Equilibrium Density-Gradient Procedure for Selection of Synchronous Cells from Asynchronous Cultures

Abstract. *A reproducible isopycnic technique, in which linear density gradients of Ficoll are used, was developed for selecting synchronous daughter cells from asynchronous cultures of* Chlorella *for biochemical studies of the cell cycle.*

Differential centrifugation of unicellular microorganisms (*1*) and animal cells (*2*) in density gradients has been used to select synchronous daughter cells from asynchronous populations for studies of the cell cycle. These gradients have been designed to stabilize the column of liquid in the centrifuge tubes and have not been sufficiently dense to behave as isopycnic gradients. In rate separation procedures the volume and concentration of the cell suspension placed on the gradient, as well as the time of centrifugation, greatly influence the separation process, and therefore difficulty often is encountered in obtaining daughter cell suspensions of reproducible homogeneity and of sufficient concentration for cell cycle experiments which require mass cultures.

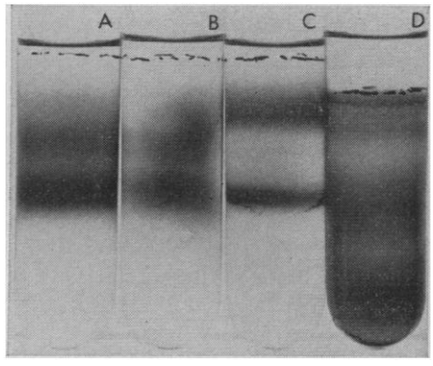

Fig. 1. Centrifugation of asynchronous cells of *Chlorella pyrenoidosa* (strain 7-11-05) to equilibrium on isopycnic gradients of aqueous Ficoll. Gradient A, 1 ml of cell suspension (1.71×10^9 cells) in culture medium was layered upon a 34-ml linear density gradient of Ficoll (25 to 45 percent) in deionized water. An additional 1 ml of 45 percent Ficoll in water was layered with a syringe on the bottom of the cellulose nitrate centrifuge tube (2.54 by 8.89 cm) to make gradient A equivalent to gradient B; gradient B, linear density gradient was identical to A with the same cell number in 1 ml of 45 percent Ficoll in water layered with a syringe on the bottom of centrifuge tube; gradient C, linear density gradient was identical to A with the cell suspension layered on top, also as in A. The suspension was composed of cells removed with a syringe from the top and bottom of the zone of buoyant cells in a gradient similar to A (see text); gradient D, 5 ml of cell suspension (8.55 by 10^9 cells) in culture medium was layered on a 30-ml linear density gradient of aqueous Ficoll (27 to 36 percent). The linear gradients were prepared and maintained at 2°C, as were the cells from their time of harvest. Centrifugation was performed in a model L2-65B, Beckman-Spinco ultracentrifuge, with a SW-27 rotor at 25,000 rev/min. Cells layered on top of the gradients came to equilibrium between 30 and 60 minutes, while those layered on the bottom required approximately 120 minutes. The dotted lines on the centrifuge tubes were used to indicate 30- and 34-ml levels during gradient preparations.

Reports that young erythrocytes are less dense than mature erythrocytes and could be separated by an isopycnic density-gradient technique (*3*) prompted us to determine whether other cell types might differ in density during their normal cell cycles. If the density of daughter cells proved sufficiently different from cells of other ages, the selection of these cells from asynchronous populations by use of isopycnic gradients would circumvent most of the problems encountered with differential centrifugation techniques.

Sucrose was used in initial attempts to prepare aqueous isopycnic gradients for separation of cells of different ages of the high temperature strain (7-11-05) of *Chlorella pyrenoidosa* (*4*). Asynchronous cells of this organism failed, however, to float in gradients containing as much as 75 percent sucrose (weight/volume). Because the pelleted cells from these gradients appeared to be plasmolyzed, it seemed probable that their high apparent density resulted from dehydration.

