SYNCHRONIZATION OF MOUSE L-CELLS BY A VELOCITY SEDIMENTATION TECHNIQUE

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ABSTRACT Differential velocity sedimentation at unit gravity has been used to separate an asynchronous population of mammalian cells into fractions synchronized in all phases of the cell cycle. Better enrichment was obtained for G_1 and S phases than for G_2-M phase. Electronic cell volume measurements of the fractions indicated that the separation was primarily dependent on cell size, and an experimentally determined sedimentation coefficient agreed very well with its predicted value. Sources of dispersion in the separation (including the contribution of cell density heterogeneity) were quantitated and found to be insufficient to explain all of the observed dispersion. Both the limitations and the applications of the technique are discussed.

INTRODUCTION

Studies of the biochemical and physiological events accompanying the progression of individual mammalian cells through their division cycle could be greatly facilitated by techniques that yield large populations of cells differentially enriched for any desired part of this cycle. At the present time, such studies use populations of cells which are artificially synchronized by one of the following techniques:

(*a*) blockage of cell growth at a particular point in the cell cycle by some inhibitory agent (1–5);

(*b*) destruction of cells in all but a small portion of the cell cycle (6, 7);

(*c*) physical selection of mitotic cells (8).

These techniques have one main disadvantage in common: synchrony is achieved at a point (or narrow window) of the cell cycle so that the measurement of any cell cycle–dependent parameter requires that the synchronized cells be recultured until they have passed through the remaining phases of the cycle. Unfortunately, dispersion in the individual generation times of the cells results in a rapid loss in the degree of synchrony, and measurements in portions of the cycle far removed from the point of induction of synchrony tend to be inaccurate. Additional disadvantages are associated with each of these techniques. Inhibition has been shown

to result in abnormal cell growth subsequent to the synchronization (3). Selective destruction results in a background of nonproliferating but otherwise metabolically active cells which make biochemical studies impossible (6). Mitotic selection averts these problems but yields few of the input cells.

We describe here a synchronization technique which is free from most of the aforementioned problems. Good evidence exists for a monotonic increase in cell volume with position in the cell cycle (8, 9). Since differential velocity sedimentation separates cells primarily on the basis of volume (10), this technique should be suitable for the continuous fractionation of an exponentially growing mammalian cell population into all phases of the cell cycle (10).

Previous attempts to synchronize cells by velocity sedimentation (at greater than unit gravity) have worked well for yeast (11, 12) and bacteria (13), but only limited success has been achieved for mammalian systems (14, 15). Recent improvements in the resolution obtainable by velocity sedimentation at unit gravity prompted us to reexamine the feasibility of using such a procedure as a synchronization technique.

THEORY

A spherical particle (and to a close approximation a mammalian cell) sedimenting through a stabilizing gradient at 1 g attains a terminal sedimentation velocity (s) given by the expression

$$s = \frac{2}{9} \frac{\rho - \rho_0}{\eta} g r^2, \tag{1}$$

where η = viscosity of gradient medium, ρ = density of particle, ρ_0 = density of gradient medium, and r = radius of particle.

If variations in ρ are small for a homogeneous cell type such as L-cells, and if one chooses a shallow concentration gradient for the supporting medium so that variations in $(\rho - \rho_0)$ and η are also small, then

$$s \simeq kr^2 = k' V^{2/3}, \tag{2}$$

where V = cell volume and k and k' = constants.

If these conditions hold, cells before and after mitosis (which differ by a factor of two in volume [9]) will also differ by a factor of $2^{2/3}$ or 1.59 in sedimentation velocity. Thus they will be separable by sedimentation. The sedimentation distribution of antibody-producing cells from mouse spleen suggested that such a model might be valid (16); however, in these experiments, interpretation of cell cycle parameters was difficult because antibody-producing cells were always a minority population. Experiments described in this paper, using a single homogeneous population of cells, give a direct confirmation of this hypothesis.

