DENSITY INVARIANCE OF CULTURED CHINESE HAMSTER CELLS WITH STAGE OF THE MITOTIC CYCLE

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ABSTRACT Isopycnic banding of Chinese hamster line CHO cells in Ficoll gradients shows that a population in balanced, exponential growth is very homogeneous with respect to density, the coefficient of variation of the density distribution spectrum being less than 5% of the mean reduced density (i.e. density minus one). Similar measurements on synchronized cultures indicate that reduced density varies by less than 2% around the life cycle. The mean density of CHO cells in F-10 growth medium is calculated to be 1.051 after correction for osmotic effects of the Ficoll gradient.

INTRODUCTION

Biochemical growth can be equated usefully to the increase in total functional mass of the cell (i.e. dry mass), but the latter parameter has been determined only by use of methods whose tediousness severely limits the number of determinations which can be made. Cell size (e.g. length, diameter, or volume) is easier to measure, and the assumption has often been made that the proportionality between size and mass is sufficiently accurate to permit the use of size as a measure of growth. The possibility that imbibition and excretion of water during some phases of the life cycle cause volume changes independent of dry mass, has been investigated in only a few cases, with conflicting results.

Recent progress in rapid electronic measurement of cell volume distributions using the Coulter principle (1–5) has greatly increased the facility, accuracy, and resolution of cell volume spectrometry. These spectra, when used with the Collins and Richmond equation (6–8), determine the rate of change of cell volume, dV/dt, as a function of volume with a precision and volume resolution each of a few per cent.[1] As a result, more searching questions can now be asked concerning the laws governing the rate of cell volume increase. Conversion of these laws to laws of

[1] Anderson, E. C., and G. I. Bell. Manuscript in preparation.

growth (in the above defined sense) requires a comparably precise knowledge of the relation between mass and volume (i.e. density). Fortunately, a similar advance has been made in the determination of distribution of individual cell densities by the isopycnic banding method (9).

The present paper is a study of the relation between cell volume and density as a function of age for the CHO line of Chinese hamster cells growing in suspension culture. It will be shown that, in contrast with yeast and bacteria, the density of individual CHO cells varies by less than 2% (standard deviation) around the life cycle, and reasons for this difference in density behavior will be proposed. The fundamental conclusion, therefore, is that to this level of accuracy cell volume is equivalent to cell mass in the description of growth of these cells.

HISTORICAL

Prescott's careful measurement (10) of amoebae, using a Cartesian diver technique, indicated a possible small change in density of that organism with age. Our calculations, based on his data, give a maximum change of 0.0013 g cm^{-3} in a density of 1.019, but his own comment is that density does not vary significantly. Sandritter, Schiemer, Kraus, and Dorrien (11) reported large density changes in HeLa cells measured by interference microscopy, but the results are not convincing since the cells grew in a most abnormal manner, swelling to as much as 8 times their birth volume at midinterphase. Schaechter, Williamson, Hood, and Koch (12) noted the constancy of refractive index of bacteria as evidence of constant density but gave no quantitative data. The most detailed data are those of Mitchison (13, 14), whose determinations of volume and dry mass (by interference microscopy) of fission yeast indicated a maximum difference in concentration of dry matter of about 12% (range 33–37 g/100 ml) around the life cycle. He found (15) differences ranging from 10 to 40%, depending on the phase relation of nuclear and cell cycles for *Streptococcus*.

There are no direct data on density of cultured mammalian cells, but a number of studies of volume vs. age are relevant. Thus, Terasima and Tolmach (16) reported that the volumes of synchronized HeLa cells slightly more than doubled between divisions and suggested the possibility of cyclic density changes with a minimum density in the period just before mitosis. A number of other similar measurements, however, have indicated that volume is a smooth, monotonic function of age with no "overshoot" of a doubled birth volume which would indicate water imbibition (17–20). The rate of volume growth, calculated from the Collins and Richmond equation, agrees with directly measured volume vs. time data (19) and shows no indication of volume decrease at any volume. However, this equation gives the time rate of volume change for a given volume averaged over all cells having that volume, regardless of their age. Since the rate of volume change has been shown to depend on age as well as on volume (19), it would be possible for cells to pass through a given volume more than once at different ages and with different growth

rates. A period of volume decrease might be concealed by the process of averaging over age.