Ficoll (*5*), a sucrose polymer of high molecular weight (~ 400,000), was next used for gradients because of its lower osmotic strength relative to a given density of sucrose. Because certain batches of Ficoll contained a factor or factors that caused clumping of cells on gradients and caused inhibition of growth after the cells were removed from the gradients, the Ficoll was routinely dialyzed as a 15 percent solution against distilled water for 4 days at 2° to 3°C. The dialyzed preparations were filtered through a 0.45-μm Millipore filter and then subjected to flash evaporation at 45°C until the concentration of Ficoll was approximately 45 percent. The actual density of the purified Ficoll was determined at room temperature with a 50-ml volumetric flask used as a pycnometer. Linear gradients were then prepared at 2° to 3°C (*6*).

When cells of *Chlorella* were cultured under a light intensity of 5500 lu/m^2, they divided into four daughter cells. Asynchronous cells cultured under this light were layered on either the top or bottom of identical linear density gradients of Ficoll and centrifuged. The entire populations of cells in both gradients were buoyant in the same density region, an indication that this is truly an equilibrium density-gradient procedure and not merely differential centrifugation of different size cells (Fig. 1, A and B). As further proof of buoyancy of these cells at their isopycnic points, a syringe was used to remove cells from both the top and bottom of the zone of buoyant cells from a third

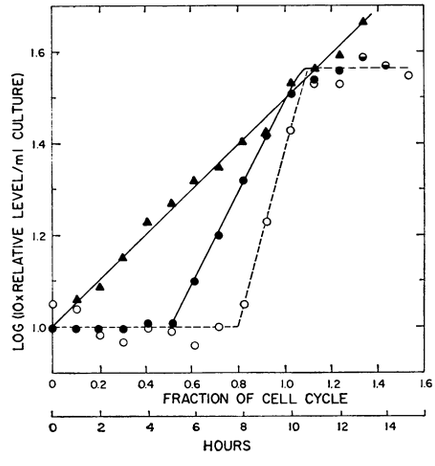

Fig. 2. Changes during synchronous growth of daughter cells of *Chlorella pyrenoidosa* (strain 7-11-05) selected from an asynchronous culture by an isopycnic density-gradient procedure. (○) Periodic increases in cell number; (●) total cellular DNA; and (▲) approximately an exponential increase in total protein per milliliter of culture. One cell cycle is defined as the time at which fifty percent of the cells have divided. The initial concentrations of cells, DNA, and protein per milliliter of culture were 140 $\times 10^6$, 9.30 μg, and 355 μg, respectively; the light intensity was 5500 lu/m^2. The culture apparatus of Hare and Schmidt (*9*) was used along with their continuous dilution procedure, which was modified by reducing the dilution rate to 13.5 percent per hour; DNA was measured by a modified diphenylamine procedure (*10*) while cellular protein was estimated by the procedure of Lowry *et al.* (*11*); linear density gradients of Ficoll (27 to 32 percent, 26 ml) in deionized water were used. The top 5 ml of buoyant cells from these gradients were those used.

identical gradient. The cells were mixed, freed from the Ficoll (by centrifugation after dilution), resuspended in fresh culture medium, and layered upon another identical gradient. When centrifuged once again, the cells were distributed into two distinct bands (Fig. 1C) corresponding to their positions on the original gradient. The return of the cells to their original position indicates that the Ficoll gradient does not alter their density significantly. The increased wall effects seen in this gradient were the result of incomplete resuspension of the cells after their centrifugation to a pellet to remove the Ficoll of the first gradient. Complete dispersion of the cells subsequently was achieved with a Dounce homogenizer without apparent cell damage.

Microscopic examination of fractions, taken progressively down a gradient such as shown in Fig. 1A, revealed that cells gradually increased in size from small morphologically uniform daughter cells at the top to larger dividing cells at the bottom of the zone of buoyant cells. Thus, *Chlorella* cells appear to increase in density as they develop through their cell cycle.

