CELL GROWTH AND DIVISION

II. Experimental Studies of Cell Volume Distributions in Mammalian Suspension Cultures

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ABSTRACT Experimental proof is given that the volume distribution spectrum of mammalian cells in suspension culture can be determined accurately with a Coulter spectrometer. Stable spectra corresponding to the predictions of a mathematical model are observed under favorable conditions of growth. Cell volume spectrometry appears to be a useful method for diagnosing the state of the culture with respect to past uniformity of growth rate and present population age distribution. In addition, it offers a method for quantitative study of the laws governing cell growth and division.

INTRODUCTION

In the preceding paper (Bell and Anderson, 1967), a mathematical model for growth and division of cells is developed which permits analysis of cell volume distributions in terms of a volume growth function, $f(V)$, and a division probability function, $p(V)$. The applicability of the model to real systems depends as much on the reliability of the experimental data as on the accuracy of the model. The determination of cell volume distributions by Coulter spectrometry is the only method offering sufficient resolution and accuracy to be practical for this purpose, but it is subject to errors from a number of sources, both physical and biological. It is important, therefore, both to establish the conditions under which accurate results are obtained and to provide proof of accuracy for a given application. This has not previously been done for mammalian cells in suspension culture.

In addition to methodology, this paper discusses the stability of the observed volume distributions and their utility as indicators of the state of a cell population. The data are taken from a library of some 1500 spectra acquired over the past 2½ years for six mammalian cell lines.

EXPERIMENTAL PROCEDURES

Volume spectra of suspension cultures are reported in this paper for Chinese hamster (CHO) cells (Tjio and Puck, 1958) grown as previously described (Tobey et al., 1966), and for Fisher's

murine lymphoma L5178-Y grown in Manson's modification of Fisher's medium. Similar spectra were observed for HeLa S3 cells in Eagle's medium, for L-929 murine fibroblasts grown in Eagle's medium plus 5% calf serum and antibiotics, and for cell lines C-13 and P-183 grown in F-10 medium. The latter two lines (kindly provided by Dr. Vittorio Defendi, Wistar Institute, Philadelphia, Pa.) are derived from the BHK21 Syrian hamster cells of Stoker and MacPherson (1964), P-183 being a polyoma transform of C-13.

Electronic Measurements

Spectra of cell volume distributions were measured by using an electronic particle counter of the Coulter type in which passage of an insulating particle (the cell) through a small conducting aperture produces a change in resistance which is related to the volume of the particle (Gregg and Steidley, 1965; Harvey and Marr, 1966). The aperture used in most of these measurements was 90 μ in diameter and 200 μ in length. This length ensures a transit time long enough to permit pulse-height saturation with the flow rates and electronic time constants used (Kubitschek, 1962). Identical spectra were obtained with apertures of this diameter as long as 400 μ and as short as 100 μ. A regulated vacuum of 34 cm Hg below atmospheric pressure was used to pull the cell suspension through the aperture at a flow rate of about 4 ml/min. Aperture currents were generally 50–100 μa, obtained from a regulated high-voltage supply of high internal impedance. The transistorized electronics were designed and built by the electronics group of this laboratory.[1] The preamplifier had a low input impedance (less than 1000 ohms) to make particle pulse-height relatively insensitive to changes in solution resistivity and a transimpedance of 300 kohms. The main amplifier consisted of two conventional feedback stages, each with a maximum gain of 100, with band width-restricting networks between stages. Integrating and differentiating time constants of 5 and 50 μsec, respectively, were selected empirically on the basis of best signal-to-noise ratio. Amplified pulses were analyzed with a RIDL multichannel analyzer modified to accept the comparatively slow pulses. (The input time constants to the lower-level discriminator in the ADC were increased, and the duration of the reset trigger pair was lengthened.) Spectra were analyzed in 100-channel quadrants, which provided adequate dynamic range and resolution for the structure observed. Spectra were recorded by photographing the oscilloscope display of the analyzer and also on punched paper tape. The tapes were later converted to punched cards (10 cards per spectrum) for filing and computer processing. Electronic stability was checked daily with a precision pulse generator, and daily calibrations of the entire system were obtained from ragweed pollen spectra of mean volume 3000 μ^3 (determined optically). The apparatus was located in a temperature controlled room held at 22 $\pm$ 1°C.