MATERIALS AND METHODS

The experiments described were carried out on a subline of Earle's L-cells designated L60T (3). The cells were routinely grown in suspension culture in spinner flasks (Bellco Glass, Inc., Vineland, N. J.) at 37°C. The medium employed was CMRL 1066 (17) lacking thymidine but supplemented with 10% (v/v) fetal calf serum (FCS) (Flow Labs, Inc., Rockville, Md.). Under these conditions, the doubling time of the cell population during the exponential growth phase was about 16 hr (consisting of a G_1 phase of 4 hr, an S phase of 8 hr, and a G_2-M phase of 4 hr). Before separation, an exponentially growing population of mouse L-cells was pulse-labeled with tritiated thymidine TdR-^{3}H (5 μCi/ml for 15 min), washed, and resuspended in phosphate-buffered saline (PBS) (18) containing 10% FCS. Two filtrations of this suspension through a glass capillary array filter (pore size: 37 μ) reduced the frequency of clumps to less than 1%. The cell concentration was adjusted to 2.5×10^5 cells/ml (a concentration well below that at which "streamers" form in the cell band [19, 20; see also discussion in reference 10]), and 20 ml of the suspension were then sedimented at 1 *g* for 3½–4 hr on a linear gradient of 15–30% FCS in PBS at 4°C in a closed cylindrical Lucite chamber (diameter 13.8 cm; height 7.9 cm) as indicated schematically in Fig. 1. Typically, 80–90% of the input cells were recovered. An aliquot of each fraction was used to determine the cell concentration and volume distribution by means of an electronic cell counter and pulse height analysis system calibrated to give absolute volume measurements (10). The remaining cells were fixed, stained, and prepared for radioautography (21) so that the percentage of labeled cells and mitotic index could be determined for each fraction.

For cell density determinations, 2×10^6 L-cells in exponential growth phase were pulse labeled with TdR-^{3}H as described previously and centrifuged to equilibrium at 4°C on a

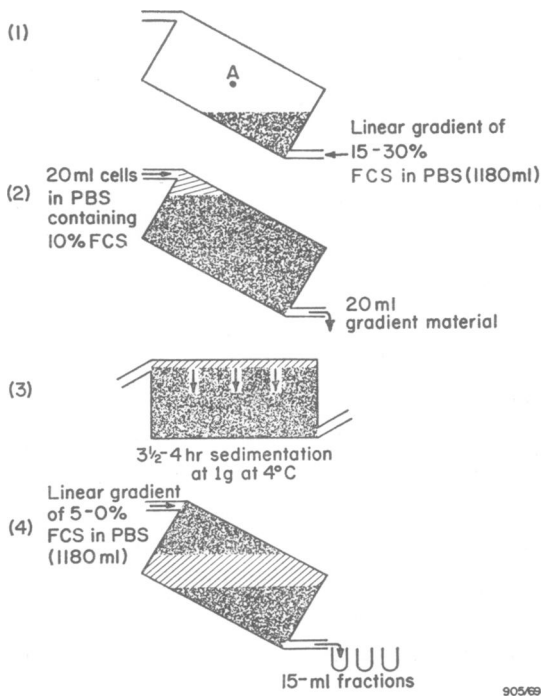

FIGURE 1 Schematic representation of cell separation procedure. With the chamber tilted roughly 30° from the horizontal about its axis *A*, a gradient is introduced from the bottom (1). The cells are then introduced from the top (2) and the chamber is carefully returned to the horizontal for sedimentation (3). Fractions are collected with the chamber in its original tilted position (4). The sedimentation chamber is described in detail in the text.

Ficoll gradient (22). The refractive index, cell concentration, and modal cell volume of each fraction were determined and the acid-insoluble tritium activity of the remaining cells was measured on a liquid scintillation counter (23).

RESULTS

Synchrony Obtained

A typical cell concentration vs. sedimentation velocity curve is shown in Fig. 2. If cell volume increased linearly with time throughout the cell cycle, one might ideally expect a sharply bounded exponential region where the boundary points (represent-

