Separation of Erythroid Progenitor Cells in Mouse Bone Marrow by Isokinetic-Gradient Sedimentation

By J. Misiti and J. L. Spivak

Isokinetic-gradient sedimentation employing a shallow linear gradient of Ficoll in tissue culture medium was used to isolate erythroid progenitor cells (CFU-e) from mouse bone marrow. Following gradient sedimentation, 34% of the total nucleated cells and 48% of the CFU-e applied to the gradient were recovered, and three distinct modal populations of CFU-e could be distinguished. The slowest-migrating population did not require exposure to exogenous erythropoietin in order to form erythroid colonies in vitro. The other two modal populations of CFU-e required exposure to exogenous erythropoietin for differentiation. One of these, constituting 64% of the hormone-dependent CFU-e recovered, migrated with the bulk of the marrow cells, whereas the other migrated ahead of the bulk of the marrow cells. This latter population, which contained 34% of the CFU-e, was recovered with 11% of the marrow cells, representing a twofold to threefold enrichment. BFU-e migrated more slowly than the erythropoietin-dependent CFU-e. Resedimentation studies suggested that the two erythropoietin-dependent CFU-e populations were distinct modal populations. When cells from the fastest-migrating population of erythropoietin-dependent CFU-e were cocultured with unseparated marrow cells, a further twofold to threefold enhancement of erythroid colony formation was obtained. Comparison of isokinetic-gradient sedimentation with discontinuous and continuous albumin density-gradient sedimentation revealed that isokinetic-gradient sedimentation was a more efficient method than the former and a more rapid method than the latter for isolating CFU-e from mouse bone marrow.

The interaction of erythropoietin with its target cells cannot be studied to advantage employing suspensions of unseparated bone marrow cells because of the cellular heterogeneity of that organ. Not only are populations of nonerythroid cells present that are capable of influencing the growth of erythroid cells, but within the erythroid cell population itself there exist subpopulations of cells that differ with respect to sensitivity and response to erythropoietin. A variety of techniques can be employed to separate nonerythroid cells from erythroid cells and to fractionate erythroid cells according to their states of maturation. Surface adherence removes macrophages, monocytes, and some lymphocytes from marrow cell suspensions, and immune hemolysis destroys orthochromatic normoblasts, reticulocytes, and more mature erythrocytes that are present in such suspensions. Erythropoietin-responsive cells have been separated from the bulk of the marrow cells by discontinuous density-gradient centrifugation, whereas continuous (isopycnic) density gradients have been employed to separate morphologically identifiable erythroblasts from other marrow elements. Velocity sedi-

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mentation at unit gravity has been widely used to isolate subpopulations of immature erythroid progenitor cells (CFU-e) and, in conjunction with immune hemolysis, to obtain marrow cell populations enriched for pronormoblasts and basophilic normoblasts.

None of these cell separation techniques is without drawbacks. Discontinuous gradient centrifugation is an inefficient technique, since the accumulation of cells at each gradient interface impedes the progress of other cells through that zone. This limits the quantity of cells that can be processed and reduces separation efficiency. For continuous density gradient centrifugation, preparation of the gradient medium must be meticulous in order to avoid artifacts in cell migration caused by the influence of the medium on cell size. Sedimentation of marrow cells at unit gravity is time-consuming, since it must be performed at 4°C in order to reduce cell aggregation and minimize metabolic suicide. The quantities of reagents required are also substantial.

An alternative approach to the fractionation of marrow erythroid cells that avoids many of the problems mentioned is the technique of isokinetic-gradient sedimentation developed by Pretlow and associates. The technique is based on the observation that the viscosity of Ficoll in aqueous solution increases as an exponential function of the Ficoll concentration. By employing a shallow gradient of Ficoll and low centrifugal force, cells are sedimented at a constant velocity that varies with the diameter and density of the particular cell. Isokinetic-gradient sedimentation is capable of a high degree of resolution; it is less time-consuming than velocity sedimentation at unit gravity, and it subjects cells to lower centrifugal force than isopycnic sedimentation. It has been used successfully for the isolation of megakaryocytes and pluripotent stem cells (CFS-s) from bone marrow, as well as for separation of the cell components of a variety of organs and tumors. We have employed isokinetic-gradient sedimentation for the isolation of CFU-e from mouse bone marrow. We report here the results of our studies and a comparison of this technique with discontinuous and continuous albumin gradient sedimentation.