Cell suspensions were diluted with isotonic saline immediately before measuring to give final concentrations of the order of 10^4 cells/ml in order to keep coincidence loss in the analyzer below 10% in all cases (maximum counting rate about 40,000 c/min). Spectral shape was time-invariant over periods long compared with total elapsed measurement time after dilution. Total counts of the order of 10^5 were accumulated in each spectrum, the criterion being that the peak amplitude (usually near channel 30) should approach 5000 per channel, thus requiring more total counts in a broad spectrum than in one which peaked sharply. At the modal channel, therefore, the statistical precision of the amplitude determination was about $\pm 2\%$, with an electronic volume resolution of about 3%. The extremes of the spectra are less reliable as a result of poorer counting statistics and of the possible presence of noncellular particulates, fragments, and clumps.

[1] "Interface" electronics to permit the use of a commercial Coulter counter with an existing multichannel analyzer are available from Coulter Electronics, Inc., Hialeah, Fla.

Optical Measurements

An aliquot of the cell suspension was placed in a special Petri dish with an optical coverslip window in the bottom (Unitron Instrument Division, United Scientific Co., Boston, Mass.) and was observed in a Zeiss inverted plankton microscope (Carl Zeiss, Inc., New York). As soon as the cells ceased drifting across the field, i.e. were in contact with the glass, but before significant attachment and distortion of shape had occurred (5–20 min), photomicrographs were taken at either ×730 or ×1160 diameters. Phase optics were used on untreated preparations, and normal optics were used on preparations stained with neutral red in saline at a final concentration of 0.01%.

Mean diameters of individual cell images were determined on an absolute basis by matching the photographic image against a projected circular spot of light of variable size with a Zeiss-Endter TGZ-3 48-channel particle size analyzer (Endter and Gebauer, 1956), calibrated against an optical graticule photographed along with the cell preparations. Cells were assumed to be spherical for calculation of volumes. From 300 to 900 cells were measured for the determination of a spectrum.

PROOF OF ACCURACY

The response of a Coulter counter to particles of varying shapes and volumes has been discussed in detail by Gregg and Steidley (1965). Potential sources of nonproportionality between volume and electronic pulse-height are cell shape, size, and membrane capacitance. Mammalian cells in suspension cultures are usually so nearly spherical in shape that no significant error is expected from this source (except perhaps for the few cells in late telophase). With respect to size, it can be calculated from the theoretical equation of Gregg and Steidley that for cells of the largest size considered here (about 20 μ diameter in a 90 μ aperture) the deviation from strict proportionality would be only 3%. Membrane capacitance effects are expected to be small and were shown by the above authors to be negligible for mouse lymphoblast cells. Additional potential sources of error are mismatch of the amplifier rise time with the transit time of cells through the aperture, nonlinearities in electronics, and excessive aperture currents. Invariance of spectral shape with respect to changes in aperture current, amplifier gain, and particle concentration (below 10^4/ml) provided evidence for lack of significant distortion from these sources. Calibration with a precision pulse generator (with pulse shape matched to that of the cells) proved the inearity of the electronics. However, the best proof of the over-all absolute reliability of the electronic counter for measurement of cell volume would be comparison with an independent method of volume determination. The method chosen for comparison was the optical determination of cell diameters. This method is limited in terms of size of sample which can be measured, and thus its accuracy does not approach that attainable with the Coulter counter. Furthermore, it must be assumed that cell diameters in planes perpendicular to the field of view are the same as those observed. However, the sources of error are quite different in the two methods, and demonstration of agreement between them would greatly increase confidence in both.