The conclusions to be drawn from these data are that yeast and bacteria show significant changes in density with age but that this effect may be smaller or absent in mammalian cells. Leif and Vinograd's (9) elegant demonstration of the variation of human erythrocyte density with age is not relevant in the present context, since the differentiated red cell does not have a simple "closed" life cycle. However, their paper was apparently the first to report the precise determination of cell density distributions by the isopycnic banding method.

THEORY

Isopycnic Banding

Objects which are allowed to settle downwards through a medium of increasing density will reach buoyant equilibrium at the depth at which the medium density is the same as their own. Objects of differing densities, therefore, will be segregated at different depths, a phenomenon known as isopycnic banding. This technique has been used extensively for both preparative and analytical work and has been reviewed at length (21). The time required for cells to reach isopycnic equilibrium in a given centrifugal field can be calculated from Stokes law and the density-viscosity data given by Boone, Harell, and Bond (22). (Pretlow, Boone, Schrager, and Weiss [23] have published tables to facilitate this type of calculation.) CHO cells in Ficoll should reach a position with 0.0006 density unit (1% on reduced density) in less than 1 min of centrifugation at 1000 *g*. This requirement is greatly exceeded in some of the following measurements in order to demonstrate that the cells are not changed by continued exposure to high fields.

Osmotic Effects

The osmotic properties of living cells have been reviewed in some detail e.g. see Dick [24] and Olmsted [25]. In summary, it can be said that the mammalian cell membrane appears to be very rapidly permeable by water, only slowly permeable by electrolytes, and impermeable by high molecular weight solutes. No pressure differences are thought to exist across the membrane, and the mammalian cell is essentially in osmotic and hydrostatic equilibrium at all times. Therefore, the cell responds rapidly (in less than 1 min) and, within limits, reversibly to changes in osmolarity of the surrounding medium by the appropriate gain or loss of water to maintain equal activity for intra- and extracellular water. Measured volume and density, therefore, should depend on osmolarity of the medium in a simple manner.

In order to raise the density of the gradient medium high enough to permit isopycnic banding, large concentrations of solute must be used. To minimize osmotic effects, the solute should have a high molecular weight. We have chosen to use

Ficoll (Pharmacia Fine Chemicals Inc., New Market, N.J.) (26), a synthetic copolymer of epichlorohydrin with sucrose having a molecular weight of 4×10^5. Boone et al. (22) have demonstrated that CHO cells remain viable (as judged by plating efficiency) following suspension in Ficoll solutions of much higher density than that required for isopycnic banding. We will demonstrate below that the volume (and, hence, density) changes which occur during banding are due to reversible osmotic effects and are the same for cells of all volumes (and, hence, ages).

Reduced Density

In the discussion of density data, the concept of "reduced density" will be useful. This is the excess density of the cell above that of water (the latter being taken as unity): $RD = \rho_c - 1$, where ρ_c is the measured density of the intact cell. The significance of this concept is derived as follows. Let m_d and m_w be, respectively, the mass of dry matter and mass of water in a cell of volume V_c. Cell density is then:

$$\rho_c = \frac{m_d + m_w}{V_c}, \tag{3}$$

and reduced density, $\rho_c - 1$, is

$$RD = \frac{m_d + m_w - V_c}{V_c}. \tag{4}$$

The "apparent volume," ϕ_d, of dry matter is usually defined (27):

$$\phi_d = V_c - V_w. \tag{5}$$

We substitute for V_c in the numerator of equation 4 the value found by solving equation 5 and, noting that $m_w = V_w$, find

$$RD = \frac{m_d - \phi_d}{V_c}. \tag{6}$$

The quantity in the numerator of equation 6 is directly measurable by the cartesian diver method and has been called the "reduced mass" by Prescott (10). The apparent volume, ϕ_d, is given by the mass of dry matter times its apparent specific volume, Φ_d, so that equation 6 can be written:

$$RD = \frac{m_d}{V_c}(1 - \Phi_d), \tag{7}$$

and we reach the important conclusion that, if Φ_d is constant, the reduced density is directly proportional to dry mass of the cell and inversely proportional to cell volume. Thus, at constant degree of hydration, reduced density must double (on the average) between cell birth and division. At constant dry mass, reduced density

will vary inversely with degree of hydration. The fractional changes in reduced density, therefore, are equal to fractional changes in quantities of primary interest in this investigation.

