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CELL CYCLE SYNCHRONIZATION OF MOUSE LIVER
EPITHELIAL CELLS BY ELUTRIATION CENTRIFUGATION

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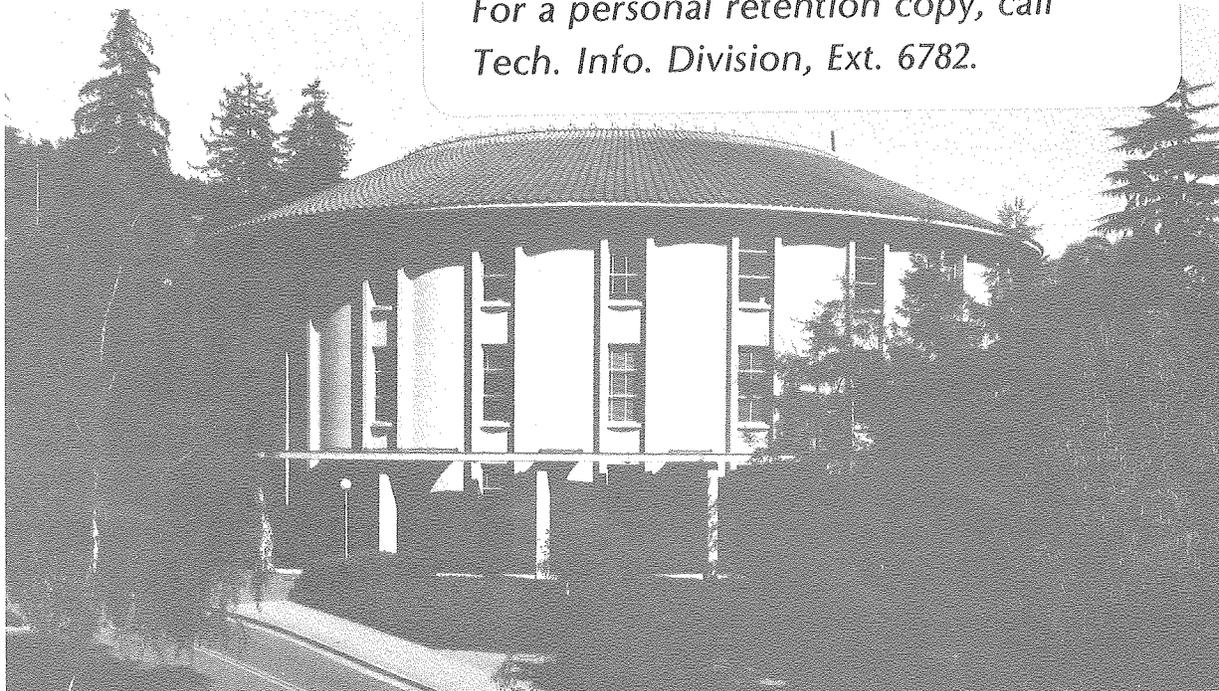
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CELL CYCLE, SYNCHRONIZATION OF MOUSE LIVER EPITHELIAL CELLS

BY ELUTRIATION CENTRIFUGATION

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ABSTRACT

Detailed methods are described for the sorting and cell cycle synchronization by means of centrifugal elutriation of an established mouse liver epithelial cell line (NMuLi). In a comparison between three different elutriation media and between two different temperatures (4° and 20° C), the NMuLi cells were found to be most reproducibly sorted in the cell cycle when run in growth medium in the absence of serum and at the lower temperature. Under these conditions, and using decrements of rotor speed calculated from an empirically derived algorithm as described in the text, an initially asynchronous population (38% G₁, 36% S, and 26% G₂M) was sorted into fractions enriched to 60% G₁, 75% S, and 50% G₂M. Of the cells loaded into the rotor, 30% were lost in the elutriation process, and about 20% recovered as aggregates. The remainder appeared in the various synchronized fractions. Epithelial cells sorted in this manner demonstrated no loss of viability, and upon replating showed significant movement in the cell cycle by 6 hrs post elutriation. The degree of synchronous movement through the cell cycle achieved by elutriation depended on the part of the cell cycle from which the original elutriated fraction came. Cells collected as late S and G₂M moved through the cell cycle with the tightest synchrony.