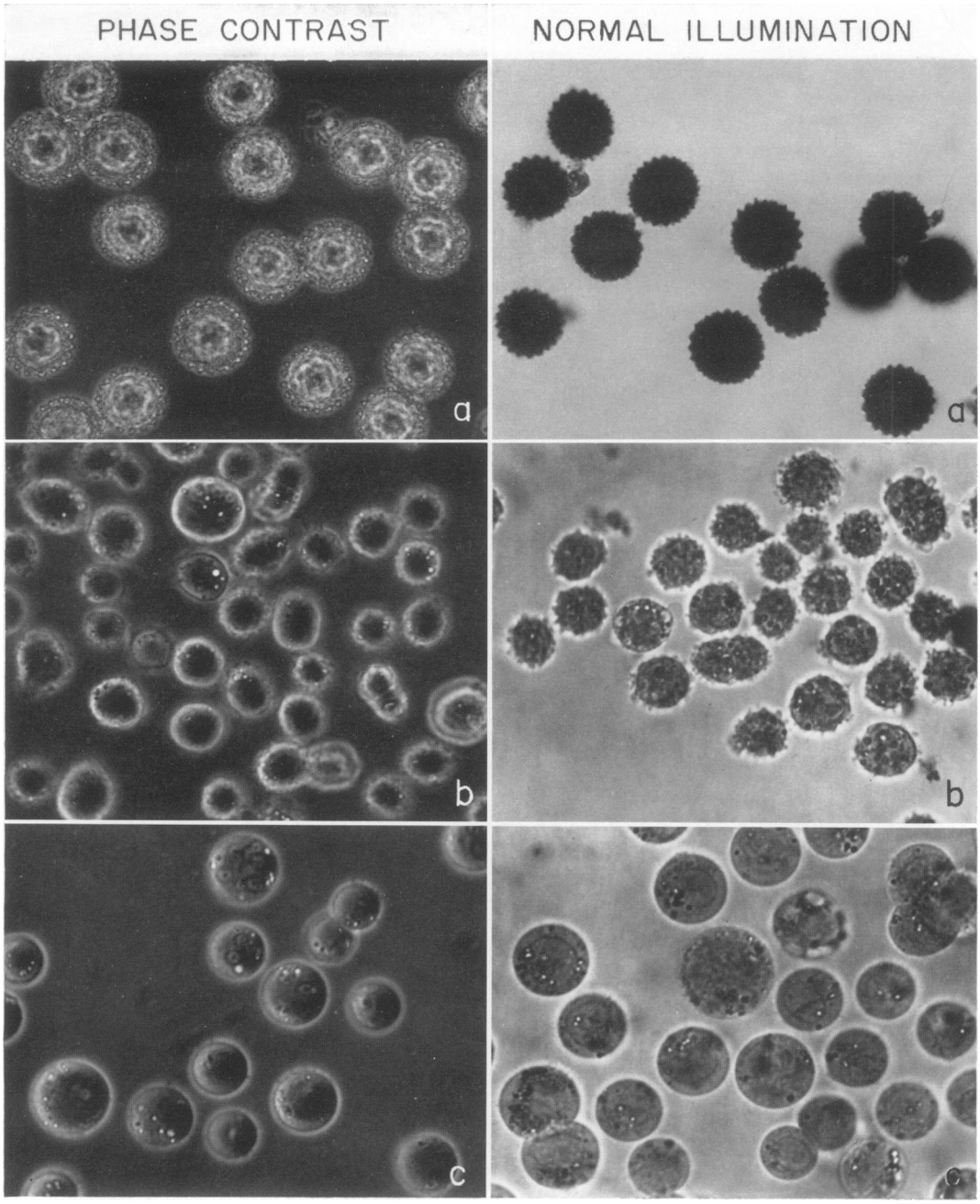

FIGURE 1 Photomicrographs of cells: phase contrast and normal illumination, neutral red stain with (a) ragweed pollen, (b) normal CHO, and (c) CHO swelled in hypotonic saline.

Chinese hamster (CHO) cells were measured as they normally occur in suspension culture and also after swelling in hypotonic medium. The latter treatment served to distend the cells into spherical form and to stretch the cell wall. The former effect made it easier to estimate optically the mean diameter of a given cell, while the latter might enhance any errors in the Coulter method due to cell wall conductivity or capacitance. Both swelled and normal cells were photographed under phase contrast and under normal illumination after staining. Typical photomicrographs are shown in Fig. 1.

