# SEPARATION OF NUCLEI REPRESENTING DIFFERENT PHASES OF THE GROWTH CYCLE FROM UNSYNCHRONIZED MAMMALIAN CELL CULTURES

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## ABSTRACT

Nuclei have been isolated from unsynchronized cultures of Chinese hamster fibroblasts after varying intervals of growth following the incorporation of thymidine -$^3$H for 20 min. These nuclei were fractionated by unit gravity sedimentation in a stabilizing density gradient of sucrose, and fractions were analyzed for the concentration of nuclei, DNA, and radioactivity. A more rapidly sedimenting population of nuclei in the $G_2$ phase of the cell cycle was separated from a group of nuclei in the $G_1$ phase, and nuclei in progressive stages of DNA synthesis (S phase) were distributed between these two regions. The fractionation of intact cells by sedimentation according to their position in the cell cycle was found to be less satisfactory than the corresponding separation of nuclei. This probably results from the continuous accumulation of mass within individual cells throughout the entire cell cycle, whereas most of the mass of a nucleus is replicated during a relatively narrow interval of the total cell cycle.

## INTRODUCTION

The sequence of biochemical events occurring during the cell cycle remains under active investigation in many laboratories. Large numbers of cells or cell components from a narrow interval of the cell cycle are often required for the analysis of this complex series of events. Synchronously dividing populations of cells are frequently employed for this purpose. Current methods used to obtain these synchronous populations are limited by the rapid decay of synchrony following its induction, possible alteration of normal cellular metabolism by blocking agents, and restriction in the total number of cells which can be obtained. Additional methods for obtaining populations of nuclei or cells from specific phases of the cell cycle would be useful in these studies. During efforts to fractionate mammalian metaphase chromosomes by sedimentation at unit gravity, we observed that two visible sedimenting bands were frequently present, and morphological examination indicated that the particles within each of these zones were nuclei. Further investigation demonstrated that this separation resulted from fractionation of nuclei according to position in the cell cycle.

## MATERIALS AND METHODS

### *Cell Culture*

The near-diploid (2n = 22) cell line (V79) which was used is a stable clonal subline of male Chinese hamster fibroblasts containing 23 chromosomes per cell (88% of the cells still contain this modal num-

ber). It was generously provided by Dr. Norman Salzman (1). The cells were maintained in monolayer culture in a modified Eagle's medium, Hu-10 (2), and they were grown in suspension culture in Ham's F-10 medium (3) with calcium omitted. Both media were supplemented with 10% fetal calf serum.

### *Radioisotope Incorporation*

Cells were incubated for 20 min with $3 \times 10^{-6}$ M thymidine-$^3$H (0.5–1.0 mCi/$\mu$mole; Schwarz BioResearch, Inc., Orangeburg, New York), a 20-fold quantity of unlabeled thymidine was added to dilute the subsequent uptake of isotope, and the suspension was centrifuged at 50-100 *g* for 5 min. The pelleted cells were resuspended in fresh, prewarmed medium containing $6 \times 10^{-5}$ M thymidine, resulting in a total reduction of about 1000-fold in the specific activity of radioisotope in the medium. It was separately determined that thymidine at this concentration does not alter the rate of cell growth.

The distribution of tritium in nuclei isotopically labeled for 20 min and "chased" for 1 hr was determined. Less than 1% of the material was soluble in cold 10% TCA or sensitive to treatment with pancreatic RNase (40 $\mu$g/ml) for 1 hr at 37°C. However, 95% of the tritium was soluble in cold 10% TCA after incubation with bovine pancreatic DNase I (20 $\mu$g/ml) for 30 min at 37°C.

### *Radioisotope Assay*

1-ml aliquots of aqueous suspension were transferred to scintillation vials, and cells and nuclei were lysed by incubation at 60°C for 20 min after the addition of 0.10 ml of a 10% solution of sodium dodecyl sulfate (SDS). The suspension was diluted with 10 ml of Packard Insta-Gel (Packard Instrument Co., Downer's Grove, Ill.) emulsifier or a 2:1 toluene:Triton X-100 emulsifier (4) containing 5.5 g of PPO and 125 mg of dimethyl POPOP per liter, and agitated on a Vortex mixer (Scientific Industries, Inc., Springfield, Mass.) to obtain a stable emulsion. Radioactivity was assayed in a liquid-scintillation spectrometer.