INTRODUCTION

The mitotic cycle of cells is the focus for a great variety of studies in cell biology, ranging from the control of cellular proliferation to mutation and heredity (Mitchison (1971)). In many cases, the ability to synchronize large cell populations in various cell cycle phases is vital to the study, as has been amply discussed by previous authors (Meistrich, Meyn, and Barlogie (1977); Mitchell and Tupper (1977)). In our studies of the action of chemical carcinogens upon the mitotic cycle of the mouse liver epithelial cell line, NMuLi, we have sought a way to obtain synchronous or cell cycle phase enriched populations of 0.5×10^8 - 1.0×10^8 cells for various biochemical studies (Becker and Bartholomew (1979)). Meistrich et al. (1977) and Mitchell and Tupper (1977) showed that elutriation centrifugation provides reasonably good synchrony with minimal perturbation of cellular functions in the process, and for these reasons we have chosen to implement an improved elutriation centrifugation system for our NMuLi cells. To our knowledge this is the first adaptation of elutriation centrifugation to epithelial cells.

METHODS

1. Cell Culture Technique

Monolayer cultures of the established epithelial cell line NMuLi, derived from the livers of Namru mice by Owens et al.(1974), were seeded in 100 mm culture dishes(Falcon Plastics, Oxnard, Calif.) in Eagle's Minimal Medium(GIBCO, Grand Island, N. Y.), denoted here as MEM, with 10% donor calf serum (Flow Laboratories, Rockville, MD), and allowed to reach saturation density(1.3×10^5 cells per cm^2). At 60 hrs prior to elutriation, the cells were transferred at a 1 to 10 dilution into roller bottles (Falcon Plastics, Oxnard, Calif.). Transfer of cells from the dishes was done by washing the monolayers once with saline GM($1.5 \text{ mM Na}_2\text{HPO}_4$, $1.1 \text{ mM KH}_2\text{PO}_4$, 1.1 mM glucose , and 0.14 M NaCl , at pH 7.4) followed by trypsinization for 3 min at 37° C with DISPO(saline GM containing 0.5 mM EDTA and 0.1 mg/ml crystalline trypsin(GIBCO, Grand Island, N. Y.)). Trypsinized cells were removed from the surface of the dishes into NEUT (saline GM containing 6.3 mM MgSO_4 , 1.1 mM CaCl_2 , 0.2 mg/ml soybean trypsin inhibitor(GIBCO), 0.01 mg/ml DNase(Worthington, Freehold, N. J.), and 0.1% bovine serum albumin(Sigma, St. Louis, Mo.).

For elutriation, medium was aspirated from 8 roller bottles and the cells were washed once with saline GM and trypsinized as described above to yield 6.0×10^8 cells in 40 ml of suspension medium for loading into the elutriator.

Prior to loading the cells, the elutriation system was flushed with 70% ethanol, followed by rinsing with sterile suspension medium.

2. Elutriation

The Beckman JE-6 elutriator rotor and associated J-21 centrifuge formed the heart of the system illustrated in Figure 1. The centrifuge rotation speed control was modified to use a ten-turn potentiometer for greater precision, as we chose to use decrements of rotor speed as the means of removing sub-populations from the separation chamber. Cell suspension medium was continuously driven through the system by peristaltic pump (Cole Parmer Masterflex, with 7014 head), and the sample was loaded through a post-pump valve at 10 ml/min via a syringe drive (Orion Instruments, Cambridge, Mass.). Fluid from the pump passed through a bubble trap prior to traversing a triflat flow meter (5-60 ml/min range, Manostat, New York, N. Y.), from which it passed directly into the elutriator rotor. The bubble trap also served to damp out the peristaltic pressure variations.

Upon exit from the rotor, fluid passed through a Beckman DB Spectrophotometer equipped with a custom-built 4 cm pathlength flow cell capable of sustaining flow rates in excess of 500 ml/min (for flushing purposes). At an illumination wavelength of 600 nm, the optical detection system easily sensed cells emerging from the rotor in concentrations of 2.0×10^3 cells/ml or greater. A valve then

enabled either collection or disposal of the effluent, the desired subpopulation being aseptically collected via a specially designed sterile transfer assembly(Figure 2).

Cells were routinely loaded with rotor speed set sufficiently high (typically 3500-4000 revolutions per minute for a counterflow rate of 25 ml/min) to minimize the washing out of whole cells. Speed decrements began from this initial loading condition, and the spectrophotometer was adjusted to 100% transmittance under these conditions. The set of speed decrements for obtaining the desired cycle phase enrichments in the fractions was determined by a modeling routine, described below, calculated during the experiment by means of a programmable calculator.