Results of the determination of mean volume for several cultures are summarized in Table I. The "optical volumes" were calculated from measured diameter, i.e., the diameter of the spot of light which best matched the cell image. The "electronic

TABLE I

COMPARISON OF MEAN CELL VOLUMES DETERMINED BY OPTICAL AND ELECTRONIC MEASUREMENTS

Condition of cells	Optical method	Optical volume	Electronic volume	Ratio, electronic/optical
		μ^3	μ^3	
Normal	Phase	866	930	1.07
		945	1050	1.11
Swelled	Phase	2130	2420	1.13
		2750	2790	1.02
				Mean 1.08
Normal	Stain	934	850	0.90
		893	790	0.88
Swelled	Stain	2200	1900	0.86
				Mean 0.88

volumes" were calculated from the Coulter spectra by calibration against ragweed pollen using, for the latter, the volume determined optically on stained preparations. The mean pollen volume was 3000 μ^3, corresponding to a diameter of 18 μ. Phase contrast measurements were not used for pollen because of the difficulty of image matching.

The electronic volumes are consistently higher (by an average of 8%) than the optical volumes determined under phase contrast and are consistently lower (by about 12%) than those determined by staining. Note that these volume errors correspond respectively to errors of plus 3 and minus 4% on cell diameter, which is the parameter actually determined by the optical method. Consistent errors of this magnitude in matching the reference light spot to the cell image seem quite possible, since the outline of the cell is neither sharp nor circular. An additional source of systematic error is in the optical determination of the volume of the ragweed pollen

used to calibrate the Coulter system. (This is a very roughsurfaced object, and determining the true effective diameter is somewhat difficult.) We believe, therefore, that this agreement is satisfactory and within the probable errors of the optical method.

Further confidence in the electronic method derives from the agreement on the magnitude of the volume increase in hypotonic medium. In the comparison with phase contrast photography, the average swelling factor (volume increase) was 2.7 by the optical method and 2.6 electronically. In the second comparative experiment, the corresponding factors were 2.4 and 2.3, respectively. Thus, changing cell volume

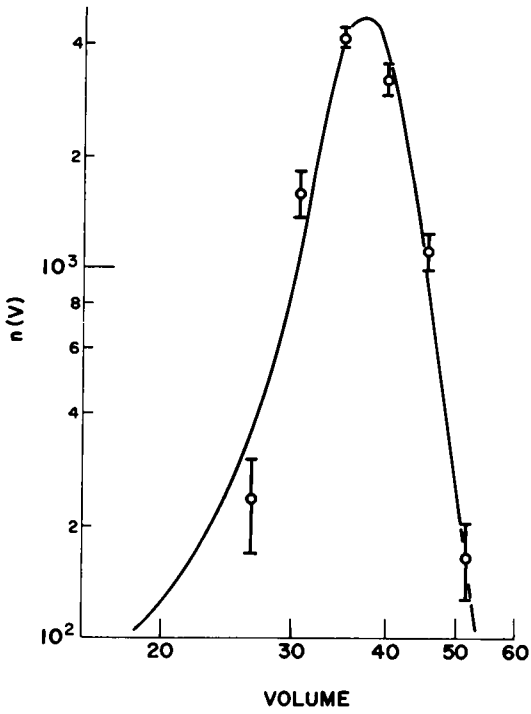

FIGURE 2 Volume spectrum of ragweed pollen determined optically (points) and electronically (curve).

and shape and stretching the cell membrane did not produce a detectable effect upon the relative determinations.

A more sensitive test of reliability for present purposes is the agreement of spectral shapes rather than of mean volumes. This test is hampered by the difficulty of optically measuring a large enough number of cells to define the spectrum with high statistical precision. However, sufficient data were collected to establish agreement on the principal features.