### *Isolation of Nuclei*

The cell suspension was chilled at 5°C, and the cells were sedimented at 220 *g* for 10 min and washed twice with cold, buffered, isotonic saline (Puck's Saline A [5]). The cells were resuspended (2–4 $\times 10^7$ cells/ml) in hypotonic buffer containing 0.01 M NaCl-0.01 M Tris HCl-0.0015 M $MgCl_2$, pH 7.4 (6). After swelling for 10 min at 0°C, the suspension was transferred to a Dounce homogenizer containing 4 volumes of pH 3 buffer (0.05 M acetic acid–0.05 M NaCl–1 mM $CaCl_2$–1 mM $MgCl_2$, pH 3.0) (1) in 0.1 M sucrose, and the cells were disrupted by 10 strokes with a tight pestle (about 25 $\mu$ clearance). The nuclei were sedimented at 500 *g* for 10 min and resuspended in pH 3 buffer containing 0.1 M sucrose. The procedure was monitored by phase microscopy, and additional homogenization was performed if a significant percentage of intact cells persisted. The nuclei were sedimented again, then washed twice in fresh buffer containing 0.1 M sucrose and 0.5% Triton X-100, and twice in the buffer from which sucrose had been omitted.

### *Fractionation of Nuclei*

The apparatus and procedures employed have been previously described (7). The suspension of nuclei ($10^8$ in 30 ml of pH 3 buffer) was rapidly pipetted into the bottom of a Lucite sedimentation chamber (except where otherwise indicated, 20 cm in diameter with a cylindrical section 6 cm high), and the introduction of a gradient of sucrose below it was immediately begun at a flow rate of 30 ml/min. A short, steep, exponential gradient introduced immediately below the layer of nuclei was followed by a linear gradient. This was achieved by passing the effluent from a 2-chambered linear gradient mixer (3.5–10% in 1400 ml) through a constant-volume mixing chamber that initially contained 250 ml of 0.5% sucrose. A second linear gradient of sucrose (10–12% in 1000 ml volume) followed. Flow was interrupted until separation occurred; then the suspension was displaced from the chamber with a solution of 12.5% sucrose at a flow rate of 30 ml/min, and 10-ml fractions were collected. All solutions contained pH 3 buffer and 0.02% Triton X-100. Concentrations of nuclei were determined with a Sanborn-Frommer cell counter (Sanborn Co., Waltham, Mass.), which detects particles by scattered light. The measurement of the concentration of nuclei prepared in this way was not affected by variation of the sucrose concentration in the medium.

The maximum load of nuclei which may be applied under the conditions of these experiments was determined in a small, four-chamber, sedimentation device. The liquid entered all chambers from a common source, and the density gradient and sample volume relative to the cross-sectional area was similar to that employed in the 20 cm diameter chamber. No streaming (7) occurred when the concentration of nuclei in the suspension applied was $3.2 \times 10^6$/ml or less, but in the range of $1.3 \times 10^7$ nuclei/ml or greater there was streaming. Even gross convective disturbances appeared at the higher levels, and many nuclei were found on the floor of the sedimentation chamber.

### *Chemical Assays*

The concentration of DNA was measured by the diphenylamine procedure of Burton (8) employing deoxyadenosine as the standard. The method was modified slightly to permit the measurement of low concentrations of DNA in fractions collected from the sedimentation chamber. Nuclei were collected on vinyl acetate membrane filters (Gelman Metricel VM-4,[1] 0.8 $\mu$ in pore size, 13 mm in diameter) by vacuum filtration and washed with 0.001 M HCl–0.001 M $CaCl_2$. The dry membranes were transferred to snap-top glass vials (14 mm I.D.) and incubated at 70°C for 20 min after the addition of 0.4 ml of 0.7 N PCA. The diphenylamine reagent was mixed with the hydrolysate, and the solution was incubated after discarding the filter. The DNA values obtained by this filter modification were 20% lower than those obtained in the conventional assay, but the measured DNA content was proportional to the number of nuclei employed with either method.

