# THE RESOLUTION OF MIXTURES OF VIABLE MAMMALIAN CELLS INTO HOMOGENEOUS FRACTIONS BY ZONAL CENTRIFUGATION

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## ABSTRACT

Large-scale separation of mixtures of mammalian cells was obtained with the A-1X zonal centrifuge rotor and density gradients consisting of Ficoll dissolved in modified Eagle's MEM suspension-culture medium. The cells remained viable as tested by plating efficiency or by motility observed with time-lapse photography. Rabbit thymocyte and HeLa cell mixtures were separated with 99 and 89 per cent purity, respectively. Mixtures of thymocytes and suspension-cultured, human acute leukemia cells (Roswell Park strain LKID) were separated with 93 and 91% purity, respectively. HeLa cells were isolated 92% pure from a mixture with horse leukocytes. A book of charts giving the sedimentation position and velocity versus time of cells in the A rotor under standard conditions of gradient composition, angular velocity, and temperature was prepared with the use of a computer program based on the differential sedimentation equation. The charts are used to estimate the centrifugation time necessary for maximum separation of cells. The success achieved in separating mixtures of cells points to the future possibility of large-scale fractionation of solid tissues, especially tumor tissues, into preparations of viable cells of a single type.

## INTRODUCTION

We are currently developing methods for the preparation of homogeneous fractions of viable whole cells from disaggregated solid tissue, particularly tumor tissue. There is a definite need for the development of such techniques. Much of the newer knowledge of molecular biology, based on the study of homogeneous cell populations such as *E. coli*, is difficult to apply to tissues, especially pathological tissues, because they are usually composed of a mixture of morphologically and functionally different cell types which are present in unknown proportions. A hepatoma, for example, consists of a variable mixture of malignant parenchymal cells, normal parenchymal cells, Kupffer cells, shown by actual count to make up 33% of the total cell population in liver (1), and necrotic cells. In addition, there are proliferating fibroblasts, endothelial cells, and a variety of inflammatory cells. To us, the isolation of a pure fraction of viable tumor cells is an essential prerequisite if more than relatively gross and qualitative biochemical studies are to be made of the average tumor at the subcellular level.

The problem of preparing pure, whole cell

fractions from a solid tissue falls into two parts: (*a*) disaggregating the tissue into a mixture of suspended single cells, and (*b*) separating the pure cell fractions from the mixture. This report describes our progress with the second and more difficult part as approached by the method of zonal centrifugation in an isoosmotic density gradient. We have used an experimental system consisting of mixtures of cells prepared from rabbit thymocytes, suspension-cultured human acute leukemia cells (strain LK1D from Roswell Park Memorial Institute, Buffalo), suspension-cultured HeLa cells, and horse leukocytes. Centrifugation was done in the A-IX zonal centrifuge rotor designed by Anderson (2), with a gradient solution composed of Ficoll, a synthetic sucrose polymer of average molecular weight 400,000, dissolved in commercially available suspension-culture medium. Mixtures of thymocytes and human acute leukemia cells constituted a model suspension representing the peripheral blood buffy coat or disaggregated lymph nodes of a patient with acute leukemia. Mixtures of thymocytes and HeLa cells represented a model for disaggregated lymph nodes containing metastatic carcinoma cells.

## MATERIALS AND METHODS

### *Preparation of Gradient Solutions*

The standard solvent used throughout to prepare Ficoll solutions was "SMEM," which consisted of Eagle's minimum essential medium with glutamine plus Earle's salts without calcium or bicarbonate and with 10 times the usual amount of phosphate ("spinner salts"). SMEM was obtained in powder form from Grand Island Biological Co., Grand Island, N. Y. After the powder was dissolved, the pH was adjusted to 7.0 with 1 N NaOH. Ficoll was obtained from Pharmacia Fine Chemicals Inc., New Market, N.J.

### *Preparation of Single-Cell Suspensions*

RABBIT THYMOCYTES: The thymuses of 3-month-old New Zealand white rabbits were minced with fine scissors in 10 volumes of cold 50:50 fetal calf serum:SMEM solution, and the released cells were strained through Nitex nylon cloth, pore size 20 $\mu$ (Ernst Tobler, Inc., New York). Cell counts were adjusted to 100 million cells per milliliter, an equal volume of cold 15% DMSO (dimethyl sulfoxide) in SMEM was added, and the cells were viable-frozen in a Linde (Linde Division, Union Carbide Corporation, New York) BF-3 apparatus at a cooling rate of 1°C per minute. The cells were stored over liquid nitrogen in screw cap vials, 200 million cells per vial, until needed. Viability after frozen storage was over 80% as determined by the trypan blue dye exclusion technique (final dye concentration of 0.17%).