Fig. 2 compares the spectrum (log-log plot) of ragweed pollen determined by the optical method (points, with 1σ error bars) and by the electronic method (drawn as a smooth curve because of the high precision and resolution of the data). The width

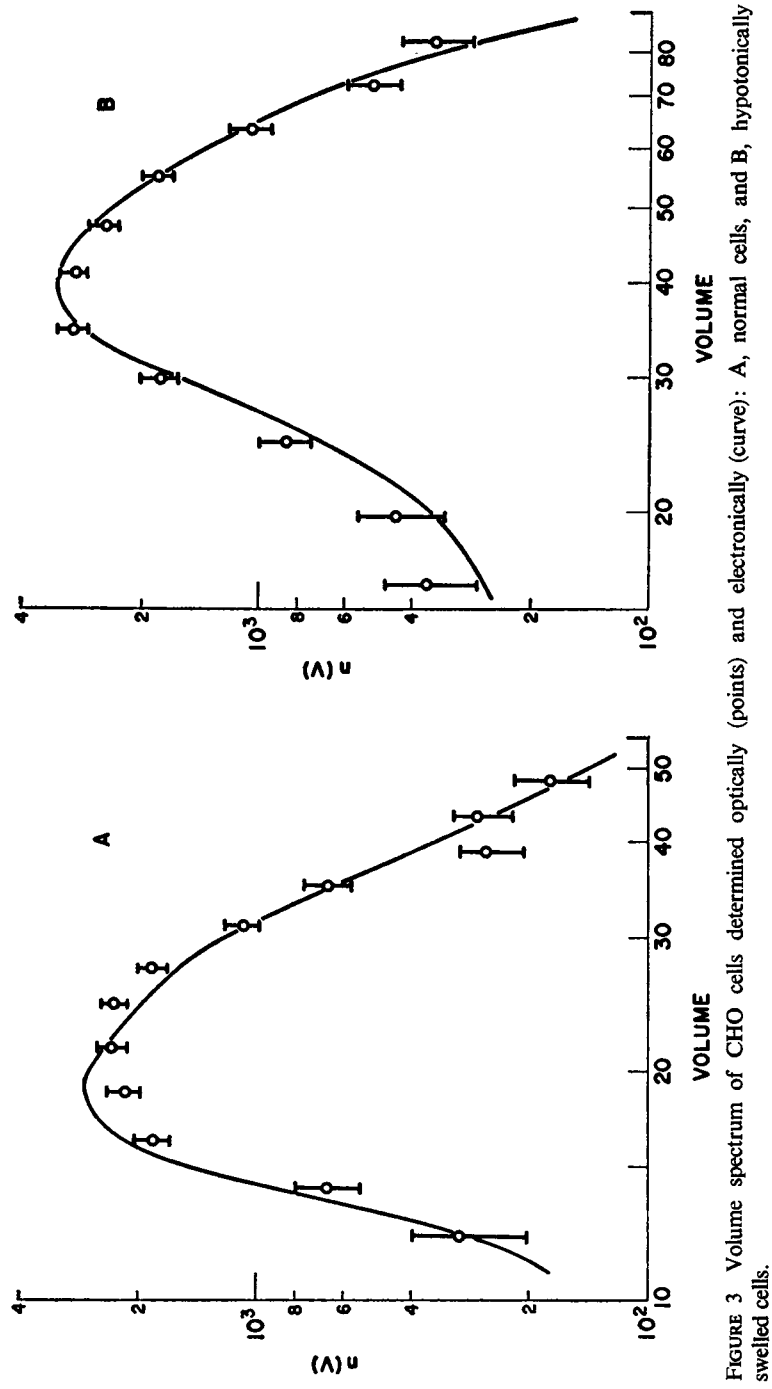

FIGURE 3 Volume spectrum of CHO cells determined optically (points) and electronically (curve): A, normal cells, and B, hypotonically swelled cells.

of the peak is the same, and the optical data suggest the same asymmetry which is present in the more precise Coulter data.

Fig. 3 A compares the spectra of normal (unswelled) CHO cells by the two methods. Again, the peak width and the steepness of the two edges are found to be the same by both methods. The possible disagreement on the details of the peak shape are probably within the experimental error of the optical method (the error bars are $\pm$ 1σ and, therefore, span only the 84% probability range of the optical results).

Fig. 3 B compares the spectra of CHO cells after swelling. The agreement in this case (the easiest for the optical method) is excellent in all respects.

