

# SEPARATION OF HE<sub>2</sub>LA CELLS BY COLLOIDAL SILICA DENSITY GRADIENT CENTRIFUGATION

## I. Separation and Partial Synchrony of Mitotic Cells

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### ABSTRACT

Using a colloidal silica density gradient, HeLa cells in mitosis were found to have a density of 1.040–1.046 g/cc, lighter than the remaining interphase cells. The mitotic cells could be harvested and cultured after centrifugation, showing growth synchrony by measurement of a peak in mitotic index 21 hr after establishing the culture. By using Colcemid or vinblastine sulfate, HeLa cells were arrested in metaphase and centrifuged on the colloidal silica density gradient. The blocked metaphase cells were lighter in density than the interphase cells but somewhat more dense than untreated cells selected by the density gradient centrifugation. Near-equilibrium conditions were established during the centrifugation of cells so that cell density measurements could be made, and the gradient medium employed was not measurably toxic to those cells tested.

The separation of mammalian cells in different phases of the cell division cycle from a heterogeneous cell population has been the objective of many studies. Whitmore (1) reviewed the separation and growth synchronization of cells and classified the methods as "natural" and "induced" synchronization. Induced synchronization has been most useful to produce large numbers of cells, however, natural synchronization may be preferred, since it eliminates the cellular alterations caused by chemically or temperature-induced synchrony. Axelrod and McCulloch (2) and Terasima and Tolmach (3) used the natural selection system of shaking the less tightly adhered cells in mitosis from monolayers. The mitotic cell yield is limited with this method but may be partly overcome by systems such as that of Lindahl and Sörenby (4), where cells in mitosis are continuously collected from a roller

bottle, harvested by centrifugation, and held at subnormal temperatures for further use.

For collection of mammalian cells at different stages of the cell division cycle from suspension cultures, Sinclair and Bishop (5), Schindler et al. (6), and Morris et al. (7) used sucrose density gradient centrifugation. This natural selection system can be readily used with large numbers of cells from suspension culture, eliminating the mechanical limitations of the collection system. The centrifugal forces employed were not harmful to the cells (6) and the gradient media allowed continued growth. Other density gradient centrifugation procedures have used Ficoll (7–9) to separate cells from various phases of the growth cycle. Density gradient media which provide enough density for equilibrium centrifugation of cells and maintain cell viability have been difficult to develop, especially if at the same time the

requirements of isosmotic conditions, proper ionic strength, and low viscosity for rapid sedimentation are fulfilled.

The present report demonstrates that colloidal silica gradients provide these characteristics plus the advantages of a self-generating gradient. A review of work using colloidal silica as gradient material was presented by Pertoft and Laurent, 1969 (10). Colloidal silica has been utilized in this study to provide the separation of mitotic cells from a heterogeneous cell population and the mitotic cells can be shown to give at least one cycle of partially synchronous growth in culture.

## MATERIALS AND METHODS

### *Cell Cultures*

HeLa, S-3 cells (obtained from Dr. J. Maizell, Jr., Albert Einstein Medical School, Bronx, N. Y.) were grown in spinner culture in Eagle's Spinner Medium (11) including spinner salts, penicillin, streptomycin, and kanamycin plus 7% calf serum. Cell population densities ranged between 2 and  $6 \times 10^5$  cells/ml in spinner culture of 0.5–2.0-liter volumes and cells were used only from cultures growing at the maximum rate of 37°C. When mitotic phase cells were required in large numbers, HeLa cell cultures were arrested in metaphase by use of Colcemid (a gift of Ciba Industria Chimica S.p.A., Milano) or vinblastine sulfate (Velban, Grand Island Biological Co., Grand Island, N. Y.) as described in Fig. 4.