The apparent specific volume, Φ_d, will depend on the concentration of dry matter to only a slight degree (28, 29) and can be taken as constant over the range of current investigation.[2] Since cell dry matter is largely (about two-thirds) protein, ϕ_d is approximately 0.75 cc/g (Chap. 19, Table I of reference 30) and, from equation 7,

$$RD \sim 0.25\ (m_d/V_c), \tag{8}$$

(i.e. the concentration of solids in the cell is roughly 4 times the reduced density).

EXPERIMENTAL METHODS

The cells used in these experiments were local subclones of Chinese hamster cells line CHO (31) grown as previously described (32). Mitotic cells were separated from monolayers by the mechanical selection principle of Terasima and Tolmach (16) using our modification (33, 34) of the technique of Robbins and Marcus (35). Differential volume distribution spectra for the cells were determined by the Coulter principle of electronic cell sizing according to the procedure previously described (4). For low viscosity solutions, the aperture used was 100 μ in diameter by 250 μ in length. Amplifier and analyzer time constants were adjusted to match the transit time of this aperture (about 20 μsec). For viscous Ficoll solutions, the aperture used was 150 μ by 100 μ, which permitted rapid enough liquid flow to match the electronic parameters for all except the most concentrated solutions. The volume pulse-height calibration curve was determined as a function of Ficoll concentration using ragweed pollen (which is similar to CHO cells in size), and small corrections (never more than 15%) were applied to the Coulter volumes as necessary. There was no significant difference in spectrum shape nor width as measured with the two apertures.

Cell density was determined by isopycnic banding linear Ficoll gradients. Approximately 10^6 cells from the exponentially growing stock cultures were separated by 30 sec centrifugation in a clinical centrifuge and resuspended in 0.5 ml F-10 medium without serum supplement. This was layered onto a Ficoll gradient whose density, in a typical experiment, varied from 1.048 to 1.075 (approximate concentrations 0.12–0.20 w/w Ficoll in F-10 medium). In some experiments a broader density range, 1.02–1.10, was used. Alternatively, cells were suspended in dense Ficoll and layered below the gradient to approach equilibrium from the opposite side. The pH of all solutions was adjusted (with small bits of dry ice) to the range 7.0–7.5 as judged by phenol red indicator. Gradient volume was 5 ml, depth 4 cm.

Samples were centrifuged in an SW-65 rotor in a Beckman Model L2-65 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) at room temperature (22°C) for periods ranging from 5 min to 3 hr in fields ranging from 900 to 20,000 *g*. After centrifugation, the gradients were separated into 10–20 fractions, and the refractive index of each fraction was measured with a Bausch and Lomb refractometer (Bausch & Lomb Incorporated, Rochester, N.Y.) (20 μl sample). The final gradients were very nearly linear in all cases (except for the

[2] The argument is that ϕ of proteins is accurately given by the sum of the specific volumes of the constituent amino acids and that the latter are essentially concentration independent to greater than 1 M.

initial and final fractions), and the presence of the cell band had no effect on measured refractive index. An aliquot of each fraction was diluted 20:1 with isotonic saline, and the total cell concentration was determined by integral Coulter counting. Recovery of cells in the band averaged 24% of the cells taken, the loss (36) being due to inefficiencies of transfer, impaction, and retention on the sides of gradient tubes (cellulose nitrate, Beckman No. 305050), and some clumping by the preliminary centrifugation. Cell volume distribution spectra were also measured in some cases by similar dilution of fractions with either saline or isopycnic Ficoll. Calculations of mean density and coefficient of variation were based on actually measured density profiles after centrifugation.

RESULTS

Osmotic Measurements

The response of CHO cells to a change in osmolarity of the environment was determined by measurement of the volume distribution spectra of exponential-phase cell populations following their suspension in different media. Solutions of saline, sucrose, or mixtures were used with stoichiometric osmolarities varying from 0.21 to 0.41. (No correction has been made here for the osmotic coefficient which, for normal saline, is about 0.93 [25, p. 29]). When mean cell volume was plotted against reciprocal osmolarity, all data gave a good fit to a straight-line relation with the empirical equation $V/V_0 = 0.38 + 0.189/m$, where V is the volume at osmolarity, m, and V_0 is the volume in physiological saline (osmolarity 0.308). This is a form of the Boyle-van't Hoff law (24) applicable to osmotic equilibria and indicating a "partial volume" of cell water (24) of 0.62 V.