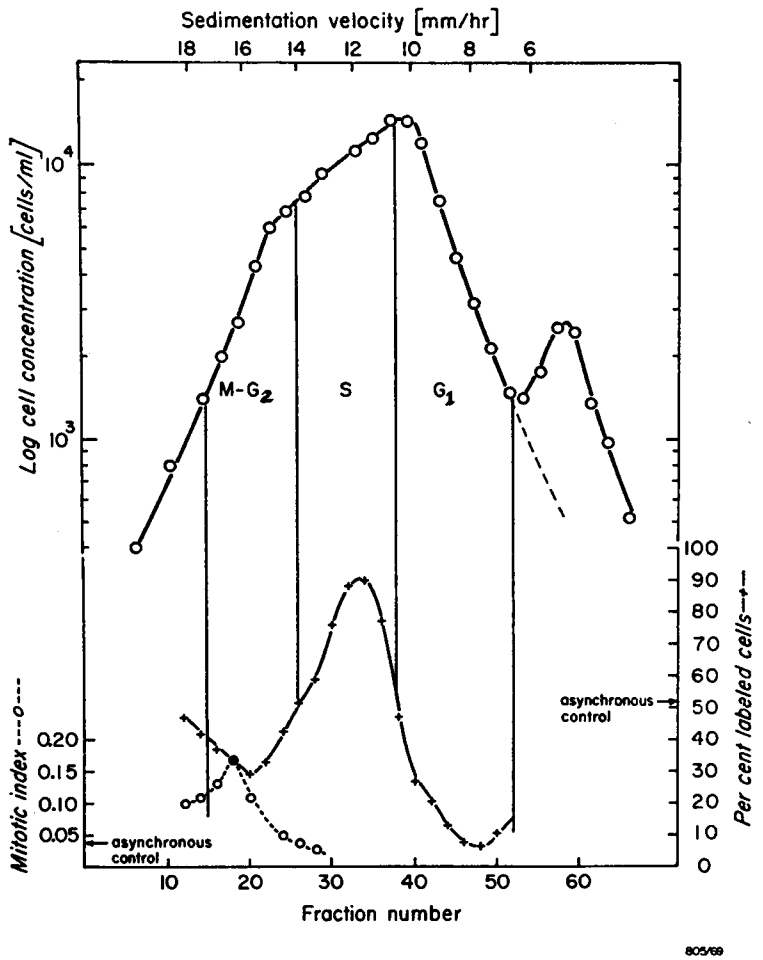

FIGURE 2 Distribution of cell concentration, per cent labeled cells, and mitotic index for a population of mouse L-cells separated by velocity sedimentation at unit gravity. The smaller peak in the cell concentration profile is composed of cell fragments.

ing mitotic cells and cells in early G_1) would have relative cell concentrations of 1 and 2 and relative sedimentation velocities of 1.59 and 1, respectively. The curve actually obtained has an exponential region but is broadened by dispersive factors which will be discussed later.

It may also be seen from Fig. 2 that the distributions of S phase cells and mitotic cells are considerably more homogeneous than that of the population as a whole. More specifically, the data on labeled cells indicates that relatively pure populations of G_1 and S phase cells can be obtained (>90%) whereas only about 70% purity can be obtained in G_2-M phase. The presence of random clumps and polyploid cells in the G_2-M region of the gradient contributes to the reduced purity obtained, and the small proportion of total cells in this region (<20%) tends to magnify the effect. In fact, the proportion of labeled cells approaches that of the asynchronous control for very large *s*, indicating that these fractions are composed entirely of polyploid cells and random clumps. The peak mitotic index obtained (17%) is comparable with most synchronization techniques (except, of course, mitotic detachment).

A measure of the proportion of cells in each phase of the cell cycle may be made directly from the data of Fig. 2. If we exclude the outermost 4% of the cell concentration distribution in Fig. 2 (the extremes representing mainly clumps and polyploid cells at large *s* and broken cells at small *s*), and if we define our S phase to be that region of the distribution in which the percentage of labeled cells exceeds the value for the asynchronous control, we obtain values of 18.4, 48.4, and 33.2% for the proportion of cells in G_2-M, S, and G_1 phases, respectively. These values are in excellent agreement with the predicted proportions for an exponentially growing population having the currently accepted doubling time and phase durations of these cells (i.e. 19, 49, and 32%, respectively).

The usefulness of any cell synchronization technique is largely dependent upon its reproducibility. We have found that cells maintained in the exponential phase of growth exhibit sedimentation profiles of the characteristic shape shown in Fig. 2.

TABLE I

REPRODUCIBILITY OF SEDIMENTATION VELOCITY OF PEAK ENRICHMENT OF S PHASE AND M PHASE CELLS

Experiment	TdR-^{3}H-labeled cells: *s* of peak enrichment	Mitotic cells: *s* of peak enrichment
	mm/hr	*mm/hr*
1*	11.6	16.6
2	11.3	16.6
3	11.5	16.0

* Data from Fig. 2.