### *Density Gradient Medium*

All density gradients were formed in a medium referred to as PEL, containing polyvinylpyrrolidone (PVP), Eagle's Minimum Essential Medium (MEM), and Ludox colloidal silica. Ludox HS 40 (40%, w/w, 52%, w/v) was purchased from E. I. DuPont de Nemours & Co., Wilmington, Delaware, and dialyzed against 20 vol of distilled water with five changes during the 6-hr dialysis period which resulted in a final 42.1% (w/v) concentration of Ludox HS. PVP (Arthur H. Thomas Co., Philadelphia, Pa., 40,000 mol wt) was prepared as a 33% solution in distilled water and dialyzed as the Ludox, resulting in a final concentration of 20.2% (w/v). Eagle's MEM (11) without serum but with penicillin, streptomycin, and kanamycin was prepared at twice the normal concentration (2 ×). The gradient medium was prepared just before use by shaking a mixture of the following components added in the indicated order: 18 parts Ludox HS (dialyzed to 42.1% w/v), 42 parts of 2 × Eagle's MEM, 25

parts of dialyzed PVP (dialyzed 20.2% solution), 2.5 parts 1 N HCl, and 2.5 parts of double-distilled water. The osmotic pressure was measured with an osmometer (Advanced Instruments, Inc., Newton Highlands, Mass.) and was between 280–310 milliosmols; the pH was 7.6 and the density, 1.055 g/cc. Densities of the medium and samples were determined by duplicate measurements in density gradient columns prepared from mixtures of kerosine and carbon tetrachloride as described earlier (12). The individual medium components can be sterilized before mixing by autoclaving, except for the 2 × Eagle's MEM which must be filter sterilized.

### *Gradient Formation*

Preformed gradients of PEL medium were formed in  $3.8 \times 10.1$  cm cellulose acetate tubes (95 ml) in an angle head Type 21 Beckman rotor (Beckman Instruments, Inc., Fullerton, Calif.). Tubes were centrifuged for 20 min at 20,000 rpm (53,664 g) in the Beckman model L-65B ultracentrifuge at 20°C, with brake on during deceleration.

### *Analysis of Cells Harvested from the Gradients*

HeLa cells were centrifuged on the preformed gradients for 20 min at 800 g as described in the Results section. 3-ml fractions were collected from gradient tubes by displacing the gradient with undiluted Ludox. Cell counts were made on each fraction using a Celloscope (model 302, AB Lars Ljungberg & Co., Stockholm) with dual threshold settings adjusted to yield counts of whole, unclumped HeLa cells. Mitotic indexes were measured by mixing a drop of the sample with one drop of 0.025% crystal violet in 1.0% acetic acid and observing directly using 40 × phase-contrast objectives. If samples could not be examined at once, mitotic indexes were determined after fixation of pelleted cells in a 5:2:3 (v/v) mixture of ethanol, acetic acid, and water before mixing with the 0.025% crystal violet stain (6).

## RESULTS

### *Gradient Medium and Cell Viability*

The maintenance of cell viability was of importance in this work on formulating the gradient medium. It was determined by monolayer toxicity studies that 5% Ludox, and mixtures of 5% Ludox and 16% polyethylene glycol or 5% Ludox and 1% Methocel (Dow Chemical Co., Midland, Mich.), were toxic and therefore not useful, despite their ability to separate cells. PVP provided protection from silica precipitation

