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Isolation of Cell Subpopulations from *in Vitro* Tumor Models According to Sedimentation Velocity[1]

Ralph E. Durand

Radiobiology Research Laboratories, Department of Radiology, University of Wisconsin, Madison, Wisconsin 53706

SUMMARY

Chinese hamster V79-171 cells grown *in vitro* as asynchronous single cells, as plateau-phase cultures, and as multicell spheroids contained subpopulations that differed in cell volume. These subpopulations could be isolated according to their sedimentation velocity at unit gravity. Asynchronous cells were found to sediment at a modal velocity of 17.7 mm/hr, whereas the modal sedimentation velocities of plateau and spheroid cells were found to be 16.0 and 15.1 mm/hr, respectively. When the composition of each subpopulation was determined using either [^{3}H]thymidine incorporation or sensitivity to ionizing radiation as an index of cell cycle position, it was found that G_1-like populations developed in both *in vitro* tumor models. Since the sedimentation velocity technique permits isolation of functionally different cell subpopulations after treatment with cytotoxic agents, it may now be possible to assay differential lethality when such agents are applied to cells growing *in situ* in a tumor-like situation.

INTRODUCTION

In vitro systems have been particularly rewarding for tumor cell biologists, due to the low cost, speed, and ease of quantification of responses in such systems. In addition, single cells in culture can be maintained in a more or less optimal environment, which can be closely monitored and is free from indirect tumor-host or immunological influences. While such cells in asynchronous growth may be advantageous for many applications, it is generally conceded that these systems represent a special case that is found rarely, if ever, in the *in vivo* tumor. *In vitro* models that retain the inherent advantages of culture systems, yet display growth characteristics more comparable to tumors *in vivo*, have been developed only relatively recently. One of these *in vitro* systems utilizes plateau phase cells and has been critically reviewed by Hahn and Little (5). Sutherland *et al.* (10) more recently developed the multicellular "spheroid." Although the 2 models differ in that the former is 2-dimensional whereas the latter is 3-dimensional, both provide a simulation of tumor growth conditions, including development of "noncycling" subpopulations of cells (3, 5, 10).

After treatment of either tumor model with a cytotoxic agent, the number of viable cells remaining can easily be determined by standard colony formation techniques. However, such an experiment yields only the net results and by itself provides no information as to whether the agent was differentially lethal to subpopulations of cells within the model.

Additional information might be obtained if it were possible to grow cells in these tumor-like situations, perform the treatment of interest, and then nondestructively separate the various subpopulations of cells and assay each for its response. One method of accomplishing this was suggested by the similarity between the 2 *in vitro* tumor systems. In both systems, some cells tend to collect a non- or slowly cycling, pre-DNA synthesis stage of the cell cycle. Since a cell doubles its volume as it progresses through the cell cycle and divides, one might expect that various subpopulations of cells could be physically separated simply on the basis of cell size using the Sta-Put techniques developed by Miller and Phillips (7). Subsequent demonstrations that partially synchronous subpopulations could be obtained from asynchronous mammalian cell cultures (6, 11) suggested that the various subpopulations of cells growing *in vitro* under tumor-like circumstances might be equally amenable to separation.

This communication deals with the separation of V79-171 Chinese hamster lung cells, grown as both plateau phase and spheroid tumor models, into subpopulations by the technique of velocity sedimentation. Since the technique allows essentially 100% recovery of cells and is nondestructive, it may have general applicability in characterization of cells from inhomogeneous cell populations.