3. DNA Histograms

Cell cycle distributions of all cell populations, both before and after elutriation, were obtained by staining the cells using the propidium iodide technique described by Crissman and Steinkamp (1973) and analyzed in a flow cytometer (Holm and Cram (1973)). A Spectra Physics Model 171 argon ion laser provided a 2.0 watt excitation beam at 488 nm wavelength. Individual histograms were normalized to constant total cell count, and gain-shifted to place the G_1 peak mode in channel 100, to facilitate visual comparison.

RESULTS

Multiple tests on NMuLi cells showed that good cell cycle enrichment required that the cells be suspended in cold (4° C) growth medium without serum. Poor results were obtained for samples of less than 1.5×10^8 or more than 8.0×10^8 cells, so this range was used in our experiments.

Of the cells loaded into the rotor, we were able to account for only 70-80%, including some 10-20% that showed up as aggregates in the last fraction collected. Reconstituted experiments, in which equal volumes of each fraction were pooled to reconstruct the initial pre-elutriated population, revealed no cycle selectivity in loss of cells during the elutriation process.

Examination of elutriated fractions by microscope showed them to be virtually free of cell aggregates and debris, indicating another advantage of the elutriation technique; i.e., "clean-up" of the sample populations. DNA histograms of collected fractions consistently showed far less low-channel noise (debris) than their pre-elutriation parent populations, and much less evidence of aggregates was apparent in the respective histograms. Cellular debris was easily detected by the spectrophotometer when 260 nm illumination was used, and the effluence of debris consistently occurred at rotor speeds far too high to permit the exit of whole cells, and subsided within 10 to 15 min after all the sample cells had been loaded into the chamber. Microscopic

examination of this collected effluent revealed a large assortment of cellular fragments, which go virtually undetected at 600 nm because the suspended particles are too small to significantly scatter light at these wavelengths.

From one trial to the next, G_1 cells (the first significant fraction detected) eluted from the rotor at varying sedimentation rates equivalent to 5-9 mm/hr at unit gravity, reflecting volumetric or density variations which we found difficult to control experimentally. However, the ratio of G_2M cell sedimentation rate to that of G_1 cells proved sufficiently constant to be used to obtain reproducible fractions enriched in G_1 , G_1 -early S, S, late S- G_2M , and G_2M phases, respectively.

Speed decrements were calculated by expressing the relationship between rotor speed (W), counterflow rate (F) and relative sedimentation rate (RS) as

$$W=k(F/RS)^{1/2}$$

where k has been determined empirically to be 1.93 for NMuLi cells, W is in units of thousands of revolutions per minute (KRPM), F is in ml/min, and RS is in mm/hr equivalent at unit gravity. To apply the model, the sedimentation rate of the G_1 cells was determined for a fixed flow rate at the highest rotor speed at which the optical transmittance dropped below 0.6 at 600 nm, corresponding to about 3,000 cells/ml. These cells were assigned a relative sedimentation rate (RS) of 1.0, and subsequent rotor speed settings

were calculated from the model by inserting the RS values covering a given desired range, typically 1.0 - 1.2, 1.9 - 2.1, and 2.5 - 2.7 for enrichment of G_1 , S, and G_2M fractions, respectively. These values were found to work best when 600-800 million cells were loaded, and to require adjustment for different sample sizes.

DNA histograms of the parent and elutriated populations collected in a typical run are shown in Figure 3. These cells were replated upon collection, and each of the different cell cycle phase enriched fractions showed movement in the cell cycle within 6 hrs post seeding (see below). Population doubling times for the replated cells were about the same as for non-elutriated NMuLi cells in these culture conditions: 14-16 hrs. The particular replat experiment for which this elutriation was done required a very large G_1 enriched sample population, and we consequently chose to sacrifice some synchrony in order to collect enough cells. Other runs have yielded virtually pure G_1 NMuLi cells from initially asynchronous populations.

In a test of the stability of this method, parallel batches from the same parent cell population were run at two different counterflow rates (15 ml/min and 25 ml/min). Fractions were collected at the same relative sedimentation rates in each case, and their DNA histograms showed them to be virtually identical in cycle phase enrichment, indicating that the method is independent of flow rate for this cell

system.