CULTURED HUMAN ACUTE LEUKEMIA CELLS (ROSWELL PARK STRAIN LK1D): These cells were kindly provided by Dr. George Moore, Roswell Park Memorial Institute, Buffalo, N.Y. They were supplied viable-frozen in 10% DMSO in SMEM, 50 million cells per milliliter, 200 million cells per vial. Viability after frozen storage was over 90%.

HELA CELLS: Suspension-culture strain S3 was grown in SMEN with 5% horse serum. Cells were harvested by centrifugation and viable-frozen in 7.5% DMSO in SMEM at a concentration of 50 million cells per milliliter. Viability after frozen storage was over 90%.

HORSE LEUKOCYTES: Blood from jugular venipuncture was collected aseptically in 500 ml plastic blood donor bags. ACD (acid citrate dextrose) anticoagulated solution (citric acid 0.8%, sodium citrate 2.2%, dextrose 2.45%), 150 ml per liter of blood, was used. The red cells were allowed to sediment for 1 hr, and the supernatant leukocyte-rich plasma was removed and centrifuged at 200 × *g* for 5 min. The pelleted leukocytes were resuspended in plasma and adjusted to a concentration of 100 million white cells; an equal volume of cold 15% DMSO in plasma was added, and the cells were viable-frozen. Viability after freezing was over 85% as determined by the trypan blue dye exclusion method. The final erythrocyte–leukocyte ratio was 5:1.

The Chinese hamster cells used for plating efficiencies were kindly provided by Dr. Mortimer Elkind, National Institutes of Health, Bethesda, Md.

### *Measurement of Mean Cell Diameters and Densities*

Cell diameters were measured with a Bausch & Lomb Incorporated (Rochester, N.Y.) calibrated ocular micrometer with phase microscopy at a magnification of 1,395. For the measurement of mean cell densities, between 10 and 20 million cells were banded isopycnically in a 10–20% continuous linear Ficoll–SMEM gradient in a Spinco SW-25 rotor for 2 hr at 10,000 rpm. Longer periods of centrifugation did not change the position of the band. 0.5 ml portions of the band were taken with a band-recovery apparatus (Oak Ridge National Laboratory, Tenn.) which consisted of a fine, flat-tipped needle and power-driven syringe assembly mounted on a fine-toothed rack and pinion. This instrument permitted precise placement of the needle tip in the center of the band and accurate band recovery. The density of the band material was measured by the gradient column method with a calibrated density gradient made with kerosene and bromobenzene. A more con-

venient method of band recovery and mean cell density determination was developed in later work. A conical brass "tapping cap" was made which fitted water-tight into the top of the 1 × 3 inch Spinco plastic centrifuge tubes containing the gradient with isopycnically banded cells. 50% w/w sucrose solution was introduced through a puncture into the bottom of the tube with a large bore needle connected to a Sigma peristaltic pump (Sigma Chemical Company, St. Louis, Mo.). The gradient with banded cells was slowly expelled upward through the cap and attached tubing and collected in 0.5 ml fractions. The assembly is shown in Fig. 1. The refractive index of each fraction was determined, and the density was obtained by comparison with previously calibrated solutions. A cell count was done on each fraction with a Coulter electronic cell counter. The mean cell density of the sample was taken as the intersection of the peak of cell counts with a line connecting the linear plot of density values on each side of the peak.

### *Measurement of Cell Viability*

The effect on cell viability of centrifugation in Ficoll–SMEM density gradients was determined by plating efficiencies in the case of Chinese hamster cells and by motility observed with time-lapse photography in the case of HeLa cells and LK1D cells. For time-lapse photography, cells suspended in SMEM with 5% fetal calf serum were placed in Sykes-Moore chambers and overlaid with sterile dialysis tubing, which flattened the cells against the chamber floor and kept them in the same optical plane. Exposures at four frames per minute for 2 hr was sufficient to determine the presence or absence of motility.

### *Measurement of Refractive Index, Density, and Viscosity of Ficoll-SMEM Solutions*

A series of Ficoll–SMEM solutions of accurately known concentration (% w/w) were prepared and assayed for the following: refractive index, with a Bausch and Lomb refractometer; density, with 25-ml calibrated glass pycnometers (Fisher Scientific Company, Pittsburgh, Pa.); and viscosity, with an Oswald viscometer (Fisher Scientific Company). All measurements were made at 20°C. The results are shown in Table I.