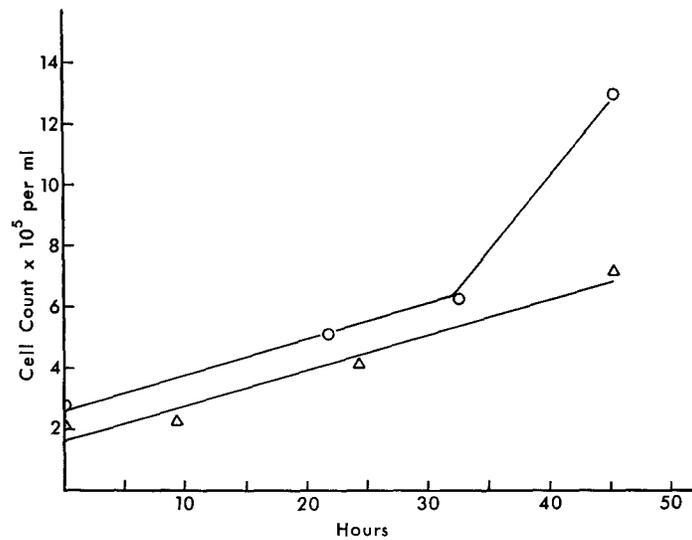


FIGURE 1 HeLa cell growth rate after exposure to PEL medium. Cells were placed on a preformed PEL gradient and centrifuged for 20 min at 800 *g*. The resulting cell band (the majority of cells) was harvested, diluted with  $2 \times$  Eagle's MEM, and pelleted at 100 *g* for 8 min. The cells were washed once in Eagle's MEM Spinner Medium and a spinner culture established at a cell density of  $3.3 \times 10^5$  cells/ml. Time of exposure was approximately 1 hr. The control cells, not exposed to PEL, had a cell density of  $2.1 \times 10^5$  cells/ml when placed in culture. Control cells,  $\Delta$ — $\Delta$ ; PEL-treated cells,  $\circ$ — $\circ$ .

and cell detachment, and was therefore used to prevent Ludox toxicity and modify the gradient shape. Eagle's medium was added to the mixture to provide cell nutrients and the proper osmotic pressure. In other toxicity tests, suspended cells were exposed to the PEL medium for 20 min during low-speed separation centrifugation, harvested by dilution in MEM, and pelleted at 100 *g*, 8 min, then washed once and resuspended in 50 ml MEM spinner medium and grown as spinner cultures. The growth rate over a 44 hr period was similar to the growth of control cells not exposed to the PEL medium, and showed approximately two doubling periods during this time (Fig. 1).

### Separation of Cells on PEL

#### Density Gradients

Density gradients were formed as discussed in Materials and Methods from PEL medium, density 1.055 g/cc. After gradient formation, up to  $0.3 \times 10^8$  cells to be separated were pelleted (100 *g*, 8 min) and resuspended in 5 ml of PEL medium removed from the top of the gradient. Cells were well dispersed using three to four strokes of a loose A-pestle in a Dounce homogenizer, then layered over the preformed gradient. Cell separation

was achieved under conditions approaching equilibrium (*vide infra*) when the centrifugation was carried out at 2000 rpm (800 *g*) for 20 min at room temperature in a Wifug (Model X-3) with a swing-out head. On termination on the run, 3-ml fractions were collected and analyzed for cell number, density, and mitotic index, as described in Materials and Methods. The data collected from one experiment are shown in Fig. 2. In Fig. 2 *a* it is evident that the gradient is non-linear but the majority of cells accumulate in a single peak. The mitotic phase cells (approximately 3.0% of the total cell population) are clearly separated from the majority of the cells and peak at a density of 1.046 g/cc, where 71% of the cells collected are in mitosis. Cell debris accumulates above this density. In other similar experiments as high as 89% of the cells were in mitosis at this density level.

The display of data as in Fig. 2 *a* is adequate for single gradients, but when several gradients are compared, the results become confusing unless the density curves are identical, for the bands or peaks shift in the tube following the steep portion of the gradient. To eliminate these shortcomings, we have plotted the per cent of total number of

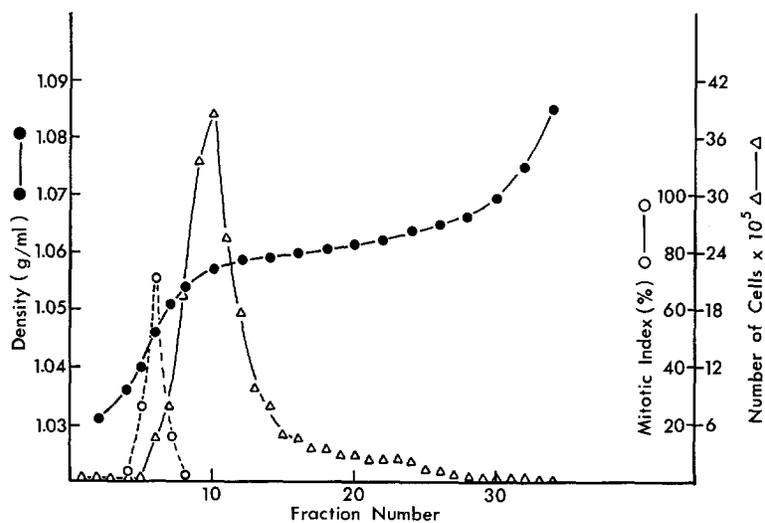


FIGURE 2 Distribution and mitotic index of HeLa cells after centrifugation on a preformed PEL gradient.