A stronger indication that the observed volume changes are the result only of osmotically determined water flow is provided by the time scale and reversibility of the changes. Following transfer to a new medium, the cells reached their equilibrium volume very rapidly (within a few minutes of mixing), remained at this

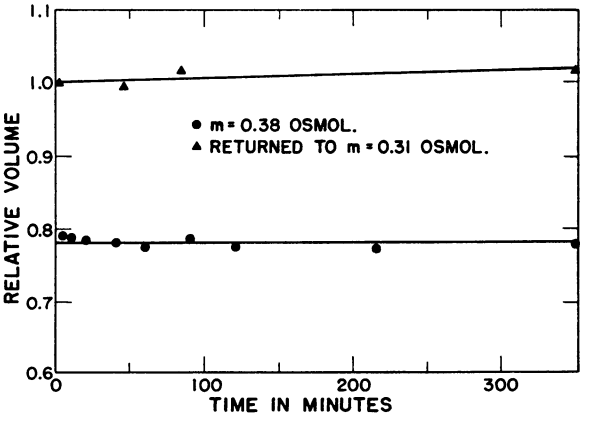

FIGURE 1 Stability and reversibility of mean cell volume at two osmolarities.

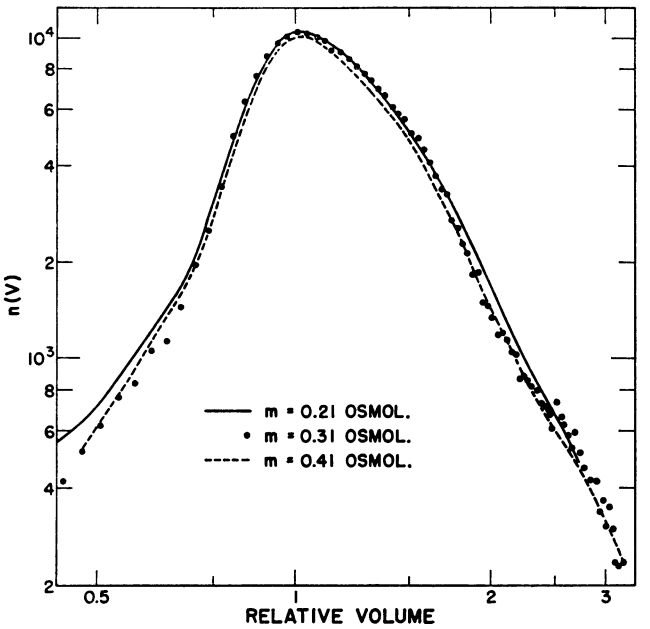

FIGURE 2 Invariance of shape of the volume distribution spectrum of CHO cells with osmolarity.

volume without further change for periods of several hours, and returned to their original volume in isotonic medium. Equilibration time is consistent with the kinetics of water transport (25, pp. 76–80) but not with the movement of electrolytes (24, p. 411). Typical data are shown in Fig. 1 in which relative mean cell volume for the population is plotted against time for transfer between solutions of two different osmolarities. (Small amounts of F-10 medium [about 10% by volume] are present in these solutions and are included in the calculated osmolarities.) The lower curve is for a population transferred from 0.31 to 0.38 osmolar saline at zero time; volume equilibration is essentially complete at the first point (3 min), and no further changes occur in 6 hr. The volume of cells returned to physiological saline at various times is given by the upper curve. The very slight (about 2% in 6 hr) upward trend of this curve is consistent with the very slow entry of salt into the cells while in hypertonic saline. The magnitude of this change is too small to invalidate the conclusion that, over periods of 1 hr or so (the duration of the banding experiments), these cell volume changes are due solely to water movement.

The above results apply to the mean volume of the population. It can also be shown that the fractional volume change is independent of age and volume of an individual cell; this is essential if density distribution, as determined by isopycnic banding, is to be applicable to the growing population. Thus, Fig. 2 shows the volume distribution spectra of exponential-phase CHO populations (the number of

cells, $n(V)$, having volume V is plotted on a logarithmic scale against the logarithm of volume) at three different osmolarities: tonicity 0.7, 1.0, and 1.4. The spectra are normalized to the same modal volume, and their identity at all volumes indicates that all cells of the population experience the same fractional expansion or contraction. Similar measurements on cells held in 0.4 osmolar saline for 40 min at room temperature and then returned to 0.3 osmolar saline showed the same invariance of spectral distribution. From these data it is concluded that, within the accuracy of the measurements, cells of different ages and volumes respond to osmotic changes by identical fractional changes in volume and, hence, by equation 7, in reduced density.