### *Zonal Centrifugation of Cell Mixtures*

The A-IX zonal centrifuge rotor (2) contains a Plexiglas and aluminum-reinforced flat cylindrical chamber of 1.3 liters capacity which measures 1.5 cm in height and 17.5 cm in radial dimension. The axis of the cylindrical chamber coincides with the axis of revolution. A linear Ficoll–SMEM density gradient was pumped into the rotor while it was revolving at 500 rpm. The speed of revolution was sufficient to stabilize the gradient as the rotor filled. After the gradient had been installed, the sample of mixed cells in suspension was pumped to the center of the rotor to form a cylindrical band around the axis of revolution. This was displaced radially with 90 ml of an overlay solution. The angular velocity was then

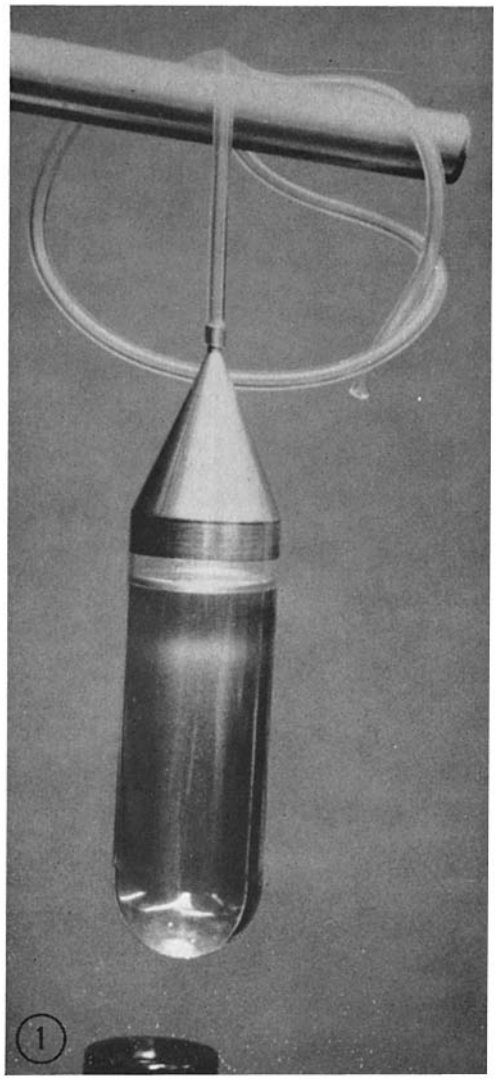


FIGURE 1 Tapping cap assembly fitted to plastic centrifuge tube containing Ficoll–SMEM gradient with isopycnically banded HeLa cells. Heavy sucrose is introduced into the bottom of the tube through a large bore needle connected to a peristaltic pump. The gradient is slowly expelled upward through the cap and collected in 0.5 ml fractions.

TABLE I

*Refractive Index, Density, and Viscosity of Ficoll—SMEM Solutions*

| Weight fraction of Ficoll in SMEM | Refractive index | Equivalent sucrose concentration* | Density at 20°C | Viscosity at 20°C in centipoises |
|---|---|---|---|---|
| | | % w/w | | |
| 0.10 | 1.34 | 11.30 | 1.03 | 8.31 |
| 0.12 | 1.35 | 13.40 | 1.04 | 12.36 |
| 0.14 | 1.35 | 15.60 | 1.05 | 17.62 |
| 0.16 | 1.35 | 17.50 | 1.06 | 24.00 |
| 0.18 | 1.36 | 19.40 | 1.07 | 33.28 |
| 0.20 | 1.36 | 21.40 | 1.07 | 45.38 |
| 0.22 | 1.36 | 23.45 | 1.08 | 61.36 |
| 0.24 | 1.37 | 25.40 | 1.09 | 80.49 |

* Concentration of sucrose in water having the same refractive index.

increased to 1,000 rpm; this caused the different cell types in the sample to enter the gradient and form separate, concentric bands at positions which depended on their diameter and density. At the end of the centrifugation period, the bands were collected in separate fractions of 20 or 40 ml each as the gradient was pumped out of the rotor from the center by displacement with a dense solution pumped to the rotor edge. The final dimensions of the gradient were usually as follows: overlay, 90 ml; sample, 40 ml; gradient proper, 1000 ml; cushion, 160 ml. Each fraction was mixed by inversion and a 5 ml aliquot was taken for observation by phase microscopy and for cell counting on a Coulter electronic cell counter. The remainder of the fraction was diluted with an equal volume of SMEM medium, and the suspended cells were collected by centrifuging for 5 min at 10,000 rpm. The supernatant fluid was aspirated, and the final trace of supernatant was completely removed from the cell pellet with a fine-tipped capillary pipette. The cell pellet was then resuspended in 0.1 ml of fetal calf serum, smeared on microscope slides, air dried, and stained with Wright's stain for differential cell counting.