MATERIALS AND METHODS

Preparation of Marrow Cell Suspensions

Swiss-Webster female mice weighing 20–25 g (Buckberg Laboratory Animals, Tomkins Cove, N.Y.) were used in all experiments. The mice were killed by cervical dislocation, and the femurs were rapidly removed. The marrow cavities were flushed with 2 ml of alpha-modified minimal essential medium (MEM-alpha) containing Hank’s balanced salt solution, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cell clumps were dispersed by repeated aspiration through a 23-gauge needle. The cells were counted in a hemocytometer and resuspended in 5 ml of MEM-alpha containing 10% fetal calf serum. The total number of nucleated cells loaded on a gradient varied from $5 \times 10^8$ to $2 \times 10^9$.

Gradient Preparation and Centrifugation

Ficoll. Ficoll (approximate molecular weight 400,000, Sigma Chemical, St. Louis, Mo.) was dissolved in Eagle’s basal medium (BME) containing 25-mM HEPES (N-2-hydroxyethylpiperazine-N-ethanesulfonic acid) buffer at concentrations of 2.7% (w/w) and 5.5% (w/w) and sterilized by passage through a 0.45-μ Millipore filter. By use of a two-chamber gradient maker (MRS, Clearwater, Fla.), an 80-ml continuous linear gradient was constructed over a 5-ml cushion of 45% (w/w) Ficoll in MEM-alpha in a sterile 100-ml polycarbonate centrifuge tube (International Equipment, IEC 2806) at rate of 1.0 ml/min.

Dispersed marrow cells ($1–2 \times 10^8$) in 5 ml of MEM-alpha containing 10% fetal calf serum were
carefully layered over the gradient using a sterile pipette. Centrifugation was performed in an IEC centrifuge (model PR-B) with an IEC 269 rotor at 4°C employing 95 g for 8 min. Acceleration was initiated gradually, reaching the peak force of 95 g over a period of 1 min. The speed of centrifugation was regulated by monitoring an electronic tachometer (Power Instruments, Skokie, Ill.). Deceleration was accomplished without braking. Following centrifugation, the gradient was placed in an ice bath, and a gradient tapping cap (Halpro, Rockville, Md.) was inserted directly into the gradient. Through this cap, a dense (70% w/w) sucrose solution was pumped into the bottom of the centrifuge tube, and samples were collected from the top of the gradient. After discarding the initial 5 ml, which contained the starting sample, 3-ml fractions were collected. A refractive index was determined on an aliquot of each gradient fraction using an Abbe-3L refractometer (Bausch and Lomb, Rochester, N.Y.) to confirm the linearity of the gradient. Each fraction was then diluted with an equal volume of MEM-alpha and centrifuged at 1000 g for 10 min at 4°C. The cell pellet was resuspended in MEM-alpha for cell counts in a hemocytometer, for preparation of Wright-stained smears for differential cell counts, and for in vitro culture. When cell sizing was performed, the individual fractions were analyzed directly with a Coulter counter (model ZH) and channel analyzer as previously described.

Albumin. A 35% stock solution (w/v) of bovine serum albumin (fraction V, pH 7.0 or pH 5.2, Miles Laboratories, Elkhart, Ind.) was prepared by dissolving 100 g of albumin in 182 ml of a sterile salt solution consisting of 121 volumes NaCl (0.168 M), 4 volumes KCl (0.168 M), 3 volumes CaCl2 (0.112 M), 1 volume MgSO4 (0.168 M), and 1 volume KH2PO4 (0.168 M) to which 4 ml of water had been added.