MATERIALS AND METHODS

Chinese hamster V79-171b lung cells were used throughout. The cells were normally maintained in asynchronous monolayer growth on 100-mm Falcon plastic Petri dishes in Eagle's basal medium plus 15% fetal calf serum using techniques previously described in detail (1, 4). Cell growth techniques for spheroids (3, 9, 10) and plateau-phase cultures (4) have also been previously described. Plateau-phase cultures of $\sim 2 \times 10^5$ cells/sq cm ($\sim 15 \times 10^6$ cells/dish) that had been fed daily were used 7 days after inoculation of 10^4 cells/dish. Single cells from dishes or spheroids were prepared using 0.25% trypsin in citrate-saline ($Na_3C_6H_5O_7 \cdot 2H_2O$, 4.4 g/liter, plus KCl, 10.0 g/liter) buffer (3, 9). The sedimentation, or Sta-Put, apparatus used was the standard SP-180 model commercially avail-

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Preliminary experiments established that cell viability was somewhat dependent upon the serum type and concentration in the separation gradient. All results reported here are from experiments in which a nonlinear gradient of 0.5 to 20% calf serum in PBS[2] was used, and in which cells remained essentially 100% viable. Cells were maintained at 4° under aseptic conditions during all separation procedures. In a typical experiment, about 40 ml of PBS followed by an equal volume of 0.5% calf serum in PBS containing 1 to 2 × 10^7 cells were introduced through the bottom of the sedimentation chamber. Next, the nonlinear gradient (1600 ml) of 0.5 to 20% calf serum in PBS was formed slowly (~35 min elapsed time) below the cell suspension. This lifted the cells to form a thin band (about 1.5 mm) at a fixed height near the top of the chamber. The cells were then allowed to sediment at unit gravity for 2.5 hr. At the end of this time, 120 fractions of 14 ml each were collected by an automatic fraction collector (~60 min elapsed time), allowing the solution to flow out through the bottom of the chamber. The 1st 20 fractions, which corresponded to the cone of the sedimentation chamber were discarded. Thereafter, every 3rd fraction was used to determine changes in 1 parameter; that is, the number of cells in Fractions 1, 4, 7, etc. were counted, whereas [^{3}H]thymidine incorporation was assayed in Fractions 2, 5, 8, etc., and cells of Fractions 3, 6, 9, etc., were plated for colony formation. The number of cells in a given fraction was determined with a Coulter Counter. Cell viability was assayed by aseptic collection of the cells from the given fractions and by placing a known number of cells into Petri dishes to determine their colony-forming ability.

The position of the cells of each fraction in the cell cycle was monitored by [^{3}H]thymidine incorporation. Cells were incubated in the presence of [^{3}H]thymidine (New England Nuclear, Boston, Mass.; stock solution, 0.05 mM; specific activity, 20 Ci/mmoles) for appropriate intervals before trypsinization and layering on the gradient. For short or pulse-labeling, 1 μCi [^{3}H]thymidine per ml medium was used, whereas 0.1 μCi/ml was used in the continuous labeling experiments. After the cells had been separated using the Sta-Put, cells from the various fractions were collected, counted, concentrated by centrifugation, and layered onto filter papers. After drying, the papers were washed twice in cold 10% trichloroacetic acid, twice in absolute alcohol, and finally in acetone before being dried and counted in scintillation vials with 10 ml of Scintisol. Results were expressed as relative cpm/cell. This does not imply that all cells of a given fraction were equally labeled, since some heterogeneity of both cell volumes and actual synthetic rates would be anticipated. Further quantitative reduction of the data thus seemed unrealistic, since only the qualitative question of which cells were in S phase was of interest.

A 2nd method of qualitatively determining the cell cycle position of cells in each fraction utilized their known radiation survival characteristics (2–4, 8). Cell populations were radiated in suspension immediately following trypsinization after monolayer growth or immediately following pretrypsinization for spheroid growth. All radiations were performed at 37° in water-jacketed spinner flasks, using a Picker Nuclear ^{60}Co teletherapy source at an effective distance of 80 cm (~150 rads/min). The radiation doses chosen yielded maximum surviving fractions of ~10^{-2}, as determined by colony-forming potential of the separated cells. Results were presented as "relative surviving fractions," since it was impossible to experimentally determine plating efficiencies of unradiated, separated cells within the same experiment. Preliminary experiments had indicated, however, that plating efficiency was essentially identical in all fractions of cells from any growth condition and was not reduced by the separation procedures.

Cell volumes were determined using the Model B Coulter Counter and its associated Model H plotter. This was calibrated using ragweed and corn pollens, with the pollen size determined microscopically with the aid of an ocular micrometer.