## RESULTS AND DISCUSSION

### *Physical Basis for the Resolution of Mixtures of Whole Cells by Zonal Centrifugation*

#### Sedimentation Behavior of Viable Cells in Ficoll–SMEM Density Gradients

The basic theoretical considerations which have been used in physically analyzing the sedimentation characteristics of subcellular particles, such as lysosomes and mitochondria (3), polyribosomes, nuclei, and glycogen particles (4), may also be applied to intact, viable, mammalian cells The ideal particle treated by classical sedimentation theory is spherical, rigid, smooth, uncharged, unhydrated, and constant in size and density (therefore osmotically inactive). If such a particle, of diameter $a$ (centimeters) and density $Dp$, is suspended in a medium of density $Dm$ and viscosity $\eta$ (poises) and subjected to centrifugation at an angular velocity of $\omega$ (radians per second), it will assume a constant terminal sedimentation velocity of $v$ (centimeters/second). At this time, three forces are acting on the particle, all along the radius of sedimentation: a centrifugal force, $Fc$; a buoyant force, $Fb$; and Stoke's force of resistance to sedimentation, $Fs$. The dependence of these forces on the variables of the system is as follows:

$$Fc = m\alpha \tag{1}$$

$$Fb = -\frac{4\pi}{3}\left(\frac{a}{2}\right)^3 (Dm)\alpha \tag{2}$$

$$Fs = -3\pi\, av\, \eta \tag{3}$$

where $m$ is mass of the particle in grams, $\alpha$ is angular acceleration in centimeters/second$^2$ (equal to $\omega^2 r$), and $r$ is radial distance of the particle from the axis of revolution in centimeters. Since the particle is sedimenting at constant terminal velocity, the sum of the centrifugal, buoyant, and Stoke's forces can be set to 0, and the equation can be solved for the sedimentation velocity:

$$v = \frac{dr}{dt} = \frac{a^2(Dp - Dm)\omega^2 r}{18\eta} \tag{4}$$

This basic differential sedimentation equation shows how the sedimentation velocity of the particle depends on particle density and, above all, on particle diameter, since diameter occurs as a squared term.

The usual mammalian cell, suspended in SMEM medium containing up to 20% Ficoll, does approximate the ideal particle, described above, in its most important aspects. It is essentially spherical, and its osmotic behavior in the above medium should remain within physiological limits. If the cell membrane is completely impermeable to Ficoll, the resulting colloid osmotic pressure at 20°C of a 20% w/w Ficoll solution should be 9.12

mm Hg, as calculated from the classical van't Hoff equation. This value is within the normal range of 5–30 mm of Hg found in human intercellular fluid, lymph, and plasma. Surface irregularities, surface charge, and surface hydration undoubtedly do give rise to hydrodynamic and electrodynamic forces, but these forces should be practically negligible relative to the large centrifugal force generated by the mass of the cell.

For controlling the conditions of centrifugation so that maximum cell separation could be achieved, it was deemed necessary to integrate equation 4, so that curves could be prepared of the distance sedimented versus time of cells of any given diameter and density. For this purpose, the conditions of centrifugation were standardized at a 10–20% Ficoll–SMEM gradient, angular velocity of 1,000 rpm, and temperature of 20°C.

With respect to the problem of integrating equation 4, $a$ and $Dp$ were constants which could be measured, and it remained to evaluate $Dm$ and $\eta$ as functions of $r$. This was done as follows. The density and viscosity of a series of Ficoll solutions were first measured; the results are shown in Table I. $Dm$ is seen to be a linear, and $\eta$ a nonlinear, function of Ficoll concentration. The Ficoll–SMEM gradient which was pumped into the A-IX rotor was linear with volume, $f = kV$, where $f$ is the Ficoll concentration and $V$ is the volume of gradient pumped into the rotor. Since $V$, as a function of radial distance, $r$, from the start of the gradient, $r_0$, is given by simple geometric relationships, the Ficoll concentrations at any radial distance $r$ in the gradient could be obtained from the following equation:

$$f = f_0 + kh\pi(r^2 - r^2_0) - 4khw(r - r_0) \qquad (5).$$

$r_0$ is radial distance to beginning of gradient; $f_0$ is Ficoll concentration, w/w, at $r_0$; $k$ is volume coefficient of the gradient in % w/w per milliliter; $h$ is height of rotor chamber, in centimeters; $w$ is width of each of four vanes in rotor chamber; $f$ is Ficoll concentration, % w/w, at any radial distance, $r$, in the rotor; $r$ is radial distance in centimeters, measured from the axis of revolution. No simple analytical function could be found which described the dependence of viscosity on Ficoll concentration. Therefore, equation 5, $Dm$ $(f)$, and $\eta$ $(f)$ were entered into a computer program which performed a numerical integration of equation 4. The data from Table I relating viscosity to Ficoll concentration were entered as a look-up program which gave interpolative values of $\eta$ $(f)$ to within 1% error. $Dm$ $(f)$ was entered as a simple linear function. For any value of $r$, $f$ was determined (equation 5), which in turn fixed the value of $Dm$ and $\eta$ for that value of $r$. A typical computer printout of sedimentation distance versus time for a cell with a diameter of 7.5 $\mu$, and a density of 1.064 is shown in Table II. A book of such tables was prepared over range of values for cell density and cell diameter so that the sedimentation position versus time, and sedimentation velocity versus time, of any given cell could be estimated.

TABLE II

*Sample Page from a Book of Computer Printouts*

Sedimentation distance and velocity versus time for cells over a range of values for cell density and cell diameter.

A-IX ROTOR.

| DIAM | DENSITY | RPM | R(0) | CRITERION |
|---|---|---|---|---|
| 7.5000E-04 | 1.0600E 00 | 1.0000E 03 | 7.5400E 00 | 1.0000E-03 |

| T (HR,MIN,SEC) | R(CM) | DR/DT(CM/HR) | CONC(WT/WT) | DENS(GM/ML) | VISC(POISES) |
|---|---|---|---|---|---|
| 0, 0, 0 | 7.5400E 00 | 4.0070E 00 | 8.5700E-02 | 1.0334E 00 | 6.1694E-02 |
| 0,15, 0 | 8.5065E 00 | 3.7025E 00 | 9.1213E-02 | 1.0356E 00 | 6.9230E-02 |
| 0,30, 0 | 9.3837E 00 | 3.3068E 00 | 9.6822E-02 | 1.0378E 00 | 7.7853E-02 |
| 1, 0, 0 | 1.0833E 01 | 2.5077E 00 | 1.0735E-01 | 1.0419E 00 | 9.6633E-02 |
| 2, 0, 0 | 1.2732E 01 | 1.4060E 00 | 1.2353E-01 | 1.0482E 00 | 1.3206E-01 |
| 4, 0, 0 | 1.4474E 01 | 5.1134E-01 | 1.4075E-01 | 1.0549E 00 | 1.7840E-01 |
| 8, 0, 0 | 1.5437E 01 | 9.2404E-02 | 1.5124E-01 | 1.0590E 00 | 2.0951E-01 |
| 8,30, 0 | 1.5479E 01 | 7.5343E-02 | 1.5171E-01 | 1.0592E 00 | 2.1100E-01 |

Standard conditions: 1,000 rpm; 20°C; 10–20% w/w Ficoll in SMEM; gradient volume, 1,000 ml; position of sample-gradient interface ($r_0$), 7.54 cm from axis of revolution.

### Characteristics of the Rate-Zonal Method of Separating Mammalian Cells by Density Gradient Centrifugation

The rate-zonal method of cell separation may be defined as one which depends on the rate at which cells sediment in a density gradient. Both cell diameter and density, principally the former, are major factors. Consider a mixture of cells *B* and *C* in Fig. 2, which have the same density but different diameters. During centrifugation the cells start off together, separate widely for a time, and then come together again as they approach their isopycnic positions. For achieving optimal rate-zonal separation, then, knowledge of the time at which maximum separation occurs is necessary. The time point of maximum separation is obtained as follows. Consider the difference between the radial positions of cell *B* and cell *C* in Fig. 2 as a function of time *t*:

$$(r_c - r_b) = f(t) \qquad (6).$$

At the time point of maximum separation, the derivative of this function is 0, and the velocities of the two cells are equal:

$$\frac{d}{dt}(r_c - r_b) = 0 \qquad (7)$$

$$\frac{dr_c}{dt} = \frac{dr_b}{dt} \qquad (8)$$

Therefore, the period of centrifugation at which maximum separation occurs is determined by plotting the velocities versus time of the cells in question as given by the computer tables (see Table II). The point at which the curves intersect gives the desired time period for maximum cell separation.

### Characteristics of the Isopycnic-Zonal Method of Separating Cells by Density Gradient Centrifugation

The isopycnic-zonal method of cell separation may be defined as one which depends on cell density only. Consider a mixture of cells *A* and *B* in Fig. 2. These cells have the same diameter but different densities. In this case the cells continue to separate until they reach their isopycnic positions. The computer tables were again useful in giving the time necessary for the cells to come within 1% of their isopycnic positions (see Table II).