More importantly, the sedimentation velocity of the peak of enrichment for both S and M phase cells is highly reproducible (as indicated by Table I).

Density Distribution of L-Cells

The cell concentration vs. density curve for a population of exponentially growing L-cells is shown in Fig. 4. It can be seen that the distribution is homogeneous with a mean of 1.051 g/cm^3 and a width at half maximum (W_ρ) of only 0.004 g/cm^3.

The similar shape of the TdR-^{3}H activity and cell concentration curves indicates that TdR-^{3}H activity per cell is roughly constant across the gradient (further suggesting that homogeneous density fractions are asynchronous).

Physical Separation Data: Agreement with Theory

Up to this point, we have been assuming that s depends only on r^2 (equation 2). To test this, we plotted log modal volume (V) vs. log s for the individual fractions

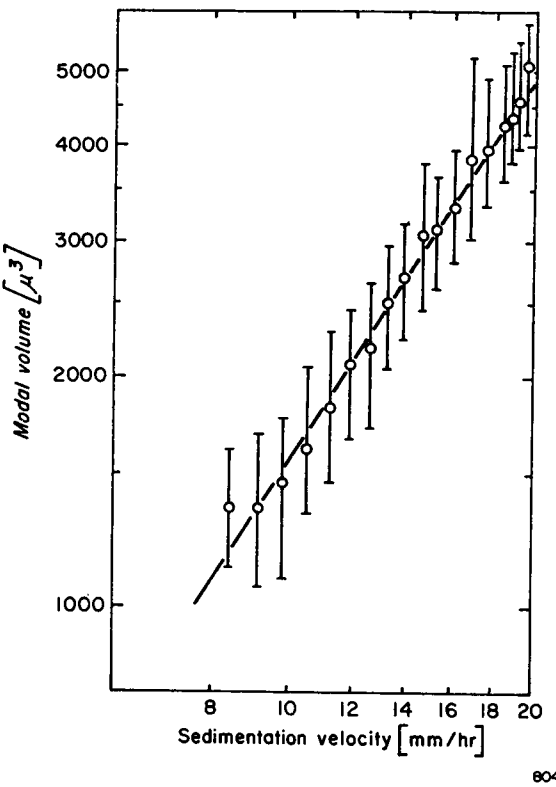

FIGURE 3 Correlation of log modal volume with log sedimentation velocity for cells fractionated as in Fig. 2. The dashed line represents a least squares fit of the data (with a slope of 1.62 for this particular experiment) and the vertical bars around each point represent the volume dispersion W for that fraction (see text for discussion).

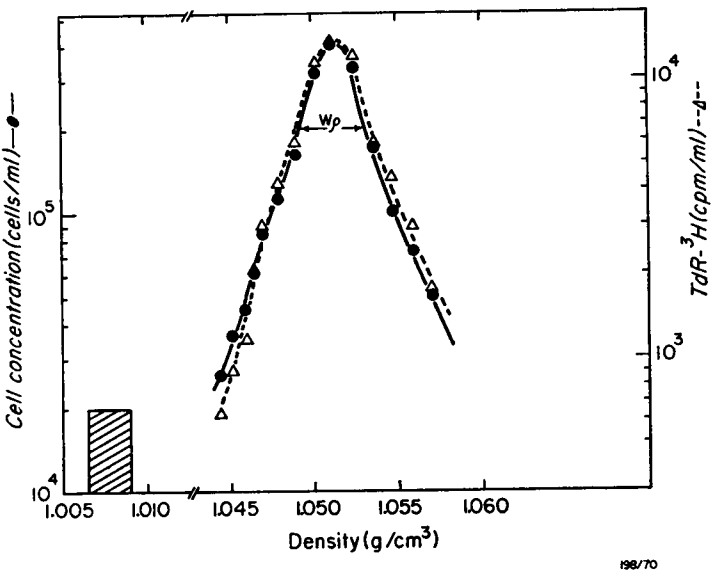

FIGURE 4 Distribution of log cell concentration and log acid-insoluble tritiated thymidine (TdR-^{3}H) activity for a population of mouse L-cells separated by equilibrium density centrifugation in Ficoll. The cross-hatched region represents the density range of the velocity sedimentation gradient (determined volumetrically) and W_ρ is the density dispersion width of the population (see text for discussion).

in the three experiments cited in Table I. The resultant straight lines (see example in Fig. 3) had a mean slope and standard deviation (as determined by least squares fitting) of 1.55 ± 0.07. This value is not significantly different from the predicted value of 1.50.