RESULTS

An asynchronous population of Chinese hamster cells includes cells randomly distributed with respect to the cell cycle and thus more or less randomly distributed in volume. The volume distribution of asynchronous cells shown in Chart 1*A* has a shoulder region of the right side of the curve reflecting the mitotic and near-mitotic populations of the cells. In contrast, Chart 1*B* shows the volume distribution of semisynchronous cells such as those found in either *in vitro* tumor model. In this case, some cells of the 17-day-old spheroids had accumulated in a G_1-like stage of the cell cycle (see Ref. 3) and consequently were found to be smaller in volume. These data indicated that the cells that had accumulated in the G_1-like part of the cell cycle had a mean cell volume that was slightly larger than half that of the

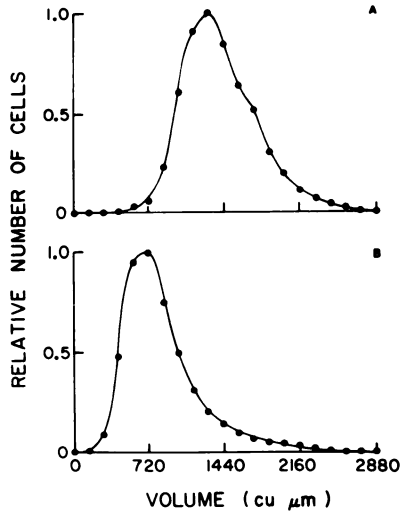

Chart 1. Relative numbers of cells as a function of cell volume for asynchronously growing single cells (*A*) or 17-day-old spheroids (*B*). Mean cell diameters were 13.9 and 12.0 μm, respectively.

[2] The abbreviation used is: PBS, phosphate-buffered saline ($CaCl_2$, 100 mg/liter; KCl, 200 mg/liter; KH_2PO_4, 200 mg/liter; $MgCl_2 \cdot 6\ H_2O$, 100 mg/liter; NaCl, 8000 mg/liter; $Na_2HPO_4 \cdot 2\ H_2O$, 1150 mg/liter).

asynchronous population. If the mean volume of these tumor-model cell populations had not correlated with independent determinations that the cells were G_1 like (3, 9), it would have been impossible to achieve useful separation.

Preliminary experiments were designed to discover whether useful separations could be achieved and whether cell viability was affected by the separation procedure. On the basis of these experiments, it was determined that maximum viability was obtained when a gradient solution of 0.5 to 20% calf serum in PBS was used. Appropriate control experiments also demonstrated that previous radiation of the cells or exposure to [^{3}H]thymidine had no influence on the cell volume or sedimentation rates at the time of separation. In separations of asynchronous cells under similar conditions, the relative position of the peak of the distribution would routinely be reproduced to ±3 fractions (*i.e.*, ±1 plotted position).

Separation of asynchronous single cells with the Sta-Put apparatus (Chart 2*A*) resulted in a sedimentation profile very similar to the volume profile (Chart 1*A*), with the peak appearing at a sedimentation velocity of ~17.7 mm/hr.

