the results.

As indicated by equation 6, the division probability $p(V)$ is given by the ratio of

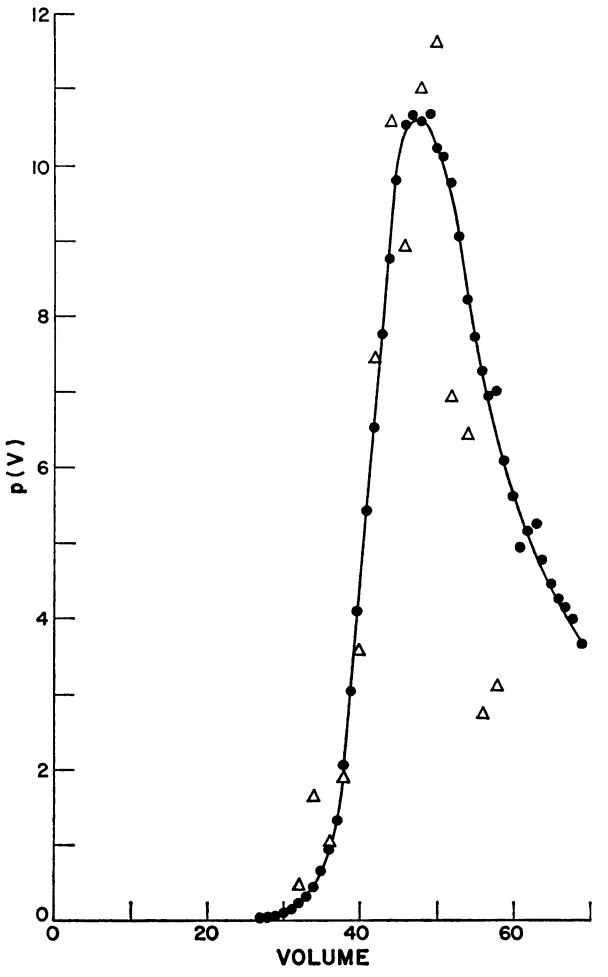

FIGURE 7 Cell division probability per mean time, $p(V) = m(V)/n(V)$, as a function of cell volume: (●) $m(V)$ from separated mitotic population and $n(V)$ from total monolayer population, and (▲) the synchronous suspension culture one generation later [$m(V)$ from the difference between two spectra measured one-half hour apart, and $n(V)$ from the initial spectrum of the pair].

$m(V)/n(V)$. The results of such a calculation are shown in Fig. 7 for the CHO culture having the steep $f(V)$ given in Fig. 5. (Similar results are obtained for cultures having exponential volume growth.) The two determinations of $p(V)$ in Fig. 7 are at least partly independent. Thus, the circles refer to an exponentially-growing culture, while the triangles are for a partially synchronous culture with an average age of one generation time. In the former case, $m(V)$ was obtained by selecting the mitotic subpopulation. In the latter case, the difference between two spectra measured 30 min apart was corrected for volume growth (using $f(V)$ from Fig. 5), and $m(V)$ was taken to be the average between the positive peak (daughter cells) and the negative

peak (parent cells) in the difference spectrum with appropriate volume normalization. The numerical agreement between the two curves is excellent up to a volume of at least 45, and they agree in indicating an apparent decline in $p(V)$ at larger volumes. Here again one must remember that the different age distributions of the two populations complicate the comparison.

Rate of Volume Dispersion

An additional line of evidence which is of great importance in interpretation of the results presented so far is the rate of volume dispersion of the synchronous populations. The very rapid age dispersion of synchronous populations is well known and is nicely epitomized in Powell's words, "Among students of synchrony, the most optimistic draughtsmen know that the condition does not long persist" (Powell, 1964).[3] It may be somewhat surprising, therefore, to observe that volume synchronization persists for a very long time indeed; well separated peaks remain even in the fifth generation. Thus, Fig. 8 is a plot of the coefficient of variation (the fractional standard deviation) of the volume spectra of two synchronous populations (open and solid points, respectively) as a function of time over the period of the first two division waves (i.e., slightly more than the 18 hr generation time of these cultures). The circles (plotted at negative times) refer to the initial mitotic populations as they were separated from monolayer by shaking, the measurements being made while the mitotic fractions still had their maximum initial value and while more than 90% of the cells were in metaphase. The upright triangles refer to the daughter population