These comparisons indicate that, under the conditions of these experiments, the electronic method senses the true geometrical volumes of CHO cells. The absolute error for mean volumes was probably less than 10%, and no significant evidence was found of distortion of the spectral distribution of volumes.

ABSENCE OF CLUMPING

A significant error could be introduced into both the mean cell volume and the spectral shape if the cells were not monodisperse but were associated in clumps. This situation is not uncommon with certain cell lines and sometimes occurs for undetermined reasons even in normally "well-behaved" cultures. It is usually immediately apparent when clumping is occurring because total cell counts become nonreproducible and show short-term variations outside counting statistics, and the apparent cell multiplication rate (slope of the concentration vs. time curve) becomes variable.

In addition to using these criteria to detect questionable cultures, we have also tested the cultures used in these experiments by two other methods. First, microscopic examination was performed, and counts were taken of the actual fraction of "multiple" cells present. This fraction was only a few per cent or less. This could not affect the shape of the spectrum near the peak but might introduce a significant distortion at the large volume end. Some of the multiples may be legitimate telophase pairs still in the process of cytokinesis, but the upper end of the spectra should be interpreted with caution. The absence of any structure at cell volumes equal to twice the modal value suggests, however, that the distortion, if any, is small.

As a second test for the effect of multiples, cell spectra were measured before and after treatment designed to dissociate multiples, including forcing through small apertures and treatment with pronase, papain, and DNase. Spectral shape was found to be usually invariant under these treatments except, of course, for long incubation with the proteolytic enzymes which resulted in obvious cell degradation.

TYPICAL RESULTS

To a good first approximation, spectral shape for exponentially growing cultures was observed to be characteristic and reproducible over periods of tens of generations in continuous culture, as shown in Fig. 4 for the spectrum of a CHO culture over a period of 230 days. (This is a log-log plot, so that spectral shape is independent of changes in cell number and

mean cell volume. Vertical displacements of the spectra are arbitrary.) All the curves consist of three more or less linear sections: one rising rapidly from small volumes, toward the mode, a second declining slowly from the mode to a shoulder at about 1.6 times the modal volume, and a third steeply falling section at the largest volumes. As shown in the accompanying theoretical paper (Bell and Anderson, 1967), these sections can be identified respectively with cells just being born, with cells growing but not dividing, and with dividing cells. These data

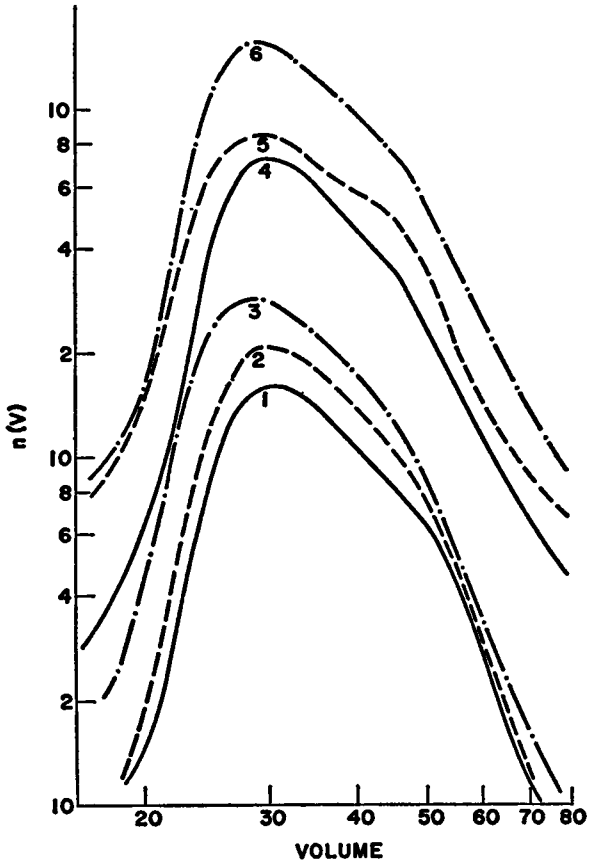

FIGURE 4 Volume distribution spectra of CHO cells in continuous batch-culture showing stable shape: curve 1, May 28; curve 2, May 30; curve 3, June 2; curve 4, January 6; curve 5, January 7; and curve 6, January 10 (arbitrary vertical displacements for clarity).

were taken from stock cultures which were being diluted every 2 days (approximately) so that the cell concentration varied from about 4×10^4 to 5×10^5. Generation times were not constant but ranged from 14 to 30 hr. The changes in modal cell volume are, therefore, not surprising, and changes in the slopes can probably be ascribed to variations in growth conditions inherent in the batch-culture method.