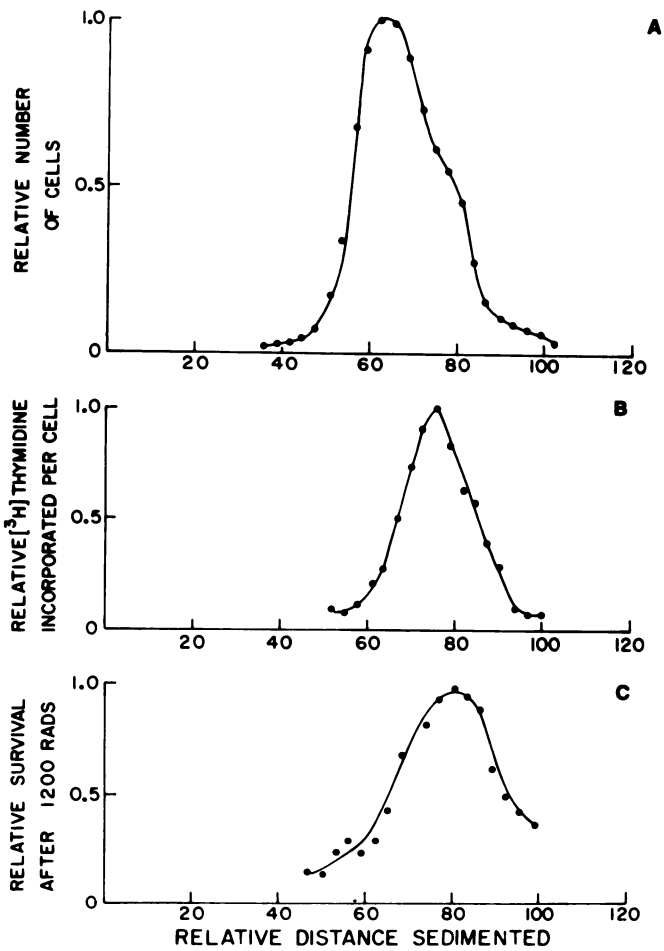

Chart 2. Sedimentation characteristics of asynchronous single cells. *A*, sedimentation profile; *B*, location of S-phase cells; *C*, relative radiation resistance of cells in each fraction. The *abscissa* is plotted in relative units (1 unit = 1 fraction collected) for convenience; in absolute units, 1 unit corresponds to a sedimentation of 0.7 mm/hr (see text) under the experimental conditions used.

Immediately prior to separation, the population of cells in Chart 2 was grown in the presence of [^{3}H]thymidine for 15 min, washed, trypsinized to form a single cell suspension, radiated with 1200 rads, and placed on the sedimentation device. Chart 2*B* shows the mean amount of incorporated tritiated thymidine per cell in each of the fractions, relative to the maximum incorporation observed. Only the larger cells of the population had incorporated [^{3}H]thymidine into their DNA during the 15-min exposure period. The distribution of labeled cells may be displaced slightly to the right on the graph, due to the fact that the labeled cells may have progressed through the cell cycle somewhat during the trypsinization and radiation procedures (≤25 min).

As would be expected, the distribution of relative radioresistance (Chart 2*C*) followed that of the distribution of cells within the cell cycle; that is, the cells in S, and particularly late S, phase were most resistant (relative survival, 1.0), whereas the smaller cells in the G_1 phase of the cell cycle were most sensitive (relative survival, 0.13). Since radioresistance at this dose (maximal surviving fraction, uncorrected for plating efficiency, was 0.010) has been found to vary by a factor of ~10 to 12 for synchronous cells (unpublished data), the factor of ~8 observed here indicates that partial synchrony was indeed achieved in each fraction.

It has previously been shown that these cells accumulate in a G_1-like phase when grown as a plateau-phase tumor model in culture dishes (4). It would thus be expected that the net cell size in such plateau-period populations would be decreased and that this subpopulation of cells could be readily separated from the cycling cells. Cells were thus grown to plateau phase under conditions of optimal nutrition (medium changed daily) and labeled with [^{3}H]thymidine for 24 hr prior to trypsinization, immediate radiation with 1500 rads, and separation using the Sta-Put (Chart 3). The sedimentation profile shown in Chart 3*A* was shifted to the left (smaller cells sizes) as expected in comparison to the *dotted line*, which shows the asynchronous distribution of Chart 2*A*. Since only every 3rd fraction collected was counted (see "Materials and Methods"), the actual shift in the peak was 5 or more fractions and corresponded to a sedimentation velocity of 16.0 mm/hr for cells at the peak of the distribution.

The cells were labeled continuously for 24 hr prior to being placed on the Sta-Put, so that both the population of cells that was then in S phase and the population that had completed S phase, G_2, and mitosis were expected to be labeled. Two discreet populations of cells did indeed contain the label (Chart 3*B*). Survival of the separated cells as assayed by colony formation after 1500 rads indicated that the smaller, G_1-like cells were more resistant (Chart 3*C*) than in the previous figure. This has previously been described (4) and was attributed to the prolonged intercellular contact. The peak survival, uncorrected for plating efficiency, was 0.0084.