When elutriated cells were reseeded in fresh medium containing fresh serum the cells moved through the cell cycle as demonstrated in Figure 4. The DNA histograms of cells harvested at various times after reseeded showed complex kinetics. The cells in the S1 fraction (containing mostly G_1 cells) moved through S with biphasic kinetics. Initially cells moved into S with a broad distribution followed at 10 hr by a wave of cells moving out of G_1 . Elutriated fractions S4, S5, and S6 contained fewer G_1 cells than S1; and cells from these fractions did not exhibit biphasic kinetics. Cells from the S1 fraction had become asynchronous in their growth by 23 to 28 hrs; whereas, cells from the other fractions maintained some degree of synchrony even after 2 generations.

DISCUSSION

If biological cells were spherical in shape, they would obey Stokes' Law, i.e.,

$$sv = (2/9) \times (g/n) \times (\text{densdif}) r^2$$

where sv = sedimentation velocity (absolute), g = gravitational or centrifugal force, n = viscosity of the medium, densdif = (particle density - medium density), and r = particle radius. By this relation, one would predict that cells possessing a given volume (at the same density) would sediment at about 1.6 times the rate of cells possessing half that volume. In our system the G_2M cells are sedimenting at 2.5 to 2.7 times the rate of G_1 cells. The fact that we find G_2M cells sedimenting at rates far greater than Stokes' Law would allow can be accounted for by any or all of the following reasons:

- 1) Shape, surface roughness and deformability of cells, and hence their hydrodynamics, differ greatly from those of rigid spheres.
- 2) Short-term aggregation of cells within the chamber may result in different hydrodynamic drag forces acting upon them.
- 3) Viscosity of the medium in the separation chamber is a function of the number of cells/ml in the chamber, among other factors.

The most likely explanation for our results is that

biological cells do not behave as rigid spheres.

The persistent presence of G_1 cells in all cell fractions implies that there is a wide variation in sedimentation rate among G_1 cells. This phenomenon can probably be extrapolated to cells in all phases of the cell cycle. Very little is known about the hydrodynamic properties of mammalian cells and essentially nothing is known about how these properties vary throughout the cell cycle. Scanning electron microscopy studies have indicated that the surface of cells is very complex and that structures such as microvilli and blebs do appear and disappear throughout the cell cycle (Porter, Prescott, and Frye (1976)). In view of these complexities it is not surprising that such complex cells as mouse liver epithelial cells have not been sorted into discrete cell cycle phases by elutriation. Our observation that greater synchrony can be achieved by elutriating in the cold probably relates to the reduced dependence of the hydrodynamic nature of the cells on the surface architecture.

Cell kinetics after reseeding the elutriated fractions indicated very little damage caused by the process. A comparison of the data from fractions containing mostly G_1 cells with the fractions from later in the cell cycle allows us to speculate on how cycle progression is regulated. The biphasic nature of the movement of the fraction enriched in G_1 suggests that these cells obey the transition probability

model for cell cycle regulation (Smith and Martin (1973); and Brooks, Bennett, and Smith (1980)). The S1 fraction contained cells with G_1 DNA content that were progressing through G_1 to S as well as cells that were in a non-proliferative state. As has been described by Brooks et al. (1980), these non-proliferative cells when reseeded into medium containing high serum would begin to proliferate with a long lag for entrance into S. Thus, the biphasic kinetics would be created by the immediate movement of proliferating cells toward and through S followed by the later movement of stimulated cells.

The lack of such complex kinetics in populations containing few G_1 cells probably reflects the lack of a random transition step in the S, G_2 , or M phases of the cell cycle for these cells. It is also of interest that cells sorted into S, G_2 , or M maintain synchrony longer than G_1 sorted cells. Even though all of these populations have passed through G_1 which contains the random transition step (Brooks et al. (1980)). The high serum level used in the reseeded can not explain totally the relative decay in synchrony since the S1 fraction was also seeded in high serum. The faster decay in synchrony for cells in this elutriated fraction may be due to such things as a faster utilization of serum components by cells stimulated from non-proliferation, or a "memory" by cells such that cells recently stimulated from non-proliferation are more likely to re-enter the non-proliferative state (G_0).