In addition to this fairly steady pattern, which was also observed with HeLa S3 and murine lymphoma cells, several types of instabilities were observed. Among these were more or less abrupt changes from one mean volume to another, as illustrated for another CHO culture in

Fig. 5. The volume increase occurred in the period May 14 to June 2 (unfortunately, no measurements were made during this time). In this case, the three component shape is preserved before and after the shift, although with large variations of slope in the region of dividing cells.

An interesting example of short-term instability is shown in Fig. 6 for an L5178-Y culture. The seven spectra taken at approximately 2-hr intervals show the passage of a "natural" parasynchronous wave due to a nonrandom initial population distribution. The apparent generation times were calculated from the slopes of the growth curve over short periods of

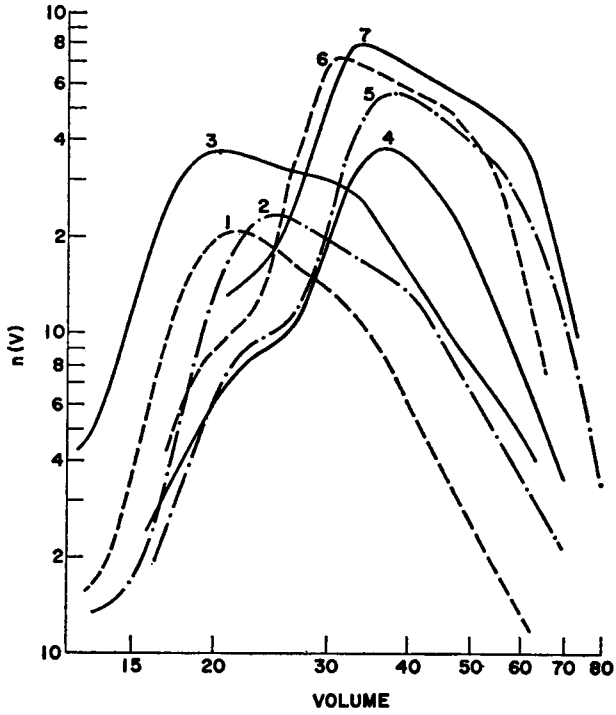

FIGURE 5 Volume distribution spectra of CHO cells in continuous batch-culture showing an abrupt volume increase: curve 1, May 4; curve 2, May 11; curve 3, May 14; curve 4, June 2; curve 5, June 10; curve 6, June 14; and curve 7, June 28 (arbitrary vertical displacements for clarity).

time (the cell concentration was determined to a precision of 0.6% every 10 min) and were found to vary from 20 hr down to 10 hr and returning to 33 hr, as indicated in the legend. As the generation time decreased, the cell population at larger volumes decreased relative to that at the mode. This effect was reversed and the peak became quite flat as the growth rate declined. Over the entire period, the mean cell volume slowly increased.

The spectra of Syrian hamster kidney cultures (C-13 and P-183 lines) showed a similar three component structure, but the width was much greater. These cells clumped badly and a high percentage of multiples was always present, making comparison with theory impossible. Murine fibroblast cells of line L-929 gave excellent spectra, an example of which is shown and discussed in the accompanying paper (Bell and Anderson, 1967).