FIGURE 2 a Density, cell number, and mitotic index vs. fraction number after a 20-min centrifugation at 800 *g* on a preformed PEL gradient.

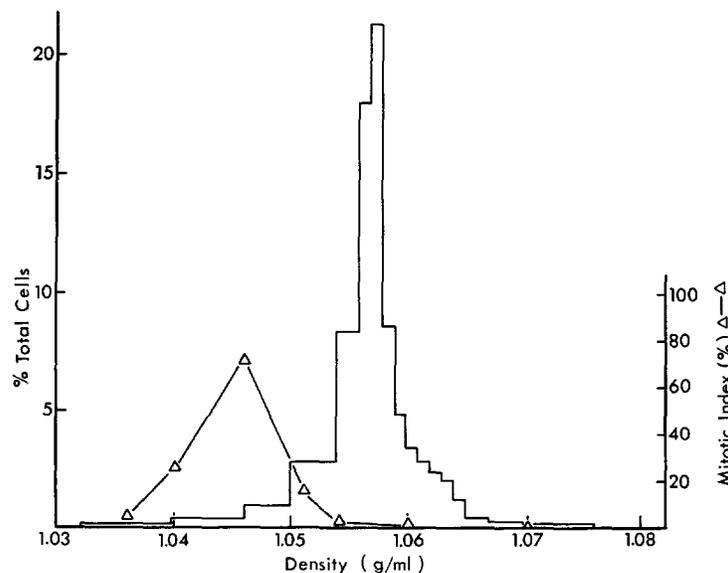


FIGURE 2 b Per cent of total number of cells in the PEL density gradient at different densities. Data from Fig. 2 a.

cells at each density (Fig. 2 *b*). This is complemented by another display which is the per cent accumulation of total cells throughout the gradient at each density (Fig. 2 *c*). Using the latter presentation, results from several gradients can be displayed and compared simultaneously.

The time of gradient centrifugation to achieve

separation was determined by centrifuging similar gradients for various lengths of time and examining them for cell distribution. The data from a 90-min centrifugation show the separation of mitotic cells from the majority of nonmitotic cells, just as in a gradient centrifuged for 20 min (Fig. 2 *c*) and therefore suggest that equilibrium

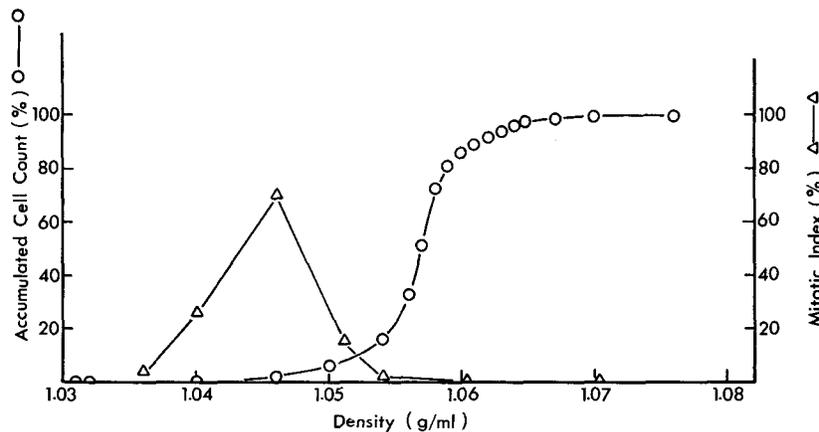


FIGURE 2 c Per cent of total number of cells accumulated and mitotic index at each density level. Data from Fig. 2 a.

is established in the shorter period. The 90-min period, however, results in a lower percentage yield of mitotic phase and a somewhat lower apparent density of the majority of the cells.