Nuclei prepared from an unsynchronized culture were assayed for DNA (8), for RNA by the orcinol method (9) with adenosine as a standard, and for acid-soluble (0.25 N HCl) and acid-insoluble protein by the Lowry phenol procedure (10) with bovine serum albumin as the standard. These nuclei contained 7 $\mu\mu$g of DNA, and the mass ratios of RNA, acid-soluble protein, and acid-insoluble protein to DNA were 0.47, 2.06, and 1.38, respectively.

## RESULTS

### *Fractionation of Nuclei by 1 × g Sedimentation*

Nuclei prepared from mammalian cells in the logarithmic phase of growth were allowed to sediment at unit gravity through a stabilizing density gradient of sucrose. A typical result is presented in Fig. 1. Two cloudy, sedimenting bands were observed after 2 hr, separated by a region of lower turbidity. A region of rapidly decreasing turbidity extended for a short distance below the lower band. There was usually a faint, very slowly sedimenting band near the meniscus which contained only cytoplasmic debris. Two peaks of DNA-containing material (the second sedimented about 50% faster than the first) were found at positions corresponding to the visible bands, although only nuclei were detected in both regions by microscopic examination. The DNA content per nucleus throughout the more rapidly sedimenting band was nearly twice that observed

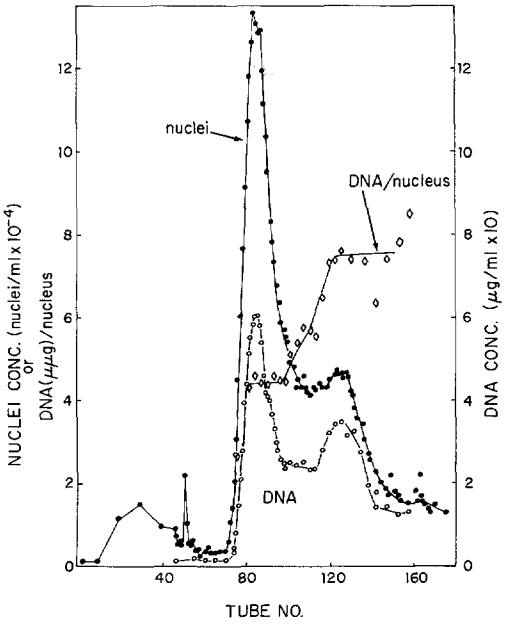


FIGURE 1 Fractionation of Chinese hamster fibroblast nuclei ($2.8 \times 10^8$ applied in 120 ml of pH 3 buffer) after 8-hr sedimentation of 25°C in a chamber 40 cm in diameter. The stabilizing density gradient is described in Methods, except that volumes were four times as great. The fraction volume was 50 ml; 10 ml of each fraction was assayed for nuclei concentration (●——●) and the remainder for DNA concentration (○——○). The calculated DNA content per nucleus is indicated (◇——◇).

across the other band, and intermediate values were found in the region between these bands. The average DNA content of the total population of fractionated nuclei was 20% less than the value obtained with unfractionated nuclei, reflecting the difference in the assay procedure (Methods).

The observed variation in turbidity across the region of sedimenting nuclei was more pronounced than the corresponding changes in concentration of nuclei in all experiments. This observation is anticipated since turbidity is generally proportional to the product of the concentration of particles and the square of their volumes, although this relationship is inexact and much more complex when applied to particles as large as nuclei.

### *Fractionation of Pulse-Labeled Nuclei*

The differing nuclear DNA content in each of the sedimenting bands as well as in the intermediate zone suggests that this separation reflects

[1] Gelman Instrument Co., Ann Arbor, Mich.

the position of nuclei in the cell cycle. This interpretation is confirmed by examination of nuclei prepared from cells that had been cultured for various intervals of time after brief exposure (20 min) to radioactive thymidine. Only the nuclei of cells actively engaged in DNA synthesis (S phase) should contain radioactivity if the culture is harvested immediately after the brief period of thymidine-$^3$H incorporation, but these labeled nuclei will have progressed to the post-DNA-synthetic ($G_2$) and finally postmitotic ($G_1$) phases after successively longer intervals of time. These Chinese hamster fibroblasts have a generation time of about 12 hr and the durations of $G_1$, S, $G_2$, and M (mitosis) are about 4.5, 4, 2.5, and 0.8 hr, respectively (1, 11).