Several improvements in the instrumentation would greatly facilitate the use of elutriation in cell cycle kinetic studies. We used a high (25 ml/min) counterflow rate to allow a correspondingly higher rotor speeds. For NMuLi cells the rotor speeds were typically 1.4 to 1.7 KRPM. We preferred these higher rotor speeds because at lower rotor speeds reproducibility and stability of the Beckman J-21 centrifuge speed control drops considerably resulting in "hunting" by the servo speed control with consequent improper removal of cell populations from the separation chamber. Also, better servo regulation of counterflow rate would stabilize against flow variations due to resistance differences along the "dispose" versus the "collect" branches of the system (see Figure 1) and those arising during the separation due to removal of cells from the chamber (and consequent viscosity change). The present method is insensitive to the total loaded sample size because of the arbitrary setting of its detection threshold to 0.6 transmission through the spectrophotometer. This has some draw backs:

When large sample populations ($>5 \times 10^7$ cells) are loaded, some level of leakage of cells from the rotor is always present, thus making ambiguous the identification of the rotor speed which corresponds to the first G_1 cells. Some of this ambiguity would be eliminated by the use of an estimate of the total size of the loaded population, and by defining G_1 cells to be

those, which began to exit after the first $n\%$ of the total have been removed: n could be determined empirically for each cell line.

Also, since replating is often involved as a subsequent step, it would be highly desirable to know what rotor-speed decrement will result in a given desired number of cells emanating from the rotor, so that plating density requirements can conveniently be met. The method could perhaps be based on the spectrophotometer reading, with some form of predictor-corrector scheme used to start decreasing rotor speed, and cease decrementing when the appropriate number of cells has exited.

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CONCLUSIONS

We have developed a reproducible scheme for selecting partially synchronous subpopulations of the mouse liver epithelial cell line, NMuLi, using centrifugal elutriation in conjunction with a model relating relative sedimentation rate to cell cycle phase. The method produced population fractions enriched in distinct cycle phases from an initially asynchronous pre-elutriated population, and the fractions show neither loss of viability nor major perturbation in their progression through the cell cycle. Improvements are to be expected in operational technique and in predictive modeling of the relation between cell cycle phase and absolute or relative sedimentation velocity for the cells in question.

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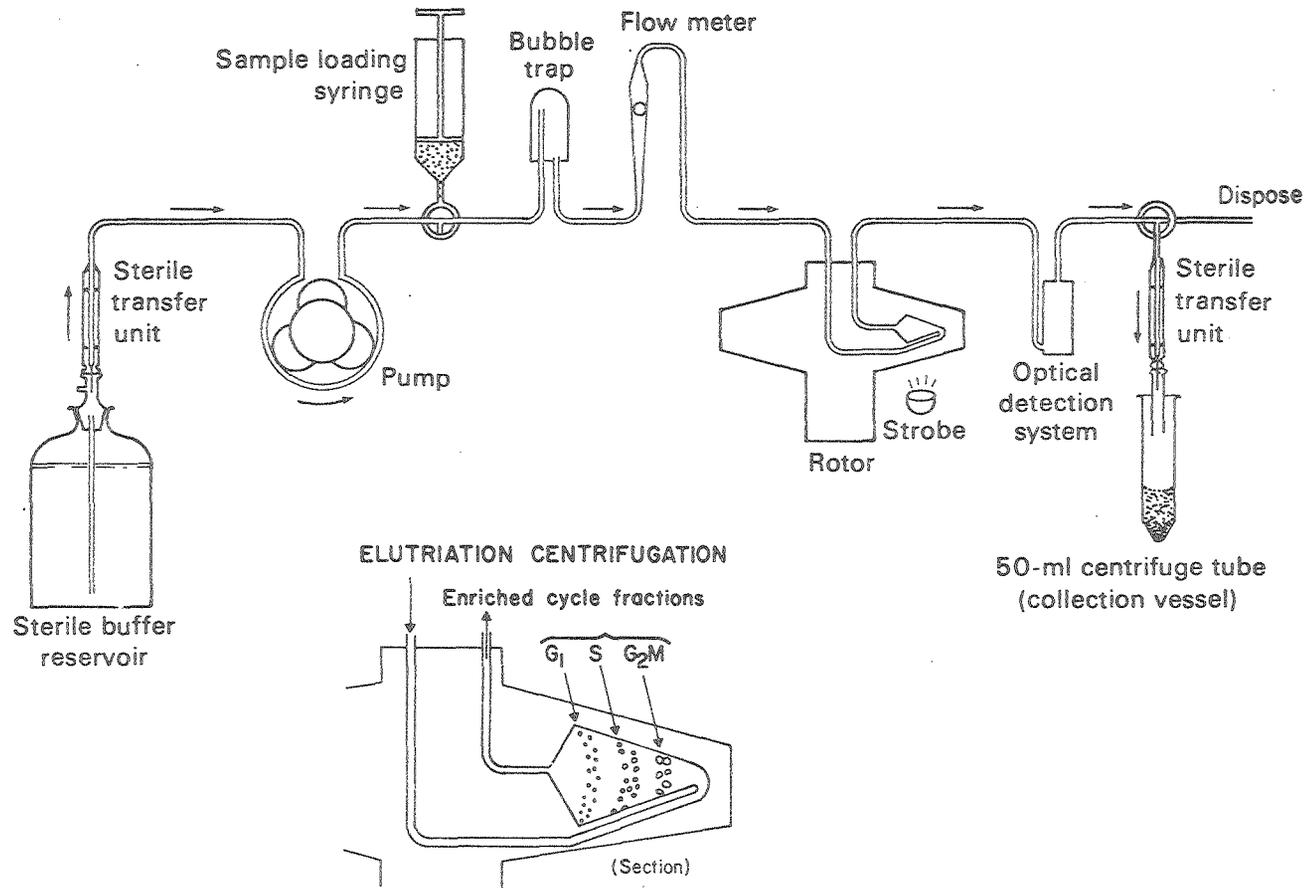
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FIGURE LEGENDS