Many of the characteristics of these cells when grown *in vitro* as spheroids have been documented previously (3, 4, 9). In agreement with these studies, the data of Chart 1*B* suggested that the internal cells of such spheroids accumulate in a G_1-like part of the cell cycle. Study of this population of cells was thus undertaken with Sta-Put

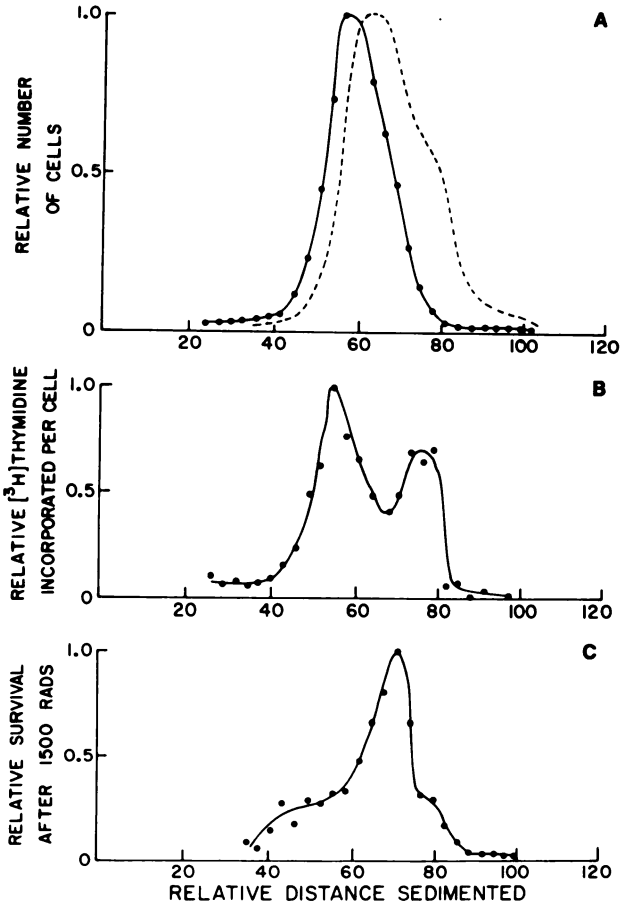

Chart 3. Sedimentation characteristics of plateau phase cells as compared to asynchronous single cells (*A*, - - -). Note that few mitotic cells were evident.

apparatus. Small spheroids containing asynchronous cells (1) were used as controls (Chart 4). Comparison of sedimentation rates of exponentially growing cells from Day 1 spheroids (Chart 4*A*) with those of exponentially growing single cells, as in Chart 2*A* (*dotted line*), indicated that there was no difference under the 2 growth conditions. A similar agreement between the relative uptake of a pulse of [^{3}H]thymidine in the various cellular fractions was observed (Chart 4*B*). The relative changes of radiosensitivity through the cell cycle after 1600 rads (Chart 4*C*) were greater in the spheroids than in the single cells (relative survivals <0.05 to 1.0, or a factor >20). This had previously been described for synchronized spheroids (2) and was attributed to intercellular contact. The enhanced survival was due to cell contact in the spheroids and lead to a peak survival of 0.012 (uncorrected for plating efficiency).

A typical population of mature spheroids, in this case 17 days old, was also prepared for Sta-Put separation (Chart 5). These spheroids were grown in the presence of 0.1 μCi of [^{3}H]thymidine per ml for 24 hr. They were then radiated at 37° as intact spheroids to a dose (2200 rads) that was known to be sufficient to kill most of the oxygenated cells within the spheroid (3, 9). Thus, surviving cells were expected to be primarily from the hypoxic fraction.