For discontinuous albumin density gradients, aliquots of the 35% stock solution were diluted with the same salt solution to the following concentrations: 33%, 31%, 30%, 29%, 28%, 27%, and 25%. To ensure reproducibility, the refractive index of each solution was determined. The discontinuous gradient was constructed by layering 3 ml of each albumin solution in a sterile 30-ml Corex tube starting with the most dense solution. Dispersed marrow cells (5 × 10⁸ to 1 × 10⁹) in 3 ml of MEM-alpha, centrifuged at 600 g for 40 min. Following centrifugation, the gradient was displaced; after the initial 5 ml that contained the starting sample were discarded, 4-ml fractions were collected as described previously for the continuous Ficoll gradient. After determination of the refractive index of each fraction, each individual fraction was diluted to 16% and 33% with the salt solution described previously. A 70-ml linear continuous gradient was constructed from the 16% and 33% albumin solution using a two-chamber gradient maker. Dispersed marrow cells suspended in 5 ml of MEM-alpha containing 10% fetal calf serum were layered carefully layered with a sterile pipette on the top of the gradient. The gradients were centrifuged at 4°C in an IEC PR-B centrifuge equipped with an IEC 269 rotor at 600 g for 40 min. Following centrifugation, the gradient was placed in an ice bath, and a gradient tapping cap (Halpro, Rockville, Md.) was inserted directly into the gradient. Through this cap, a dense (70% w/w) sucrose solution was pumped into the bottom of the centrifuge tube, and samples were collected from the top of the gradient. After discarding the initial 5 ml, which contained the starting sample, 3-ml fractions were collected. A refractive index was determined on an aliquot of each gradient fraction using an Abbe-3L refractometer (Bausch and Lomb, Rochester, N.Y.) to confirm the linearity of the gradient. Each fraction was then diluted with an equal volume of MEM-alpha and centrifuged at 1000 g for 10 min at 4°C. The cell pellet was resuspended in MEM-alpha for cell counts in a hemocytometer, for preparation of Wright-stained smears for differential cell counts, and for in vitro culture. When cell sizing was performed, the individual fractions were analyzed directly with a Coulter counter (model ZH) and channel analyzer as previously described.

In Vitro Assay of CFU-e

Based on a determination of cell concentration, the volume of each gradient fraction was adjusted to provide a final concentration of 10⁷ nucleated cells per milliliter. Aliquots from each fraction were cultured in vitro by employing the plasma clot culture system described by McLeod et al. In brief, 10⁷ nucleated cells in a volume of 0.1 ml were suspended in a mixture containing 0.2 ml of fetal calf serum, 0.1 ml of deionized bovine serum albumin, 0.1 ml of beef embryo extract, 0.1 ml of citrated bovine plasma, 0.1 U of erythropoietin (sheep plasma step III, 7 U/mg protein, Connaught Laboratories, Willowdale, Ontario, Canada) in 0.1 ml of NCTC 109, and 0.3 ml of NCTC 109. Aliquots (0.1 ml) of the mixture were plated in the wells of a Microtiter plate (Linbro Chemical, New Haven, Conn.) and allowed to clot. The plasma clots were incubated at 37°C in a high-humidity 5% CO₂-95% air atmosphere for 48 hr. A benzidine- and hematoxylin-stained squish preparation of each plasma clot was obtained as previously described, and the number of benzidine-positive colonies containing eight or more cells was enumerated on three separate clots cultured from each gradient fraction.
Preliminary studies of in vitro erythroid colony formation by CFU-e revealed a linear relationship between the number of nucleated marrow cells plated and the number of erythroid colonies formed. The dose of erythropoietin employed (0.01 U/clot) was also shown in preliminary experiments to stimulate maximal colony formation with 10^5 nucleated marrow cells.

RESULTS

Distribution of Marrow Cells in an Isokinetic Gradient

Based on the observations of Pretlow et al., we employed a centrifugal force of 95 g for isokinetic-gradient sedimentation of mouse marrow cells. However, the time of centrifugation was varied in order to determine the duration of centrifugation that would provide adequate distribution of marrow cells through the gradient without excessive loss of cells on the gradient cushion. As shown in Fig. 1, centrifugation at 95 g for 8 min provided satisfactory separation of the marrow cells, and centrifugation for 11 min was associated with an increasing accumulation of cells on the cushion. On the average, 34% (range 32%-38%) of the total nucleated cells applied on a gradient were recovered.

Cell Composition of Gradient Fractions

The cell compositions of the individual gradient fractions as determined from Wright-stained smears are shown in Fig. 2. Lymphocytes had the slowest migration of all the identifiable nucleated marrow cells. Monocytes and granulocytes had approximately the same mobility. A population of large mononuclear cells with a high nuclear/cytoplasmic ratio migrated slightly ahead of the other formed elements. Although some of these cells may have been proerythroblasts, more mature erythroid precursors were not identified in this population.