In the usual situation, the cells in a mixture differ both in diameter and in density. Comparison of curves of sedimentation position versus time prepared from the computer tables permits a decision as to which method of centrifugation to use and also gives the centrifugation period required to achieve maximum cell separation.

## *Viability of Cells after Exposure to Ficoll–SMEM Solution and after Zonal Centrifugation*

### Effect of Exposure to Ficoll–SMEM Solution

Chinese hamster cells were suspended in 10 and 25% Ficoll–SMEM solution for 1 hr at 20°C with gentle stirring, following which their plating efficiency was determined. Table III shows that their plating efficiency was essentially the same as that of control cells suspended in NCTC 109 with 10% fetal calf serum.

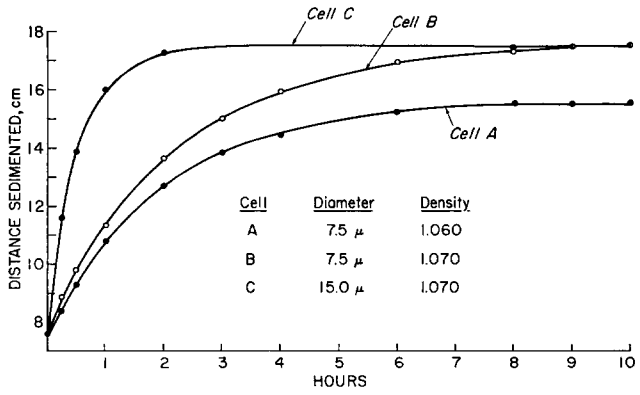


FIGURE 2 Differences between rate zonal and isopycnic zonal centrifugation.

TABLE III

*Plating Efficiency of Chinese Hamster Cells after Exposure to Ficoll-SMEM Solution for 1 Hr at 20°C*

| Medium | Colonies appearing per 100 cells plated | Average plating efficiency | Colonies appearing per 300 cells plated | Average plating efficiency | Colonies appearing per 1000 cells plated | Average plating efficiency |
|---|---|---|---|---|---|---|
| NCTC-109 with 10% fetal calf serum | 28 | 22.3 | 79 | 29.6 | | |
| | 19 | | 99 | | | |
| | 20 | | 89 | | | |
| 25% w/w Ficoll in SMEM | 38 30 | 29.1 | 92 | 27.5 | 337 | 33.4 |
| | 28 35 | | 73 | | 346 | |
| | 19 25 | | | | 320 | |
| 10% w/w Ficoll in SMEM | 39 | 38.3 | 98 | 36.1 | 328 | 37.6 |
| | 38 | | 116 | | 519 | |
| | 38 | | 111 | | 280 | |

TABLE IV

*Mean Cell Densities and Diameters of Rabbit Thymocytes, Human Acute Leukemia (LK1D) cells, and HeLa cells*

| Cell type | Mean density | Mean diameter | $\sigma$ | No. of cells counted |
|---|---|---|---|---|
| | | $\mu$ | | |
| Rabbit thymocytes | 1.060 | 6.34 | 0.70 | 500 |
| LK1D cells | 1.070 | 14.4 | 2.7 | 500 |
| HeLa cells | 1.064 | 18.7 | 2.6 | 500 |

### Effect of Zonal Centrifugation

HeLa cells and LK1D cells were sedimented in the A-IX rotor for 1 hr at 1,000 rpm and 20°C in a discontinuous 10–20% Ficoll–SMEM gradient. There was no detectable difference in motility of the cells before and after the sedimentation procedure during a 4 hr period of observation by time-lapse photography.

## *Experimental Separation of Mixtures of Viable Whole Cells*

### Sedimentation Curves of Rabbit Thymocytes, Human Acute Leukemia (LK1D) Cells, and HeLa Cells

Table IV presents the mean cell diameter, measured by ocular micrometry, and the mean cell density, measured as described under Methods, for the rabbit thymocytes, human acute leukemia (LK1D) cells, and HeLa cells used in this study. With these values, the anticipated sedimentation behavior of the three types of cells in the A-IX rotor under the standard conditions discussed previously, as obtained from the computer tables, is presented in Fig. 3. These curves show that the rate-zonal method is best for separating the thymocytes from both LK1D cells and HeLa cells. The centrifugation period given by the tables for maximum separation of thymocytes and HeLa cells was 50 min, and for thymocytes and LK1D cells 60 min.