Nuclei were isolated from cells harvested 5 min as well as 1, 1 ¾, and 8 hr after the 20-min interval of thymidine-$^3$H incorporation, and the results are presented in Fig. 2. Nuclei that had incorporated thymidine-$^3$H were present in the more rapidly sedimenting band after a 1 ¾ hr chase (Fig. 2 C), whereas radioactivity was nearly absent from the slower band. There was a progressive increase in radioactivity in the region between the two bands, indicating that nuclei in successive stages of DNA synthesis were also partially separated. After an 8-hr chase (Fig. 2 D), most of the radioactivity was found in the upper band, demonstrating that the nuclei in these bands do represent different phases of a common cell cycle. The persistence of a markedly reduced level of label in the lower band may result from an injudicious selection of the duration of the chase, but some heterogeneity of the generation times of individual cells is an equally plausible possibility. Any cells with a slightly prolonged generation time that incorporated tritium during early S phase would not have reached mitosis, whereas cells with shortened generation times could have proceeded from late S phase through mitosis to S phase or even $G_2$.

The upper and lower limits of the sedimentation rates of nuclei in $G_1$ and $G_2$ phase, respectively, have not been rigidly defined, but the results of a very short chase (Fig. 2 A) show that the sedimentation profile of nuclei in the S phase extends into both of these bands, as anticipated. Since the fraction of $G_1$ nuclei greatly exceeds that of $G_2$ nuclei in the cell cycle, the contribution of nuclei in S phase to the region in which $G_2$ nuclei sediment is more striking. The total time interval of 25 min between the initiation of thymidine-$^3$H incorporation and the isolation of nuclei is also a significant fraction of the entire $G_2$ interval and accentuates the apparent overlap of nuclei from S phase and $G_2$. Some aggregates of nuclei, as well as intact cells and nuclei with considerable adherent cytoplasmic debris, sediment at even greater rates than the $G_2$ nuclei. The systematic increase in specific activity in the region between tubes 140 and 180 might result from the sedimentation of intact (or nearly intact) cells from the S phase in this region, but this remains speculative. The differences in pattern of distribution of nuclei observed in Fig. 2 C as compared with A, B, and D probably can be attributed to differences in conditions of preparation and sedimentation (see Discussion).

The fact that these sedimentation profiles result from nuclei in differing phases of a common cell cycle was also demonstrated by the introduction of a block in the cycle. Cells were exposed to thymidine-$^3$H for 20 min, and 1 hr after the completion of the pulse Vinblastine sulfate (0.2 $\mu$g/ml) was added to the culture medium and the cells were isolated 5 hr later and disrupted. Most of the radioactivity was found in very slowly sedimenting fractions near the top of the gradient, and microscopic examination of these fractions demonstrated only metaphase chromosomes and no interphase nuclei. No radioactivity and practically no nuclei were observed in the position of the usual sedimenting band of $G_1$ nuclei. Some unlabeled nuclei sedimented in the usual S phase region, and a band of $G_2$ nuclei persisted which contained a small portion of the total radioactivity.

### *Effect of Inadequate Disruption of Cells*

The sedimentation pattern of nuclei was reasonably consistent in most experiments employing identical conditions. However, the recovery of nuclei and radioactivity in the previously described region of interest was directly related to the effectiveness of cell disruption and the removal of adherent cytoplasm from the nuclei. In one experiment, cells were cultured for 8 hr after a 17-min interval of thymidine-$^3$H incorporation. Very mild conditions of hypotonic swelling and homogenization were employed, and nearly equal numbers of nuclei and intact cells were found by microscopy. Sedimentation of this homogenate resulted in the separation of four visible bands within 2 hr. The sedimentation velocities of the upper two bands corresponded to those of $G_1$ and $G_2$ nuclei. Micros-