Cell populations suspended in various concentrations of Ficoll in F-10 medium over the range of interest (up to 0.18 weight fraction) show a similar volumetric behavior with respect to relaxation time, stability, and reversibility, although the data are somewhat less extensive and precise due to difficulties of volume spectrometry in the very viscous solutions. Spectral shape was also independent of Ficoll concentration, indicating that the fractional volume change was independent of volume and that there was also no significant change in volume distribution with time or intensity of centrifugation. For determination of relative changes in density around the life cycle, banding in Ficoll would appear to be reliable. For determination of absolute cell density under conditions of growth, correction must be made for the change in volume in Ficoll shown in Fig. 3. The abscissa is the weight fraction of Ficoll in the medium, and the ordinate is the volume of cells relative to the volume of cells in the same medium without Ficoll. The circles refer to solutions of Ficoll in F-10 without serum (0.28 osmolar), and the triangles refer to solutions of Ficoll in 0.63% saline (0.21 osmolar). (Thus, the reference volume at zero Ficoll is, in the latter case, 1.2 times that in the former.)

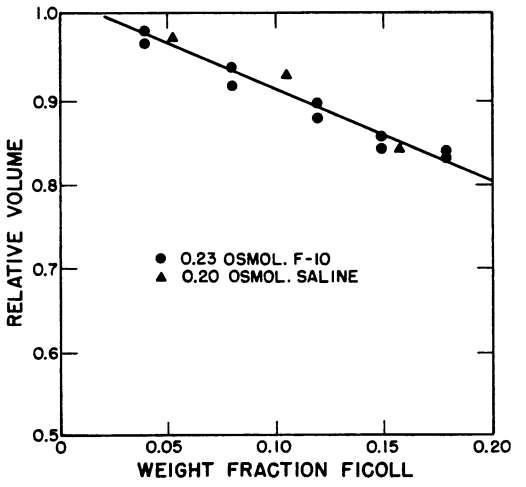

FIGURE 3 Effect of Ficoll concentration on relative mean cell volume of CHO cells in F-10 and in saline.

There is little or no difference in relative effect of Ficoll in the two solutions. In Ficoll of isopycnic banding density (1.06 g/cm^3, 0.16 weight fraction), cell volume is 15% less than in the absence of Ficoll. By equation 7, the reduced density of growing cells, therefore, should be 0.85 times the isopycnic reduced density. Accuracy of this conclusion depends on the constancy of the apparent specific volume, Φ_d. This is questionable over the entire range of Ficoll concentration, and the absolute density of growing cells, therefore, is uncertain by this factor. The relative densities of cells within a band are subject to a very much smaller error since, as will be seen, they are found only over a very narrow range in Ficoll concentration.

Density Determination

A typical isopycnic band (developed by centrifugation for 5 min at 10,000 *g*) is shown in Fig. 4 in which the fraction of cells found in a given density band is plotted as a histogram against density range of the band. (The gradients were very nearly linear, and the fixed volume fractions corresponded closely to equal density increments.) When the results are plotted on a cumulated probability scale against

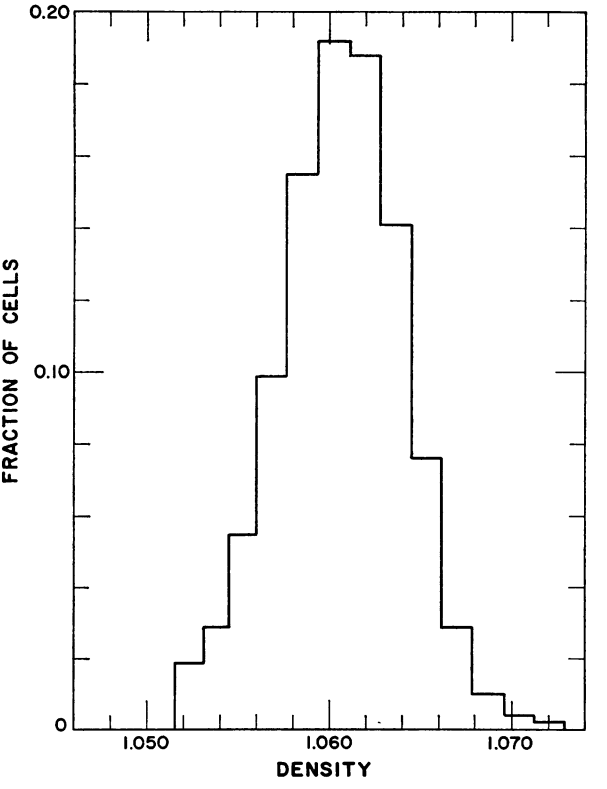

FIGURE 4 Isopycnic banding of CHO cells in Ficoll:F-10 gradient.