### Separation of Rabbit Thymocytes and HeLa Cells

A sample consisting of 230 million HeLa cells and 750 million thymocytes in 40 ml of 7% Ficoll–SMEM was processed on the A-IX rotor for 30 min under the standard conditions described previously. Although the centrifugation time for maximum separation given by the computer tables was 50 min, centrifugation for 30 min meant only that separation was less than maximum by tube 1 (40 ml) owing to the large difference in size between the two cells. The results of the separation experiment are shown in Fig. 4. On the basis of the total cell count per tube and the differential cell counts (1,000 cells) done on Wright's-stained smears of the cell pellets from the two peaks, 30% of the thymocytes was recovered 99% pure in tubes 4–6, and 95% of the HeLa cells was recovered 89% pure in tubes 26–28.

### Separation of Rabbit Thymocytes and Human Acute Leukemia (LK1D) Cells

In this experiment the sample consisted of 2.0 billion LK1D cells and 2.0 billion thymocytes in 50

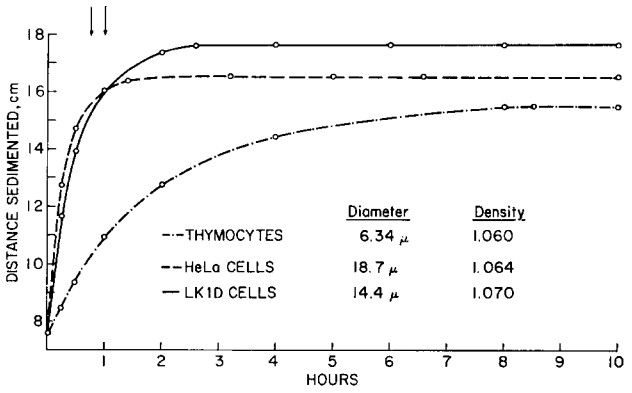


FIGURE 3 Sedimentation curves for thymocytes, HeLa cells, and LK1D cells in a Ficoll density gradient, A-IX rotor, 1,000 rpm.

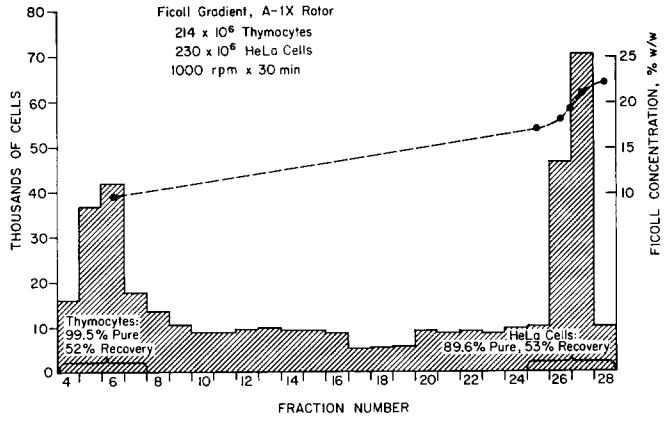


FIGURE 4 Separation of rabbit thymocytes and HeLa cells in Ficoll–SMEM, A-IX rotor.

ml of 8% Ficoll–SMEM solution. The rotor was loaded at 500 rpm, maintained at 1,000 rpm for 1 hr, and unloaded at 500 rpm into 20-ml fractions. The degree of separation of the two types of cells is shown in Fig. 5. 42% of the thymocytes was recovered 93% pure in the first peak, and 66% of the LK1D cells was recovered 93% pure in the second peak. In this case differential cell counts were also made directly on the cell suspensions in each fraction by phase microscopy. Since previous ocular micrometry on each cell type had shown that there was no overlap in the range of cell diameters between the thymocyte and LK1D populations. measurement of the cell diameter was used to assist in the differential count, although the two cell types could easily be distinguished on the basis of morphological differences alone: the thymocytes resembled small lymphocytes whereas the LK1D cells appeared as large malignant lymphoblasts with prominent nucleoli. Differential counts on Wright's-stained smears of cell pellets from each tube were in close agreement with the counts done by phase microscopy. In this experiment the Ficoll gradient was 10–18%. The position of a thymocyte peak in a 10–20% Ficoll gradient would have been at tube 11, according to the computer tables. This position compares satisfactorily with the position of the actual peak in tube 13, if one takes into account the fact that the gradient was slightly shallower.