A gradient was then designed to allow floatation of daughter cells and sedimentation of most intermediate-age and mother cells (Fig. 1D). Such gradients could be heavily loaded [up to approximately 2 g of cells (fresh weight) per 35 ml of gradient] to insure a good yield of uniform daughter cells. The daughter cells taken from such gradients exhibited a high degree of synchrony, a periodic (step) increase in total cellular DNA, and approximately an exponential increase in total cellular protein during a synchronous cell cycle (Fig. 2). When the pelleted (older) cells were taken along with the buoyant daughter cells, the cell number increased exponentially as in the untreated asynchronous culture, an indication that the Ficoll density-gradient procedure did not of itself induce synchrony.

This isopycnic density-gradient procedure potentially offers another rapid experimental approach to biochemical investigations of the cell cycle. If cells are buoyant in the gradient materials employed and if they increase continuously in density as they develop through their cell cycles, it should be possible to reveal how biochemical parameters change during a cell cycle merely by making cell counts and biochemical analyses down the zone of buoyant asynchronous cells.

To perform such studies, however, the cultural conditions must be closely controlled. *Chlorella* cells of all ages increased in density as their growth rate was increased by illumination with a higher light intensity. Under 22,000 lu/m^2 the cell cycle length remained about the same, but the division number of the cells increased to approximately eight. The density of dividing mother cells increased to the point that they could not be floated in the highest density of Ficoll tested (58 percent), but daughter cells and those of intermediate age still could be floated in gradients running from 25 to 40 percent Ficoll.

One advantage of this equilibrium centrifugation procedure is that the cells are maintained at 2° to 3°C from the time of harvest until they are again suspended in culture medium that had been equilibrated to the temperature necessary for initiating cell growth. At this low temperature the metabolism of the cells is not as likely to be perturbed as may be the case in intermittent-illumination procedures (*7*) used to synchronize *Chlorella*.

From the review by Morse (*8*) dealing with methods being tested and used for fractionation or separation of cells from tissues or suspension cultures, it appears that equilibrium centrifugation as a means of selection of synchronous daughter cells for cell cycle studies has not been reported.

THOMAS O. SITZ, ALAN B. KENT*
HAROLD A. HOPKINS
ROBERT R. SCHMIDT
Department of Biochemistry and Nutrition, Virginia Polytechnic Institute, Blacksburg 24061

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* Present address: Department of Chemistry, Florida State University, Tallahassee 32306.

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Quantitation of Strain BALB/c Mouse Peritoneal Cells

Abstract. *Total and differential counts of the peritoneal cells of male and female BALB/c mice aged 10 days to over 2 years demonstrate that the increase in cell number that occurs in mice over 2 months old is due entirely to an increase in lymphocytes. The number of peritoneal macrophages in BALB/c females is maintained at a constant level for 22 months. The stability of the macrophage population in contrast to the increase in numbers of lymphocytes suggests that the body pools of these two cell types are not related.*

Mouse peritoneal cells are now being used in studies concerned with the uptake and processing of exogenous materials (*1*), especially antigens (*2*). It has been recognized that this cell population consists mainly of macrophages and lymphocytes, and it is known that the proportion of cells is altered by the introduction of foreign materials into the peritoneal cavity (*3–5*); to our knowledge, no information is available concerning the variation in the number of each cell type that is related to age, sex, and strain (*6*). The results presented here indicate how age and sex influence the normal peritoneal cell population of the strain BALB/c mouse.

Data were derived from conventional and germ-free mice obtained from the breeding facilities of the National Institutes of Health. The total number of peritoneal cells was estimated by a modification of the method employed by Hauschka *et al.* (*7*) and Klein and Revesz (*8*), in which the cells were washed out of the peritoneal cavity by repeated rinsings with phosphate-buffered saline into graduated cylinders containing aqueous solutions of citric acid and crystal violet in amounts such that the final concentration in the washings was 0.1*M* and 0.04 percent, respectively. Between 5 and 25 ml of saline were used for rinsing, according to the age of the animal. The gradu-