#### *Growth of Mitotic Phase Cells and Production of a Synchronous Culture*

Mitotic phase cells could be harvested from samples yielding high proportions of mitotic cells (Figs. 2 a, c) after a 1:2 dilution in Eagle's MEM and centrifugation for 10 min at 100 g cells were washed once in Eagle's Spinner MEM (37°C) and resuspended in volumes to provide a cell concentration of between 1 and  $5 \times 10^5$  cells/ml and placed at 37°C in spinner flasks. The final yield of mitotic cells was as high as 18.0% of the cells in mitosis in the original, nonsynchronized culture. Cell counts and mitotic indexes were measured as shown in Fig. 3, where the first peak in mitotic index at 9.5% is shown 21 hr after establishing the culture and the cell number in the spinner culture rises as the mitotic index drops. These data indicate that this method is capable of producing a synchronous culture of HeLa cells. Since there was not a doubling of cells during the period of increased mitotic activity (21–24 hr), there was either incomplete synchrony or cell death at this time.

#### *Density Patterns from Cultures with Induced Mitotic Phase*

To continue examination of cells in mitosis for their shift in density, HeLa cell cultures were ar-

rested in the mitotic phase by the use of Colcemid or vinblastine. Spinner cultures containing 200 ml of HeLa cells at  $1.4 \times 10^5$  cells/ml were treated with either Colcemid at a concentration of 0.05  $\mu$ g/ml or vinblastine sulfate at 0.01  $\mu$ g/ml for a period of 16 hr (modified from the methods of Stubblefield and Klevecz [13] and Kim and Stambuk [14]). The mitotic indexes for these cultures were 68% for Colcemid-treated, 60.5% for vinblastine-treated, and 2.0% for untreated control cultures. Cells from each culture were pelleted at 100 g for 8 min, resuspended in 5 ml of PEL gradient medium from the top of each preformed gradient (one 95-ml PEL gradient for each culture) with three to four strokes in a loose Dounce homogenizer. Gradients were centrifuged at 2000 g for 20 min in the Wifug centrifuge at room temperature, then harvested, and 3-ml fractions measured for density, cell number (excluding any cell aggregates), and mitotic index. The number of cells in aggregates of over 25  $\mu$  accounted for less than 10% of the total number of cells. The results (Fig. 4, bottom), show that the metaphase-arrested cultures have average cell densities lighter than the control, as expected from the data in Figs. 2 a–c. Fig. 4, top shows the distribution of these mitotic cells in the density gradients. It is apparent from these data that the majority of mitotic phase cells isolated from control cultures fall within a density range of 1.040–1.046 g/cc. The mitotic phase cells after a Colcemid or vinblastine block have a much wider density range of from 1.040 to 1.060 g/cc.

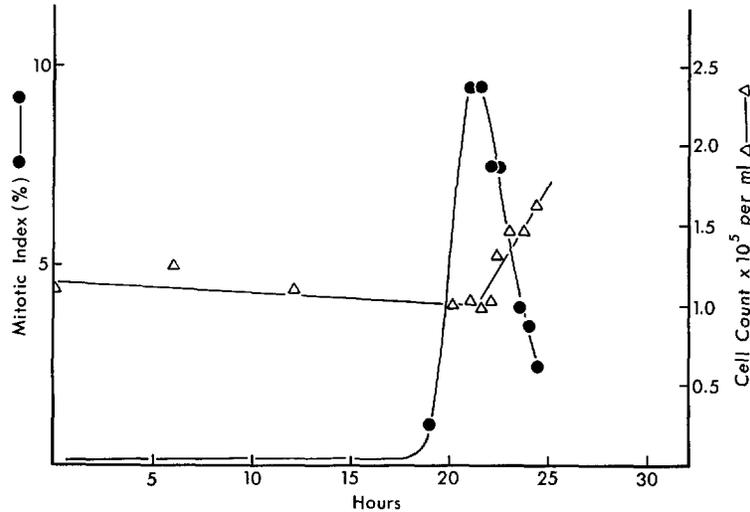


FIGURE 3 Synchronous culture of HeLa cells. The culture was established by separating mitotic phase cells on a PEL density gradient as in Figs. 2*a-c* and subsequent harvest and growth in spinner culture with measurement of cell number and mitotic index.