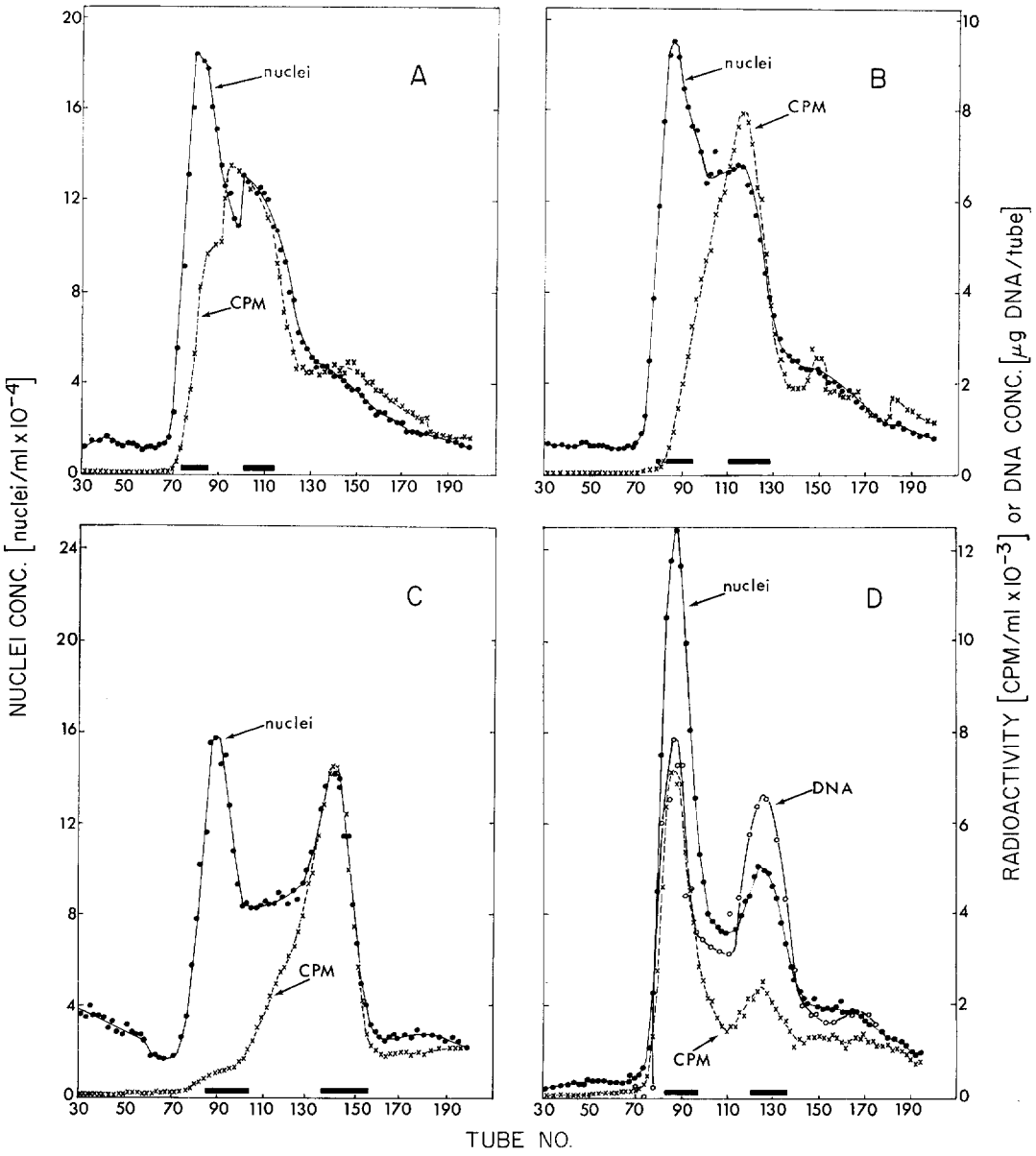


FIGURE 2 Fractionation of nuclei ($10^8$ [A, B, D] or $5 \times 10^7$ [C] in 30 ml of pH 3 buffer) after 7-hr sedimentation at 24°C in a chamber with a 20-cm diameter. Nuclei were isolated from cells that had been incubated for 20 min in the presence of thymidine-$^3$H prior to growth in the absence of $^3$H for 5 min (A), 1 hr (B), 1¾ hr (C), and 8 hr (D). The stabilizing density gradient used in A, B, and D is described in Methods. In C, the constant volume mixer initially contained 250 ml of 0.3% sucrose, and the two chambers of the linear gradient device contained 800 ml of 1% and 800 ml of 3% sucrose. For the last portion of the gradient, these were refilled with 1400 ml of 5% and 1400 ml of 7% sucrose. In plotting the concentration of nuclei and radioactivity, the experimental values have been multiplied by two in C to compensate for the smaller load of applied nuclei. All solutions contained pH 3 buffer and 0.02% Triton X-100. The distribution of nuclei (●——●), radioactivity (×-----×), and DNA (○——○) in fractions of 10 ml volume is shown. The bars indicate the positions of the visible sedimenting bands, as determined by serial measurements of band location and extrapolation to the time of collection.

copy confirmed this interpretation and revealed some overlap between the band of $G_2$ nuclei and $G_1$ cells, but the lower two bands contained predominantly intact cells. These intact, or nearly intact, cells were obviously not identical with cells that had not been exposed to pH 3 buffer, detergent, and the conditions of homogenization.

### *Fractionation of Intact Cells*

In work reported by other investigators (12–15), cell populations have been partially fractionated according to their phase in the growth cycle on the basis of differences in their rates of sedimentation, and the technique has been exploited to obtain parasynchronous cultures. We have examined cells that were labeled with thymidine-$^3$H, chased for 2 hr, chilled to 5°C to retard further progression through the cell cycle, and sedimented through a sucrose gradient in isotonic salt solution at 26°C. The cells do not progress through the cell cycle at this temperature during the interval of sedimentation (16). They sedimented as a broad band, and there was only minimal visual evidence of partial splitting into two bands late in the experiment (about 3 hr). However, the distribution of radioactivity within this broad band indicated that significant fractionation of cells had occurred according to position in the cell cycle. The major part of the radioactivity cosedimented with the most rapidly sedimenting portion of the broad band. Intact cells which were sedimented after an 8-hr chase exhibited a similar sedimentation profile, but the specific activity (Cpm/cell) was significantly greater among the less rapidly sedimenting cells than it was in the more rapidly sedimenting region. In both cases, the resolution between cells in $G_1$ and $G_2$ phases was much less pronounced than was observed with nuclei (Fig. 2 D).