TABLE I

APPARENT ISOPYCNIC DENSITY OF LOG-PHASE CHO CELLS FROM SUSPENSION CULTURE

Acceleration	Duration	Product	Mean density*	Coefficient of variation‡
$\times g$	*hr*	$\times g \cdot t/1000$	g/cm^3	
900	0.08	0.07	1.0557	5.9
	0.25	0.22	1.0583	5.5
	0.70	0.63	1.0586	5.6
2500	0.50	1.25	1.0609	5.1
	1.00	2.50	1.0628	5.3
	2.00	5.00	1.0620	5.3
10,000	0.08	0.80	1.0595	4.9
	0.08	0.80	1.0605	5.6
	0.08	0.80	1.0628	5.9
	0.08	0.80	1.0618	5.2
	0.08	0.80	1.0619	5.2
	0.17	1.70	1.0603§	4.8
	1.00	10.00	1.0593§	5.6
20,000	0.08	1.60	1.0627	4.8
	0.08	1.60	1.0584	6.2
	1.00	20.00	1.0606	4.7
				Average = 5.3

* At 22°C in 0.16 w/w Ficoll in F-10.

‡ Expressed as per cent of reduced density.

§ Cells introduced at bottom of the gradient.

density, the data fit a straight line, indicating an essentially normal distribution with a coefficient of variation of 5.8% of the mean reduced density. Mean density for the band of Fig. 4 is 1.0605 (very close to the median 1.0615). An outstanding feature of the density distribution is the extreme narrowness of the band.

Repetitions of this measurement over a 6 month period using exponential-phase CHO cultures derived from laboratory stocks led to the results shown in Table I. In 14 of 16 determinations, cells in low density medium were introduced at the top of the gradient. In two cases, cells were first suspended in high density medium and introduced at the bottom of the gradient to approach equilibrium from below, but no difference in isopycnic density was found. Sedimentation conditions for the first two runs are marginal with respect to those estimated for complete equilibration, and the apparent densities are indeed slightly low. Therefore, these two determinations are omitted in the calculation of average density. The average of the remaining results is 1.0609.

The average banding density is very constant, covering a range of only 1.0584–

1.0628 over a 30-fold range in sedimenting conditions (the product of acceleration times duration, column 3, Table I). There is no trend of apparent isopycnic density as acceleration is varied from 900 to 20,000 *g* and centrifugation time is varied from 5 min to 2 hr. This stability indicates that exposure to high *g* forces in the presence of up to 0.16 w/w Ficoll does not produce any continuing changes in cell density and also proves that the cells have indeed reached isopycnic equilibrium. Reproducibility of the result can be quantitated from the scatter of the 14 measurements about their mean; the standard deviation is 0.0015 g/cm^3, corresponding to a coefficient of variation of 2.5% in reduced density.

Turning now to the density distribution spectra of the individual populations, we find that the coefficients of variation range from 4.7 to 6.2% of the reduced density. Experimental shortcomings of the procedure (e.g. slight turbulence during centrifugation, thermal mixing, mixing during fractionation, etc.) all act to broaden the distribution. The same is true of the effect of increasing Ficoll osmolarity on cell density. The latter can be treated mathematically in terms of effective density gradient (9, 37), which is the difference between the rate of change of solution density with distance and the rate of change of cell density with distance which results from increasing Ficoll concentration. Both these rates of change are positive so that effective density gradient is less than physical density gradient. Since resolution is inversely related to the effective gradient, a reduction in the latter will broaden the distribution. We estimate (from the data in Fig. 4) that, in a physical gradient of 0.007 g cm^{-3}/cm, the density of CHO cells is increasing at the rate of 0.0012 g cm^{-3}/cm so that the effective gradient is 0.0058 g cm^{-3}/cm. This should cause a broadening of some 20% compared with the true population distribution. All factors operate to broaden the apparent bandwidth. The true density variation, therefore, is probably less than the minimum observed.