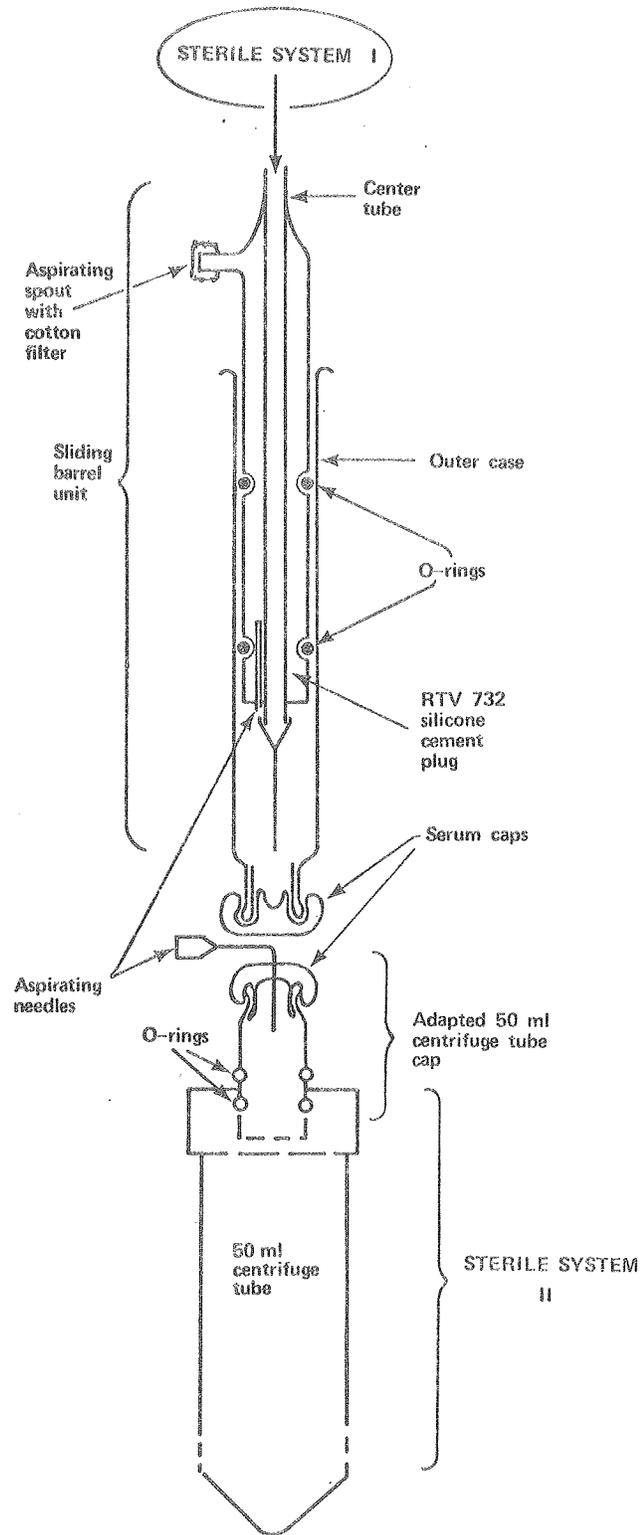
1. Schematic Diagram of Elutriation Centrifugation System.
2. Sterile Transfer Device. To transfer cells from the elutriator (sterile system I) to a collection vessel (sterile system II), the two serum capped units are forced together and the needle driven through both caps by downward pressure on the inner tube of the sliding barrel unit. Aspirating needles maintain constant pressure during the transfer procedure. After transfer, the inner tube is withdrawn while the caps are still pressed together so that no contact occurs at any time between the needle and the atmosphere (patent applied for).
3. Cell Cycle Distributions After Elutriation of a Growing Population. Original population (upper left) was sorted into 8 collected fractions at the relative sedimentation (RS) rates indicated. RS = 1.0 was assigned to the first cells leaving the rotor (details given in text).
4. Kinetics of Cell Cycle Transit of Elutriated Fractions. NMuLi cells after elutriation were seeded in fresh MEM containing 20% donor calf serum. Samples were harvested beginning 6 hrs after seeding and prepared for analysis of cell cycle distribution by flow cytometry. The numbers in the upper right corner denote the time in hours after seeding that the sample was harvested. S1, S4, S5, and S6 are the same as in Figure 3 and refer to the elutriation fraction. All DNA histograms are normalized to the same area and G₁ peak position.