## DISCUSSION

Nuclei isolated from rat liver homogenates have been fractionated previously by unit gravity sedimentation (17) and by low-speed centrifugation (18) in the presence of stabilizing density gradients. The fractionation appeared primarily to resolve the nuclei of different cell types (17), namely stromal and hepatic parenchymal nuclei, although there was also some resolution of diploid and tetraploid (17, 18) parenchymal nuclei. Apparently, the fractionation of nuclei according to position within the cell cycle has not been investigated previously with nuclei isolated from a homogeneous cell type in exponential growth, although low-speed centrifugation has been applied to unsynchronized intact cells (12–14). The pulse-chase results with thymidine-$^3$H as well as measurements of nuclear DNA content in the present study demonstrate that nuclei from the $G_1$ and $G_2$ phases of the cell cycle are separated by unit gravity sedimentation.

The usual subdivision of the cell cycle into $G_1$, S, and $G_2$ phases is strictly defined by mitosis and the earliest and latest points of chromosomal DNA synthesis, whereas fractionation of nuclei or cells by sedimentation is dependent solely on differences in the total mass of these particles in the absence of changes in their shape or density. The concentration of nuclei (or cells) at any specific point in the sedimentation profile is inversely related to the rate of increase in mass of individual nuclei (or cells) at a corresponding position in the cell cycle. Peaks would not be observed in the sedimentation profile if the rate of growth of individual nuclei (or cells) were constant over the entire cell cycle. The accumulative synthesis of histones in mammalian cells is closely related temporally to the synthesis of DNA (19-22), and this replication of roughly half to two-thirds of the total nuclear mass occurs over a time interval of only one-third of the total cell cycle (11), giving rise to distinct peaks in the sedimentation profile. On the other hand, the much more continuous accumulation of mass in whole cells throughout the entire cycle (23–27) results in a broader distribution of all cells which are not in S phase. Consequently, there is less separation between cells in late $G_1$ and early $G_2$ phases of the cell cycle.