The relative displacement of the distribution was slightly more than the plateau phase cultures, as the peak indicated a sedimentation velocity of ~15.1 mm/hr (Chart 5*A*). Uptake of [^{3}H]thymidine was confined to the larger cells; that is, the cells that were dividing over the 24-hr interval of interest could be distinguished from the internal G_1-like population of cells (Chart 5*B*). A similar finding has previously been reported using autoradiographic techniques (3, 10). The relative cellular survival at the radiation dose used (absolute survival, uncorrected for plating efficiency, 0.0062) indicated that the smallest (G_1-like) cells survived best (Chart 5*C*), and this was ascribed to the protective effect of hypoxia on the internal cells (3, 9). At lower doses, one would expect more oxygenated cells to preferentially survive, due to the relative cell numbers in each subpopulation.

DISCUSSION

The results presented in this paper indicate that subpopulations of cells from *in vitro* tumor models can be rapidly and easily separated on the basis of cell sedimentation rate at unit gravity. Under the conditions of the separation, cells sedimented at a rate that was determined primarily by their

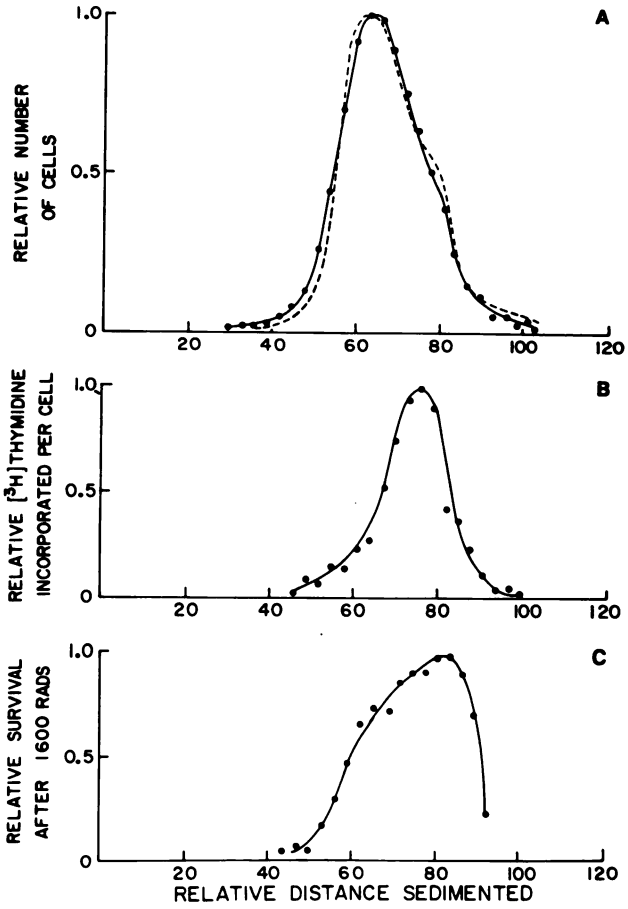

Chart 4. Sedimentation characteristics of asynchronous cells grown as small spheroids and compared to asynchronous single cells (*A*, - - -). All data were virtually identical to those of the single cells, with the exception of relative survival (see text).