DISCUSSION

The importance of accurate measurements of cell volume distributions cannot be overemphasized if the results are to be used for quantitative analysis of the dynamic properties of growth and division. Mathematical sophistication of a model is no protection against experimental errors and, indeed, may magnify them and give unmerited weight to the conclusions. Control of the errors to which cell volume spectrometry is subject is possible only when the conditions of measurement are properly defined and maintained. In this paper, we have demonstrated the reliability

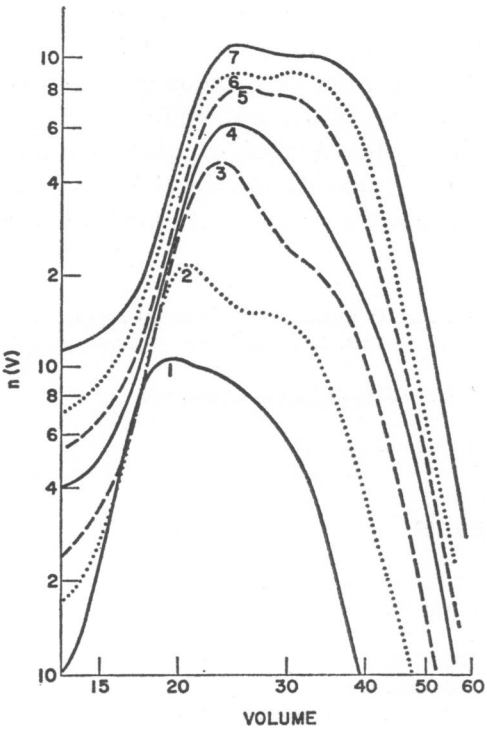

FIGURE 6 Volume spectra of murine lymphoma cells showing short-term instability associated with partial synchrony: curve 1, at 10:29 (time of measurement) with 19.3 hr (apparent generation time); curve 2, at 13:29 with 14.4 hr; curve 3, at 16:26 with 10.0 hr; curve 4, at 17:50 with 10.0 hr; curve 5, at 19:42 with 10.2 hr; curve 6, at 21:21 with 19.0 hr; and curve 7, at 23:14 with 33.0 hr (arbitrary vertical displacements).

of the Coulter spectra as far as possible given the limitations of the optical method. Trust in their accuracy to a greater degree must be based on tests of internal consistency and verification of any conclusions by independent means.

With respect to the spectra themselves, it can be concluded that (*a*) most cultures show a characteristic three component spectral shape which can be stable over many generations and which closely resembles that predicted by the mathematical model; (*b*) perturbations and distortions frequently develop, especially during long-continued culturing; and (*c*) close control of growth conditions is necessary to ensure an accurately time-invariant spectrum. When attained, the stable spectra come closest to the mathematical model.

These conclusions indicate that the spectral distribution of cell volumes is a sensitive indicator of the state of the culture. The observed stable distributions are consistent with the mathematical model developed in the preceding paper, suggesting that spectral shape is determined by growth and division functions and distribution of cell ages around the life cycle. Attainment of a time-invariant volume spectrum of theoretical shape can, therefore, be taken as sensitive evidence that the culture has attained a known age distribution and growth pattern, an essential requirement for biochemical studies of the life cycle. Stabilization of distribution requires several generations of growth under invariant conditions before convergence occurs. Thus, a single spectrum determination may yield as much information as close observation of the gross number growth curve of the culture over a number of generations.

In addition to offering a powerful control parameter for selection of appropriate cultures for life cycle analysis, cell volume spectra offer a method for quantitative study of the laws governing cell growth and division. Analysis of volume spectra in terms of theoretical models should serve to determine the functional form of these laws, the numerical values of the coefficients, and the variations of these parameters with conditions of growth.

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REFERENCES

BELL, G. I., and E. C. ANDERSON. 1967. *Biophys. J.* **7**:329.
ENDTER, F., and H. GEBAUER. 1956. *Optik.* **13**:97.
GREGG, E. C., and K. D. STEIDLEY. 1965. *Biophys. J.* **5**:393.
HARVEY, R. J., and A. G. MARR. 1966. *J. Bacteriol.* **92**:805.
KUBITSCHEK, H. E. 1962. *Rev. Sci. Instr.* **33**:576.
STOKER, M., and I. MACPHERSON. 1964. *Nature.* **203**:1355.
TJIO, J. H., and T. T. PUCK. 1958. *J. Exptl. Med.* **108**:259.
TOBEY, R. A., D. F. PETERSEN, E. C. ANDERSON, and T. T. PUCK, 1966. *Biophys. J.* **6**:567.