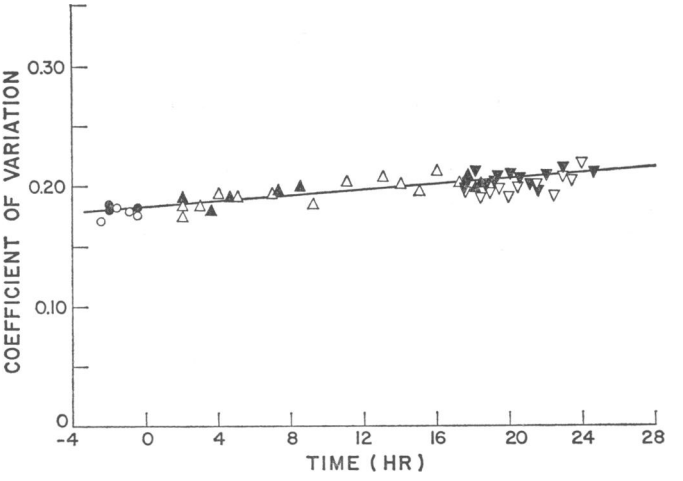

FIGURE 8 Coefficient of variation (relative standard deviation) of the volume spectra of two synchronous cultures (open and closed points) as a function of time after first division: (○ and ●) cells still in initial mitosis; (△ and ▲) first generation daughter cells; and (▽ and ▼) second generation daughter cells.

[3] Quoted with permission from Cambridge University Press.

after the first division wave and the inverted triangles to the population after the second division wave. At times, two peaks are present in the spectra and two generations can be evaluated at the same time. The important feature of the data is the very slow rate of dispersion, the coefficient of variation increasing by only 0.02 (from 0.18 to 0.20) in the first generation. For comparison, the coefficient of variation of generation time for these cells is about 0.13 (Anderson and Petersen, 1964). The observed limited rate of volume dispersion sets severe limits on possible growth laws.

DISCUSSION

Observation of an exponential rate of volume increase is not surprising, since first-order kinetics in which the rate of increase is proportional to the amount present is one of the simplest rate laws. This would be expected if the limiting factor for cellular growth were, for example, the total amount of "machinery" (mitochondria, ribosomes, enzymes, etc.) for processing substrates. A number of investigators have previously reported exponential rates of increase of cell volume and mass for both bacterial and mammalian cells (Collins and Richmond, 1962; Schaechter et al., 1962; Zetterberg and Killander, 1965) but, in addition, other rate laws have been reported (Mitchison, 1961; Harvey et al., 1967; Kubitschek, 1968). In a complicated and sophisticated system such as the cell with many degrees of freedom and many essential subsystems interlocked by feedback regulation, both positive and negative, it would not be surprising if different parameters became rate-limiting under different environmental conditions, in different clones or even in different portions of the life cycle. The loose coupling between cell division and cell volume (Prescott, 1964) and the observation of multiple states of balanced growth (Schaechter et al., 1958) are evidence that the cell is not limited to any one pattern of growth. Thus, our observation of at least two volume growth laws can be taken as a warning against oversimplification. It appears highly significant, however, that in both cases the lack of volume dispersion implies that the volume growth rate is fundamentally proportional to volume. Even in the absence of a complete solution for $F(\tau, V)$, it seems profitable, therefore, to consider in more detail the general implications of dispersionless growth.

A lack of volume dispersion should be expected for any population of cells for which $F(\tau, V) = aV$ (Bell and Anderson, 1967; Bell, 1968). Such a growth law will lead to zero dispersion independent of the division law assuming only that a cell divides into two equal daughters. It is not surprising, therefore, that for populations in which $f(V)$ was nearly proportional to V, as in Fig. 4 *B*, there is little volume dispersion. For such a population, the assumption that $F(\tau, V)$ is very similar to $f(V)$ and proportional to V would lead to zero dispersion. However, for a population with a growth law such as that shown in Fig. 5, the dispersion is also found to be slight. From the results shown in Fig. 6, in which the modal volume (experimental points) is compared with $V(t) = V_0 + \int_0^t f(V)\, dt$, it would have been tempting to

postulate that $F(\tau, V) = f(V)$. This cannot be true, since this growth law would lead to rapid dispersion. For example, if the volume-time curve of Fig. 6 applies to each individual cell of a population in such a way that the cell is born on the curve at the time t_0 corresponding to its birth volume V_0 and reaches a volume $V(t)$ after an elapsed time (age) $\tau = t - t_0$, then one can estimate the rate of volume dispersion of the population from the rate at which a given initial volume range spreads out after being shifted some time t along the curve. Thus, a cell born with a volume 18% above the mean ($V_0 = 23.6$, $t_0 = 8.3$ hr) would have reached at an age of 10 hr ($t = 18.3$ hr) a volume $V(t)$ of 35.4, which is 42% above the population mean volume ($\overline{V} = 25.0$) at 10 hr. This rapid dispersion is in glaring contradiction to the observed coherence of the volume distribution experimentally shown in Fig. 8.

Thus, it is interesting to inquire more generally what sorts of growth laws will give zero volume dispersion in a synchronous population. Any growth law which gives a cell volume,

$$V(\tau) = V_0 g(\tau) \qquad g(0) = 1, \tag{10}$$

where V_0 is the birth volume, will lead to no dispersion of the volume spectrum due to growth; at a time t after the initial division, each cells' volume will simply have increased by the factor $g(t)$. This volume growth corresponds to a growth rate,

$$F(\tau, V) = \frac{dV}{d\tau} = V \frac{g'(\tau)}{g(\tau)}$$

or

$$F(\tau, V) = Vh(\tau), \tag{11}$$

where $h(\tau) = g'(\tau)/g(\tau)$. In these equations, $g(\tau)$ and $h(\tau)$ are somewhat arbitrary functions of τ, subject, however, to the constraint that $g(\tau)$ should have a value near 2 when τ is near the mean generation time so that, on the average, cell volume doubles in a generation.