In addition, an experimental sedimentation coefficient (k' in equation 2) may be determined from the intercept of a log V vs. log s plot. The mean value of the intercept for the three experiments cited led to a value for the sedimentation coefficient k of 0.22 mm hr^{-1} μ^{-2}. A value for k may be independently calculated from the parameters in equation 1. Using the mean density of the L-cells and the gradient medium (Fig. 4), and assuming a viscosity equal to that of water at 4°C, we again obtained a value of 0.22 mm hr^{-1} μ^{-2} for k.

Dispersion and Limits of Resolution

The vertical bar on each point in Fig. 3 indicates W, the width at half-height of the volume distribution of that fraction. We define a volume dispersion parameter δ equal to W/V. δ is then a measure of the resolution of the physical separation procedure. The range of variation of δ (from Fig. 3) is 0.34–0.57. Factors contributing to δ include cell density differences, the finite width of the starting band, the volume of the fractions collected, the resolution of the electronic cell detector, and miscellaneous other factors which cannot be quantitated (such as gradient mixing dur-

ing loading and unloading). The density dispersion width of the L-cell population (W_ρ in Fig. 4) corresponds to a δ value of about 0.07, much less than the observed values. The finite volume of the fractions and width of the initial cell band (1.3 mm) also contribute very little to δ ($\sim$0.02). Hence, the factors considered are clearly insufficient to explain all of the observed dispersion, although it should be noted that the electronic cell detector system gives rise to an apparent dispersion of 0.25–0.30 (as determined by measurements of homogeneous pollens).

Apart from the limitations of the physical separation, there are other factors restricting the resolution in cell synchrony obtainable by this technique. The possibility of any major cell cycle dependence of density variations can be ruled out by the fact that there was no significant correlation of either acid-insoluble tritiated thymidine activity (Fig. 4) or cell volume with cell density. A more fundamental limitation is the distinct probability that cells at a particular point in the cell cycle will have a heterogeneous distribution of sedimentation velocities (due primarily to volume heterogeneity). This would result in a broadening of the true distribution of any cell cycle–dependent parameter assayed by this method. The quantitative extent of this heterogeneity has proved hard to estimate for L-cells, but measurements of the closely allied volume heterogeneity of another line of synchronized mammalian cells (9) have indicated that it may indeed be significant.

It should also be noted that optimal resolution of this technique is restricted by "streaming" (19, 20; see also discussion in reference 10) to cell load concentrations of the order of 10^6 cells/ml or less for this cell type (determined empirically). Hence, higher yields at comparable resolution may be obtained only by increasing the cross-sectional area of the sedimentation chamber proportionately (a chamber now exists which could separate $\sim 1.5 \times 10^8$ L-cells at good resolution and it appears that larger ones could be constructed).

Application of Technique

The synchronization technique described here may be adapted to preparative use if a sterile glass chamber is employed. Under such conditions, we obtained plating efficiencies of about 40% for fractionated L-cells in all phases of the cell cycle (compared to about 60% for an unfractionated asynchronous control). In addition, we UV-irradiated an exponentially growing cell population and measured the plating efficiency of the separated fractions.[1] The resulting pattern of cell resistance to UV as a function of position in the cell cycle was qualitatively and quantitatively comparable with that obtained by another synchronization technique (24).

SUMMARY

In summary, the technique described in this paper has three distinct advantages over other synchronization procedures, the first two of which are unique.

[1] Rauth, A. M., and H. R. MacDonald. Unpublished data.

(*a*) Synchronization is achieved at all phases of the cell cycle at the same time so that the effect of desynchronization due to dispersion in individual cell generation times is avoided.

(*b*) Differential cell cycle effects can be determined by treating the asynchronous population and assaying the synchronized fractions after separation, thus ensuring identical treatment for each fraction.

(*c*) The technique is technically simple and appears not to interfere with normal cell viability, thereby allowing biochemical studies to be made (subject to restrictions in yield).

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