Distribution of CFU-e in an Isokinetic Gradient

The sedimentation profile of CFU-e was determined by subjecting a suspension of marrow cells to isokinetic-gradient sedimentation and culturing aliquots of each fraction in vitro. The results of a representative experiment are shown in Fig. 3. Three distinct modal populations of CFU-e could be distinguished along the
gradient: one population migrated in the lightest part of the gradient (fractions 1–3), a second migrated with the bulk of the marrow cells (fractions 8–11), and the third migrated in front of the bulk of the marrow cells (fractions 13–16). This sedimentation profile was highly reproducible.

The CFU-e that migrated at the top of the gradient differed from the other two populations of CFU-e with respect to maturity and erythropoietin requirement. After 48 hr of culture in vitro, the colonies formed from these cells consisted of tight clusters or large aggregates of hemoglobinized red cells, most of which had completely extruded their nuclei, and the colonies or aggregates present per clot were usually too numerous to count. In addition, erythroid colony formation from
fractions collected in this area of the gradient did not require the addition of exogenous erythropoietin to the culture medium. This population of CFU-e is probably composed of cells triggered to differentiate in vivo by erythropoietin before being harvested by us for in vitro experiments.

There was no difference with respect to erythropoietin sensitivity or colony morphology between the other two populations of CFU-e. Both required exposure to exogenous erythropoietin for in vitro colony formation, and the colonies consisted of 8 to more than 50 benzidine-positive nucleated cells. On the average, total recovery of CFU-e applied to the gradients was 48% (range 40%–57%). Figure 4 depicts the distribution of erythropoietin-dependent CFU-e throughout a typical gradient as a percentage of the total erythropoietin-dependent CFU-e recovered from the gradient. The division of the CFU-e into two modal populations is clearly evident. Approximately 64% of the CFU-e recovered migrated with the bulk of the marrow cells, and approximately 35% of the CFU-e migrated ahead of the bulk of the marrow cells. Compared with a yield of 200–400 CFU-e/10^5 unfractionated nucleated marrow cells, the fastest-migrating population of cells contained 600–800 CFU-e/10^5 nucleated marrow cells, a twofold to threefold enrichment. This population of erythropoietin-dependent CFU-e was recovered with 11% (range 8%–14%) of the total nucleated marrow cells. The fractions 13–16 (Fig. 3), which contained the fastest-migrating population of CFU-e, did not contain a modal population of any particular cell type but rather consisted of a mixture of large immature mononuclear cells (38%), granulocytes (24%), lymphocytes (24%), and monocytes (14%).
Induction of hemolytic anemia with phenylhydrazine did not alter the sedimentation behavior of the two modal populations of CFU-e. The CFU-e population that initially migrated with the bulk of the marrow cells (fractions 7–12, Fig. 3) migrated in the same fashion (data not shown) during resedimentation. Owing to the small quantity of cells in the fastest-migrating population of CFU-e, resedimentation of this population could not be performed.

Size Analysis of Marrow Cells Fractionated by Isokinetic-Gradient Sedimentation

The sizes of the cells within the individual fractions following isokinetic-gradient sedimentation were analyzed in order to determine whether or not there was a correlation between cell size and migration. As illustrated in Fig. 2B, four distinct modal populations could be discerned on the basis of cell size. Although there was overlap between these populations, larger cells tended to migrate faster than smaller ones. No conclusions can be drawn concerning the sizes of the three modal populations of CFU-e that we observed, since there were too few cells in these populations to have a discernible influence on mean cell size within the gradient fractions.

Effect of Mixing Fractionated Marrow Cells With Unfractionated Marrow Cells

Since it is possible that the enrichment observed with the fastest-migrating population of CFU-e represented the removal of a cell population that inhibited erythropoiesis, mixing experiments were done. Marrow cells were subjected to isokinetic-gradient sedimentation, and the cells in each fraction were cultured for CFU-e alone or in the presence of \(2 \times 10^4\) unseparated marrow cells. In the presence of the unseparated marrow cells, the profile of CFU-e obtained by isokinetic-gradient sedimentation did not change. However, a further twofold to threefold enhancement of erythroid colony formation was obtained in the fractions containing the fastest-migrating population of CFU-e that could not be accounted for directly by the CFU-e present in the added unseparated marrow cells (Table 1). Since no inhibition of CFU-e proliferation was observed, it seems likely that the