The sedimentation profile of nuclei from nonsynchronized cultures was reproducible, subject to certain limitations. The most significant factor limiting the resolution of nuclei from various phases of the cell cycle was incomplete homogenization of cells, since this resulted in the overlap of nuclei differing intrinsically in mass, but containing variable amounts of adherent cytoplasm. Several different methods for the disruption of cells and the isolation of nuclei were explored. The most satisfactory preparations of nuclei, in terms of minimal cytoplasmic contamination as determined by microscopy and chemical analyses, were obtained by Dounce homogenization at pH 7.4 after osmotic shock (6) or by lysis of the cells in the

presence of the nonionic detergent Triton X-100 (0.5% concentration) in the same hypotonic buffer. These methods were not routinely employed in the present study because they did not preserve metaphase chromosome structure and adversely affected the measurement of nuclei concentration with the Sanborn-Frommer particle counter. In one preliminary sedimentation experiment with nuclei prepared by the detergent procedure above, the nuclei sedimented as visible bands which were sharper and better resolved, with a smaller amount of very rapidly migrating material than was observed in other experiments. The sedimentation rates were reduced about 20%, consistent with an increase in diameter of these nuclei of a similar magnitude. It appears probable that significantly better resolution of nuclei from different stages of the cell cycle can be obtained than has been presented if improvements are made in the conditions of isolation of the nuclei. The shape and magnitude of the stabilizing density gradient also affects the sedimentation profile, and this may have been the major factor creating the observed differences in sedimentation between Fig. 2 C and the other profiles in Fig. 2. Differences in the conditions of homogenization and in the number of nuclei applied to the sedimentation chamber may have been contributing factors, but the result indicates the necessity of controlling the conditions of both homogenization and sedimentation.

Other factors limiting the degree of resolution are disruption or distortion of nuclei during cell homogenization, aggregation of nuclei preceding or during sedimentation, and experimental conditions associated with the sedimentation. The most important consideration in this latter category is the prevention of regions of density inversion in the chamber by maintaining a sufficiently positive density gradient in the medium at all levels and by avoidance of overloading. Localized regions of instability produce "streaming," and more generalized areas produce gross evidence of convection. The required density gradient and its accompanying viscosity gradient also limit resolution, particularly in circumstances in which the difference in density between the medium and the particles (such as cells) is relatively small. Dispersion with respect to the length of the cell cycle undoubtedly resulted in apparent reduction in the resolution of nuclei pulsed with thymidine-$^3$H especially after long intervals of chase, but this is a special problem in this type of isotopic experiment.

Sedimentation appears to be a useful method for investigating the sequence of many nuclear events in the cell cycle. The times of synthesis of a variety of nuclear enzymes, histones, or other proteins or species of RNA are readily susceptible to study by either chemical assays or incorporation of radioisotopes into these nonsynchronized cultures. Events occurring during S phase can be readily detected by specific activities after pulse labeling with thymidine-$^3$H or with double isotopic labeling. The requirement for synchronized cultures is circumvented, thereby avoiding problems caused by limitation in numbers of cells or nuclei, the possibility of alteration of normal cell metabolism by the blocking agents employed to produce synchronous populations, and the rapid decay of synchrony as a function of time following its induction. Theoretically, fractionation of nuclei by sedimentation could be used to determine the fraction of the total cells in each phase of the cell cycle and consequently the relative duration of each of these phases. This procedure would differ radically from the conventional methods employed for this analysis, and it would provide complementary information. This latter objective would require a population of nuclei with no adherent cytoplasm and would be complicated by the continuity of nuclei in S phase with nuclei from both $G_1$ and $G_2$ phases and the consequent overlap occurring at these contiguous regions due to any fluctuations in the rate of synthesis of any nuclear components other than DNA and histones (such as RNA and nonhistone proteins) during the cycle. This factor assumes even greater significance when intact cells rather than nuclei are examined. However, a fractionation of cells can be obtained which is adequate for many purposes.

The results reported here also illustrate the often neglected fact that the difference sedimentation rates of individual cells of a given cell type, varying as a function of position in the cell cycle, must be considered when populations comprising cells (or nuclei) of more than one type are fractionated by sedimentation.

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