Cell volume has been shown previously (17–20) to be well correlated with cell age. It follows, therefore, that if cell density were to change during a particular phase of the life cycle then certain layers of the isopycnic band would contain relatively more cells of the volume corresponding to that age (i.e. the volume distribution spectra would be narrower). Such an effect is not observed. We have measured the volume spectra of fractions from several bands and find that all have comparable widths, the coefficients of variation ranging from 0.41 to 0.49 compared with 0.39 for the initial exponential populations. Individual spectra have similar shapes, and there is no indication of enrichment of any particular volume range.

While results so far suggest that density is constant around the life cycle, the possibility remains that some minority of cells in a limited portion of the life cycle deviates significantly from the mean density. In particular, since only a few per cent (3.5% for exponentially growing cells with a 16 hr generation time) of the population are in mitosis at any given time, mitotic cells might be difficult to observe by methods used so far. For this reason, another set of measurements was

carried out which offered a much higher resolution for specific phases of the life cycle. Synchronized populations were prepared by mitotic selection and cultured in suspension. These populations are initially extremely well synchronized (90–98% of the initial population being contained within a 15 min segment of metaphase). Synchrony inevitably disperses so that, at the next division, the standard deviation of population age distribution is 15%. However, these populations permit very accurate determination of the density distribution of narrow populations of M and G_1 cells and of increasingly broader populations over the rest of the life cycle. However, the determination is complicated by the necessity of obtaining a certain minimum size population of cells to ensure continued exponential growth rate when the original mitotic cells are replanted in the suspension culture. Unless the cell concentration is above 5×10^4/ml, generation time and rate of volume increase may not be equal to those in maximum exponential-phase growth. Since a minimum volume of 100 ml is also desirable, a total of at least 5×10^6 mitotic cells is required to maintain the culture. It is necessary, therefore, to detach cells from the monolayer at least five times, a process requiring 50 min if the spacing between separations is 10 min. To preserve sharp initial synchrony it is necessary, therefore, to chill the mitotic cells to 0° as they are collected, to pool the successive collections, and to initiate division and growth by rewarming the entire suspension when sufficient cells are obtained. It has been shown previously (33) that this procedure gives a very homogeneous population which resumes growth without significant lag. A problem arises in the present connection, however, in that the process of chilling the cells results in a detectable increase in density. This is demonstrated in Table II in which the measured densities of mitotic and log-phase cells are compared for populations which are layered on the gradient with and without a preliminary chill to 0° for 2 hr. In both cases, there is a significant increase of 6–8% in reduced density due to chilling, but in neither case is there a significant difference in density between log-phase and mitotic populations.

Results of density determinations on cells of various ages are shown in Table III; in these cases, the initial mitotic populations were chilled during collection. The doubling time of the parent exponential-phase population was 16 hr distributed as

TABLE II
EFFECT OF CHILLING ON THE APPARENT DENSITY OF CHO CELLS

Cell population	Density*	
	Chilled	Not chilled
Log-phase	1.0655	1.0616
Mitotic	1.0651	1.0602

* Measured at 22°C in 0.16 w/w Ficoll in F-10.

TABLE III

APPARENT ISOPYCNIC DENSITIES OF SYNCHRONIZED CHO CELLS (CHILLED DURING COLLECTION)

Age of population	Calculated fraction in Phase*				Mean density‡	Coefficient of variation§
	M	G_1	S	G_2		
hr					*g/cm³*	
Mitotic	0.95	0.05	0	0	1.0630	3.4
3.0	0	1.00	0	0	1.0640	5.3
6.0	0	0.90	0.10	0	1.0626	3.2
9.0	0	0.43	0.54	0.03	1.0613	4.9
11.0	0	0.21	0.60	0.19	1.0616	3.7
				Average ±SD =	1.0625 ± 0.0011	
Mitotic	0.95	0.05	0	0	1.0651	6.7
2.7	0	1.00	0	0	1.0680	5.2
4.5	0	0.96	0.04	0	1.0662	5.5
9.5	0	0.39	0.56	0.05	1.0667	6.2
				Average ±SD =	1.0665 ± 0.0012	

* Calculated from the data of Enger et al. (38).
‡ At 22°C in 0.16 w/w Ficoll in F-10.
§ Expressed as per cent of reduced density.