SCHMATIC DIAGRAM OF LCB ELUTRIATION CENTRIFUGATION SYSTEM



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Figure 1
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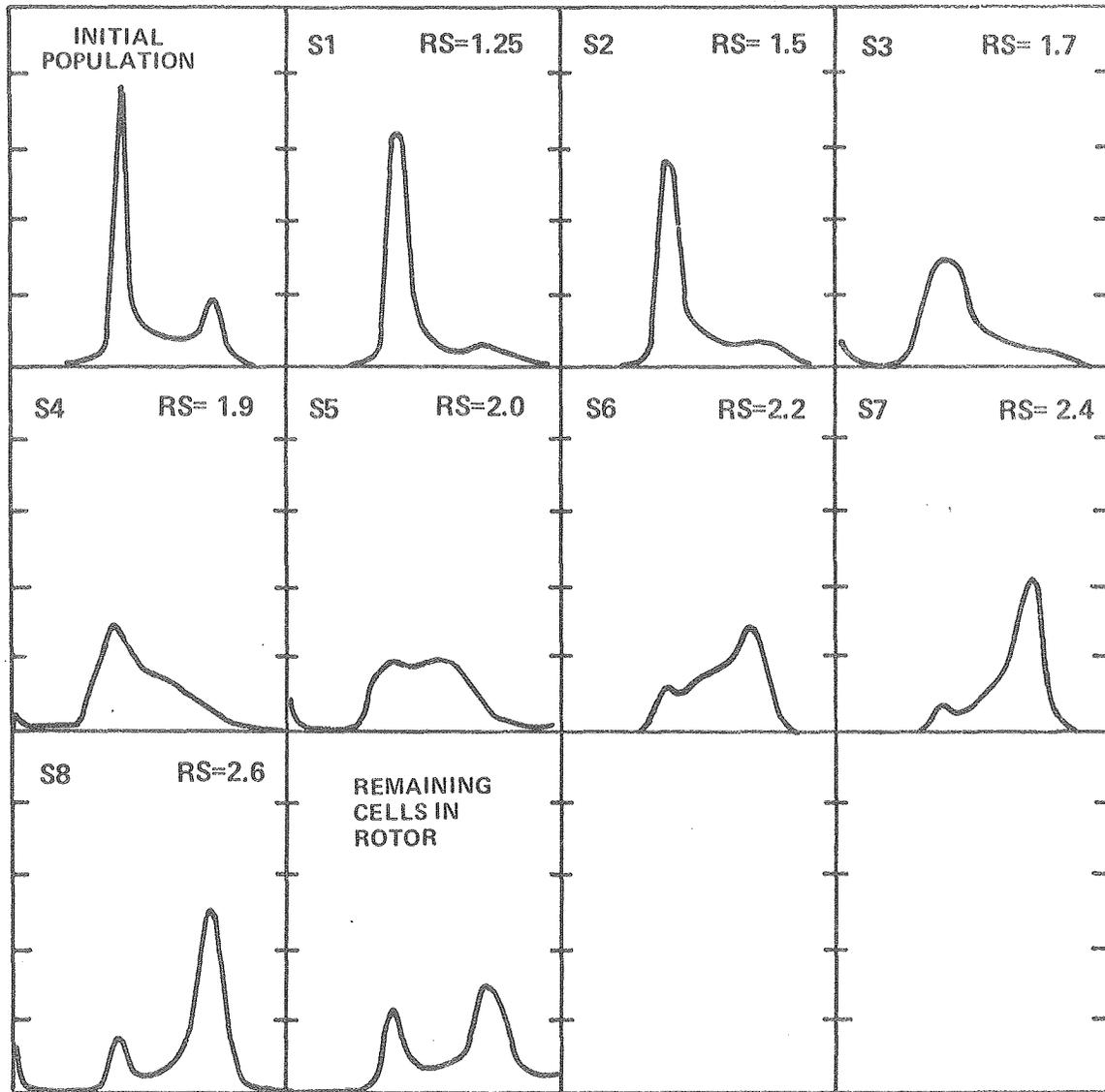