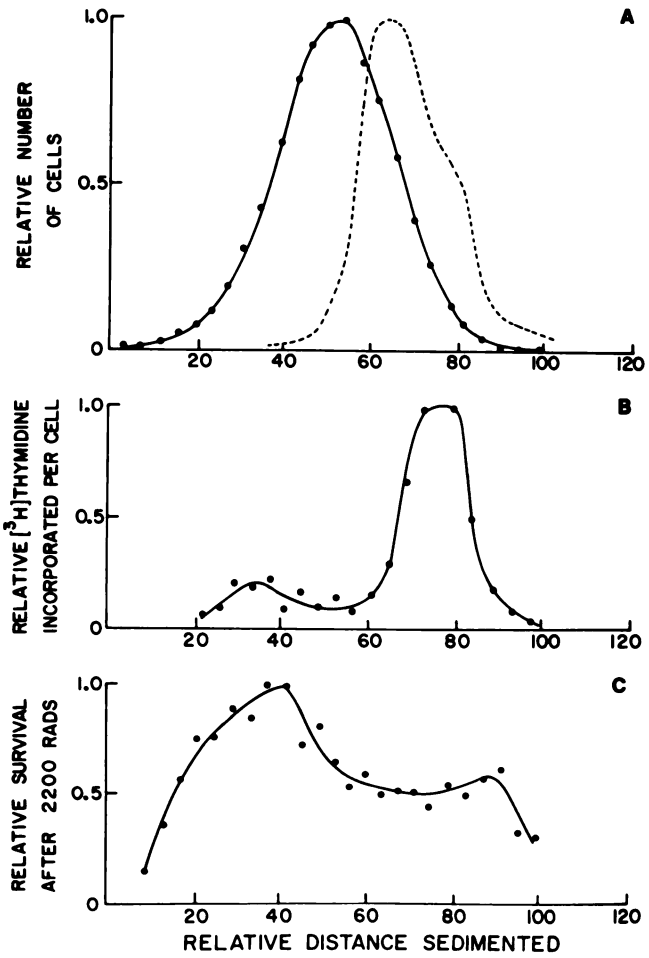

Chart 5. Sedimentation characteristics of cells from 17-day-old spheroids compared to asynchronous single cells (*A*, - - -). Note the absence of [³H]thymidine in the smaller cells after 24-hr labeling (*B*) and the preferential survival of the smaller (hypoxic) cells in *C* (see text).

size; however, theoretical considerations indicate that density also makes a minor contribution (7). Density changes may have occurred in the internal cells of the spheroids, since the smaller cells of spheroids sedimented more slowly than "equivalent" cells from plateau phase cultures. In addition, the hypoxic cells appeared to be smallest, suggesting that, fortuitously, both size and density changes may occur in this population and contribute to an even better degree of separation. This is significant, in that the "functional capacity" of these oxygen- and nutrient-depleted cells can now be observed more unambiguously.

Preliminary experiments (not reported here) established that essentially 100% of the cells introduced into the gradient apparatus were normally recovered. The sedimentation profile was found to be independent of the number of cells introduced into the apparatus for total cell numbers of $< 10^8$ (see Refs. 7 and 11 for a discussion of "streaming"); all results reported here used 5- to 10-fold fewer cells than the upper limit (see "Materials and Methods"). In addition, the viability of untreated cells was found to be independent of their sedimentation velocity; that is, cells from all fractions had an equal plating efficiency whether grown under standard or tumor-like conditions. This point was of particular importance for the cells obtained from spheroids, because the smaller internal cells were known to be hypoxic at the time of assay (9). This latter result demonstrates the ability of cells to retain a high growth potential despite localization in a nonpermissive growth region.

Since other methods of selection generally involve either mechanical or chemical manipulation of the cells prior to the selection process, one can never be completely sure that such cells are indeed representative of the population as a whole. This is of particular importance in the tumor models, because intercellular contact between viable cells has been shown to alter cell response to a least 1 cytotoxic agent, ionizing radiation (1, 4). Using the present technique, the treatment of interest can be applied to the cells *in situ*, and these cells can subsequently be separated for analysis, avoiding the ambiguities that arise whenever synchrony is achieved by selectively killing or perturbing part of the population before treatment.

Conversely, the techniques described here are subject to a number of limitations. A prerequisite is, of course, the necessity of obtaining a true single-cell population. This precludes the possibility of assaying response *in situ* and consequently introduces additional variables into an experiment. The separation technique requires more than 4 hr at 4°, which was not found to be cytotoxic alone, but may interact with other lethal agents. Since cell volume, density, and position in the 1.5-mm starting band on the gradient will contribute to the eventual position of the cell, the degree of separation is necessarily limited. Further, one would expect that even cells at exactly the same stage of the cell cycle would have a given volume distribution; hence, volume would not be expected to be the best indication of specific cell cycle position. Despite these limitations, the separation efficiency shown here suggests that technique may prove to be useful, particularly in the case of mixed cell types or mixed populations.

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