## Separation of Horse Leukocytes and HeLa Cells

This separation was performed to test the feasibility of separating circulating malignant cells from leukocytes in the peripheral blood of cancer patients. The horse leukocytes exhibited a tendency to aggregate into groups of two to eight cells on standing in SMEM for more than a few minutes. This tendency was virtually eliminated by using SMEM solutions which contained 30% ACD solution, v/v, titrated to pH 6.5, instead of pH 7.0, with NaOH. The 40 ml sample consisted of 2 billion leukocytes and 2 billion HeLa cells in SMEM

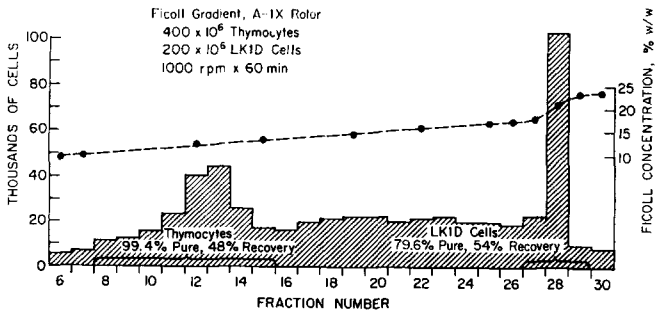


FIGURE 5 Degree of separation of rabbit thymocytes and LK1D cells in Ficoll–SMEM solution, A-IX rotor.

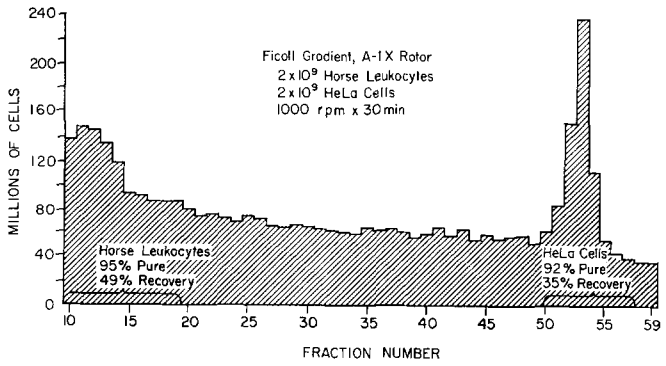


FIGURE 6 Separation of horse leukocytes and HeLa cells, Ficoll–SMEM solution, A-IX rotor.

containing 30% ACD solution, v/v, and 6% Ficoll, w/w. The gradient consisted of 8–16% Ficoll–SMEM solution containing 30% ACD. The loading speed was 500 rpm. After sample loading, the rotor was maintained at 1,000 rpm for 22 min, then unloaded at 500 rpm over a period of 20 min. 20-ml samples were collected. Results are shown in Fig. 6. The peak in tubes 49–56 represents a recovery of 35% of the HeLa cells in a state of 92% purity.

## GENERAL DISCUSSION

The relatively successful resolution of known mixtures of viable mammalian cells into homogeneous fractions by density gradient centrifugation has been demonstrated. These results are encouraging with regard to the potential applicability of the methods described to the general problem of preparing pure fractions of viable whole cells from disaggregated solid tissue, particularly tumor tissue.

The outstanding advantage of the A-IX rotor for separating whole cells is its large capacity. Separations can be done on a large enough scale so that biochemical studies can be more easily performed on the purified cell fractions or on their subcellular components after further fractionation. The large band capacity of the A-IX rotor allowed us to handle up to $4 \times 10^9$ cells without difficulty. For preparative work, the radial geometry of the A-IX rotor eliminates *wall effects* and thus provides another advantage over conventional methods of centrifugation with cylindrical tubes in swinging bucket rotors (4). Wall effects can be appreciable in a cylindrical tube. If $r_1$ is the distance of a band from the axis of revolution at the start of centrifugation and $r_2$ its distance at the finish, the percentage of the band hitting the wall during centrifugation is $100\ (1 - r_1^2/r_2^2)$. In the conventional $1 \times 3$ inch plastic tube in a Spinco SW-25 rotor, in which a band sediments from the top of a 25 ml gradient to the midpoint of the tube, the per cent of the band hitting the wall exceeds 50%.

When large amounts of pure whole cells are not needed, however, we have found that the use of density gradients in swinging bucket rotors has some advantages. Multiple experiments can be performed in a shorter time, and sterility is easier to maintain. The latter factor is of particular importance when cells are isolated for subsequent tissue culture.

In summary, we have shown that mammalian

cells tend to obey the sedimentation equation when they are centrifuged in Ficoll density gradients made up in suspension culture medium, and further that they appear to remain viable for at least 4 hr under these conditions. A satisfactory resolution of mixtures of rabbit thymocytes, human acute leukemia cells, and HeLa cells was obtained. This points to the future possibility of the large scale fractionation of solid tissues, especially tumor tissues, into preparations of viable cells of a single type.

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