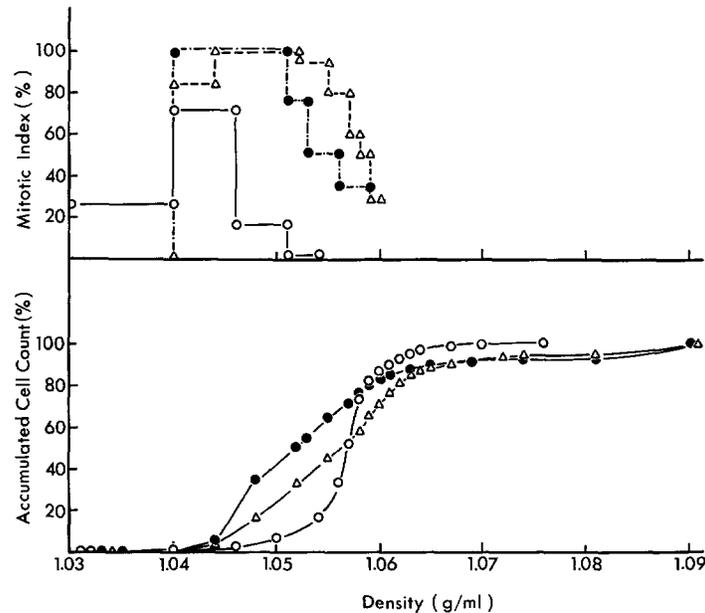


FIGURE 4 Separation of mitotic phase HeLa cells arrested by Colcemid or vinblastine sulfate on a PEL density gradient. (*Bottom*): distribution of cells in the PEL density gradient. Cultures of HeLa cells were treated with Colcemid (0.05  $\mu\text{g}/\text{ml}$ ) or vinblastine sulfate (0.01  $\mu\text{g}/\text{ml}$ ) for 16 hr before centrifugation on a PEL gradient as in Figs. 2*a-c*. Mitotic indexes of culture just before density gradient centrifugation were: control, 2.0%; Colcemid-treated, 68.0%; and vinblastine sulfate-treated, 60.5%. (*Top*): Mitotic index of cells at each density level harvested from the PEL gradient in Fig. 4, bottom. Control,  $\circ$ — $\circ$ ; vinblastine sulfate-treated,  $\bullet$ — $\bullet$ ; and Colcemid-treated,  $\triangle$ — $\triangle$ .

#### DISCUSSION

The PEL density gradient medium was developed as a low-cost, nontoxic medium which would provide a density range useful for cell separation.

Sucrose, used by Sinclair and Bishop (5) and others (6, 7), is inexpensive and nontoxic in low concentrations, but when a denser gradient is needed for analysis of cell density, the osmotic

pressure becomes too high, a factor which will affect the cell size. Colloidal silica has a low osmotic pressure in water even at the highest concentrations and isosmolar conditions can be achieved by adjusting the proportions of  $2 \times$  Eagle's medium and water in the medium. PEL medium provides enough density so that equilibrium conditions are reached when separating the mitotic growth phase from the majority of cells. The separations in this study are therefore based on differences in cell density and not on cell size, as in previous work (5-7). The same medium has also been used in preliminary experiments to separate KB-cells and L-cells and HEP-2 cells with the same good resolution.

The HeLa cells used in this work and HEP-2 cells studied subsequently sediment to very predictable densities in the PEL gradients under conditions described above, with the mitotic phase cells consistently found at densities between 1.040 and 1.046 g/cc. The densities of mitotic phase cells which have been held in metaphase arrest by Colcemid or vinblastine sulfate are higher than for mitotic cells selected from the heterogeneous population. To explain this effect, it is possible that metaphase cells are more dense than those in other mitotic phases; however a more likely reason is that the greater densities are produced by the Colcemid or vinblastine.

It was also demonstrated that a partially synchronized culture of HeLa cells could be established from mitotic phase cells separated by the PEL gradient technique. Yields of cells collected in this manner are limited by the low number of mitotic cells at any given time in a normal culture (between 2 and 4%). We have successfully separated up to  $3.0 \times 10^8$  HeLa cells on a single 95 ml PEL gradient. The limitation is then the centrifugal and harvesting capacity of the laboratory for the simultaneous processing of the gradient tubes to yield mitotic phase cells.

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