Inasmuch as growth was observed to lead to very little volume dispersion, we may postulate a dispersionless growth law of the form given by equation 10 or equation 11. Experimental values of $g(\tau)$ may then be found from the experimental values of $V(\tau)/V_0$ as given, for example, for the modal volume in Fig. 6. We have tried fitting the data of Fig. 6 with dispersionless growth laws and a variety of functional forms have been found to be in reasonable agreement with the data, including:

$$h(\tau) = a\tau^{1/2}$$

$$h(\tau) = a\tau \Big/ \left(1 + \frac{a\tau^2}{2}\right)$$

(which gives $F(\tau, V) = aV_0\tau$ with V_0 the birth volume) and

$$\begin{cases} h(\tau) = 0 \text{ for } \tau \leqslant \tau_0 \\ h(\tau) = a \text{ for } \tau > \tau_0, \end{cases}$$

(i.e., a constant time delay τ_0 following birth after which the volume grows exponentially). Once $F(\tau, V)$ has been postulated, such as by one of the above choices for $h(\tau)$ together with equation 11, then the age distribution of the unsynchronous exponential population may be derived using the known $m(V)$ and equation 11 of Bell (1968). The function $N(\tau, V)$ is thereby found (actually only for those values of τ before any division) and hence $f(V)$ may be computed from equation 3. This $f(V)$ may be compared with the experimental results such as those in Fig. 5. We thus find, for example, that a rather good fit to $f(V)$ for small V is obtained using the last of the above growth laws with a time lag τ_0 of 7% of the generation time, or about 1.3 hr for this culture. Such a delay is not inconsistent with the data of Fig. 6. The above procedure will give $f(V)$ from $F(\tau, V)$ only for volumes which are small enough that no appreciable cell division is taking place.

In principle, it is possible to obtain $F(\tau, V)$ directly from measurements of the volume spectrum of a synchronous population as a function of time. From the difference between successive spectra, F is found directly if cells are not dividing. In practice, however, our experimental results to date have not yielded values of $F(\tau, V)$ which are sufficiently precise to be useful in clarifying the extent to which volume growth can be represented by equation 11.

The situation with respect to division probability is similar. We are not yet able to evaluate $P(\tau, V)$ as a function of both parameters, but the agreement between the two determinations of $p(V)$ shown in Fig. 7 (one for an exponential population and the other for a partially synchronous culture with an average age of about one generation time) suggests that the results are correct in their general features. The abrupt rise of $p(V)$ above a threshold volume could result if there were a minimum time required for a newly born cell to complete all preparations for division, or it could result if there were a critical volume requirement. Until $P(\tau, V)$ is known it is not possible to distinguish between these alternatives.

The apparently real decline in $p(V)$ at large volumes is unexpected. It implies that the larger (and, on the average, older) cells are less efficient in their progress toward division, an effect which parallels their reduced rate of volume increase. The decline in $p(V)$ would act as a dispersive force upon the stability of the population in that large cells would be likely to grow still farther away from the average volume before dividing. The decline in $f(V)$ would have the opposite effect. Since $p(V)$ falls relatively more rapidly than $f(V)$, the ratio $p(V)/f(V)$, which is the probability of a cell dividing per unit volume at V, also has a maximum value and declines at larger volumes. The maxima in all three functions occur at about 2.4 times the average birth volume for this culture. The fraction of cells which grow past V without division is given by $\exp - \int_0^V [p(V)/f(V)]\, dV$, and evaluation of this integral shows

that only about 20% of the population passes the maximum.

It is interesting that volume growth rate and division probability both reach their maximum values at about the same volume, since one might have expected that the former would have declined earlier as the cell turned its resources to the process of division. That this does not occur is perhaps further evidence of the degree of independence of these two fundamental processes.

A growth rate function very similar to that given in our Fig. 5 has been reported by Harvey et al. (1967) for bacteria, also on the basis of the Collins and Richmond equation. They did not measure the spectrum of dividing cells but showed that over an important volume range $f(V)$ did not depend strongly on the particular $m(V)$ which they assumed. The agreement with our results for mammalian cells suggests that growth laws of this type may be applicable to a variety of cell types; however, we would question the validity of their calculation of the distribution of generation times since they assume that $f(V)$ is applicable to all cells of volume V regardless of their age. Our measurements on volume dispersion show that this cannot be generally true for mammalian cells at least and, thus, emphasize the importance of the two-parameter formulation of the Bell model and the need for experimental data adequate to permit determination of the functions $N(\tau, V)$, $F(\tau, V)$, and $P(\tau, V)$.

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