8.4 hr in G_1, 4.1 hr in S, 2.7 hr in G_2, and 0.8 hr in M. The fraction of sample in each phase is given in columns 2–5 of Table III (calculation based on Fig. 7 of Enger, Tobey, and Saponara [38]). (It is not possible to obtain a predominantly G_2 population from mitotic selection because of the rapid dispersion of the population with age.) The experiments covered 70% of the life cycle before they were terminated, and it is clear that no significant density changes with age occurred. Thus, the standard deviation of a single density determination was calculated to be ±0.0014 in Table I, and the scatter of the individual means of Table III about their grand mean is ±0.0012. Expressed as the coefficient of variation of reduced density, the latter corresponds to ±1.8%. There are probably no true density variations with age over the M, G_1, and S periods of the life cycle larger than twice this number. A limit on possible variations in G_2 cells (13% of cells in an exponential-phase population) can be set from the width of the density distribution of the total population (Fig. 4, Table I) and is certainly less than 5%.

DISCUSSION

We have demonstrated the uniformity of density of CHO cells in an exponentially growing suspension culture by two methods: the density distribution spectrum of the exponential-phase population which was shown to have a coefficient of variation for individual cells of less than 5% of the reduced density, and the direct

measurement of a synchronized population of several ages which showed coefficients of variation of less than 2% of reduced density for changes in mean density at various ages. In particular, the density of mitotic and early G_1 cells was shown to be identical, a fact confirmed by our previous observations that these cells divided exactly in half, which would not be the case if mitotic cells were in an unusual state of hydration from which they recovered during cell division.

The primary value of these observations is to endow measurements of cell volume with a more fundamental relationship to the basic processes of cell growth. To the extent that density is indeed constant, cell volume is directly proportional to cell dry mass, and cell growth laws deduced from volumetric studies such as, for example, by the Collins-Richmond equation are directly applicable to the rate of increase of cell dry mass. The importance of these conclusions results from the comparative ease with which volume distribution spectra can be determined by electronic particle sizing and from the high precision with which growth rate can be calculated with the Collins-Richmond equation.

"True" density, on an absolute basis, of the growing CHO cell is not well established by these studies. There may well be variations of several per cent in mean reduced densities of different cultures nominally of the same line grown under apparently identical conditions. In addition, the apparent isopycnic density of 1.0610 as determined by our method is the buoyant density of cells when suspended in a Ficoll:F-10 solution whose total osmolarity appears to be about 0.37 as deduced from the equilibrium volume of CHO cells. One would calculate from this that in growth medium F-10 (0.28 osmolar) the reduced density would be 15% less, leading to an estimate of 1.052 for the mean density of CHO cells in F-10 medium at 22°C. Boone et al. (22) have reported isopycnic densities of 1.060, 1.070, and 1.064 for rabbit thymocytes, LKID, and HeLa cells, respectively. These densities were not corrected for osmotic effects of the Ficoll gradient and, thus, should presumably be compared with our analogous number of 1.0610. The agreement is satisfactory and suggests a certain degree of uniformity among mammalian cells of these different types. The populations studied here were in exponential, balanced growth at nearly the maximum attainable rate, and no effort was made to vary this rate. If mammalian cells had a ribosomal content which depended on growth rate, as has been reported for bacteria by Schaechter, Maaløe, and Kjeldgaard (39), then density may vary similarly.

The constant density which we observed for mammalian cells around the life cycle is in contrast with density variations of 10–40% found for yeast and bacteria (13–15). This difference may be due to the absence of a rigid outer wall in the mammalian cell. When a difference in activity of water develops across the cell membrane, water moves to restore osmotic equilibrium by changing the solute concentration, and the cell volume changes. In bacteria, on the other hand, the rigid wall prevents gross water movement by fixing the volume so that, instead,

a hydrostatic pressure develops sufficient to equalize the activity of the water. Thus, as the dry mass of a bacterium increases, concentration and density can rise with hydrostatic pressure maintaining osmotic equilibrium, but in the mammalian cell water must move to maintain equilibrium. Thus, the density change of a bacterium or yeast is determined by the ratio of mass increase to volume increase and, hence, by the phase relation between "nuclear" and "cell" cycles as pointed out by Mitchison (15). In mammalian cells, volume is not determined independently of dry mass by a capsule, and the range of density variability is thereby reduced.

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