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Figure 2

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Elutriation fractions from NMuLi epithelial cells normalized DNA histograms

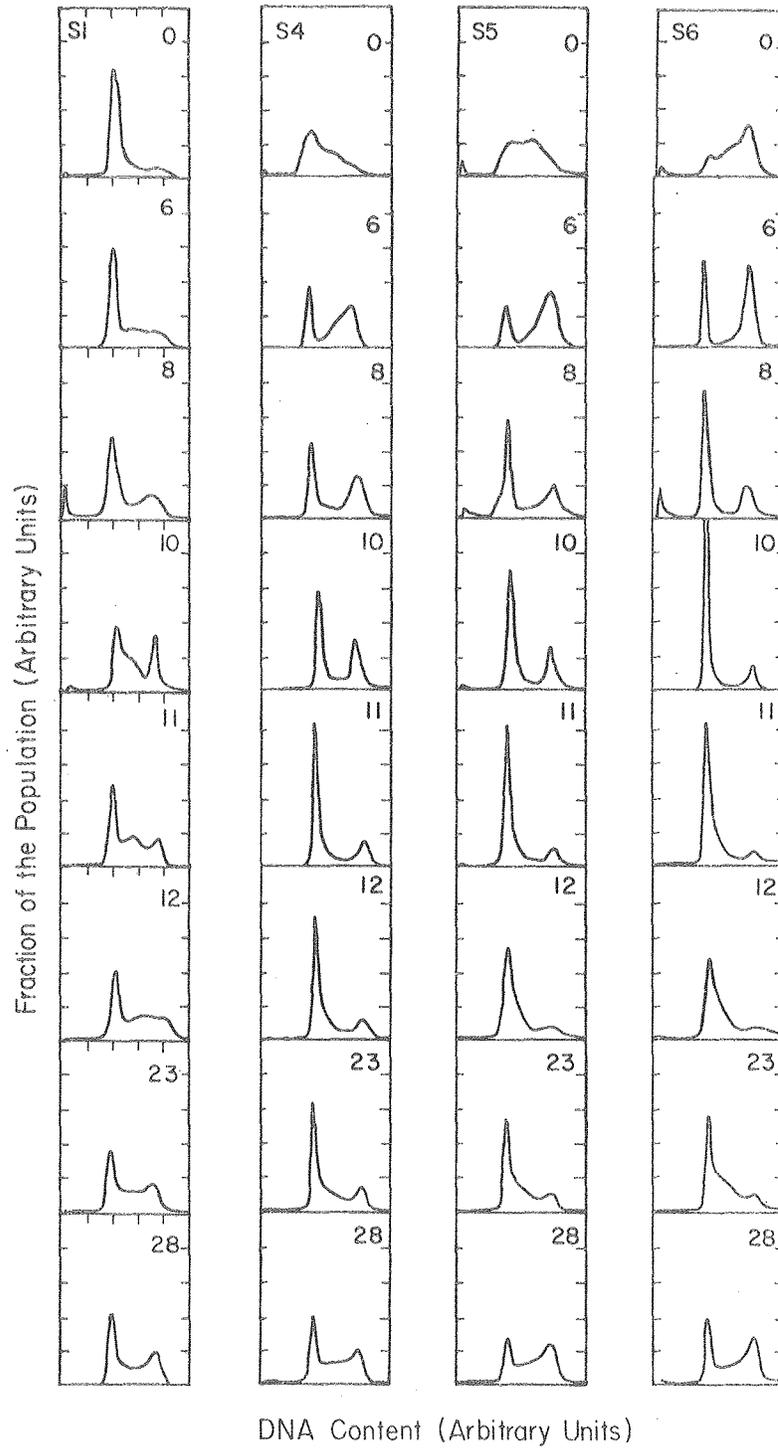


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Figure 3

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Cell Cycle Kinetics of Elutriated Fractions



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Figure 4

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