<table>
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<th>Experiment Number</th>
<th>Rapidly Sedimenting CFU-e</th>
<th>Unfractionated Marrow Cells</th>
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<td>(1 \times 10^8)</td>
<td>(4 \times 10^4)</td>
<td>997 ± 63†</td>
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*Mouse bone marrow cells (\(2 \times 10^8\)) were sedimented on an isokinetic gradient. Aliquots of \(1 \times 10^8\) cells from the peak fraction containing the fastest-migrating population of CFU-e were cultured in vitro in plasma clots alone or together with \(2-4 \times 10^4\) unseparated mouse bone marrow cells. The values for CFU-e per culture represent means ± SEM of colonies present in three separate cultures for each experimental point.

†\(p < 0.005\).
enrichment of CFU-e obtained by isokinetic-gradient sedimentation was not due to removal of an inhibitory cell population.

Distribution of BFU-e in an Isokinetic Gradient

Having defined the sedimentation behavior of the CFU-e, it was of interest to determine the behavior of BFU-e following isokinetic-gradient sedimentation. For these experiments, the fractionated cells were cultured in plasma clots as described for the CFU-e, but the incubation period was extended to 8–9 days, and the concentration of erythropoietin in the incubation medium was increased to 2 U/ml. The sedimentation behavior of marrow BFU-e is indicated in Fig. 3. As expected from previous studies employing velocity sedimentation at unit gravity, these cells migrated more slowly than the erythropoietin-dependent CFU-e and were present in significant numbers only in fractions 5–8.

Distribution of CFU-e on a Discontinuous Albumin Density Gradient.

Sedimentation of marrow cells through a discontinuous albumin gradient was not an efficient method for separating CFU-e from the bulk of the marrow cells. As shown in Fig. 4, although a significant number of CFU-e accumulated at the gradient interface between albumin solutions with densities of 1.086 to 1.094, they represented only 18%–20% of the total CFU-e recovered. Furthermore, the degree of enrichment obtained for this population of CFU-e was only 1.5-fold.

![Graph](image-url)
SEPARATION OF ERYTHROID PROGENITOR CELLS

Density (gm/cm³)

1.064 1.072 1.081 1.086

Fig. 5. Distribution of nucleated bone marrow cells and CFU-e following sedimentation in a continuous albumin density gradient at pH 7.0; 2 × 10⁶ cells were applied to the gradient.

Distribution of CFU-e in a Continuous Albumin Density Gradient

Sedimentation of marrow cells through a continuous albumin density gradient at pH 7.0 yielded two modal populations of CFU-e (Fig. 5). A threefold to fourfold enrichment was obtained for the fastest-migrating population of CFU-e. However, in contrast to the situation with isokinetic-gradient sedimentation, neither population was significantly separated from the bulk of the marrow cells. Sedimentation at pH 5.0 did not alter the migration of the CFU-e,₂ but there was a reduction in erythroid colony growth, presumably because of exposure at an acidic pH.

DISCUSSION

The progression of an erythroid cell from its most primitive state to complete maturation is a complex process during which the cell is subjected not only to the influence of hormones other than erythropoietin but also to the influence of other cell populations residing in the bone marrow. The application of techniques for cell separation has been of considerable assistance in dissecting the complexity of the maturation process. For example, Clissold,¹ employing a discontinuous albumin gradient, was able to subdivide a morphologically homogeneous population of proerythroblasts from the bone marrow of anemic rabbits into subpopulations that varied with respect to response to erythropoietin. Using velocity sedimentation at unit gravity, Adamson and his associates have demonstrated that within the marrow CFU-e population there is heterogeneity with respect to sensitivity to certain growth and developmental hormones. Cell separation methods have not been applied to the same extent for study of cell-cell interactions within the marrow, but some provocative work has been done. Using the simple technique of surface adherence, Aye demonstrated that nonerythroid cells present in human marrow stimulate erythroid cell proliferation in vitro. Rinehart and associates, employing Ficoll-Hypaque density gradients, demonstrated that circulating monocytes suppress the in vitro proliferation of circulating human BFU-e. Wagemaker, employing both isopycnic gradients and velocity sedimentation at unit gravity, identified a cell population in mouse marrow that enhances the in vitro growth of BFU-e.
In applying cell separation methods to the study of erythropoiesis, an investigator has a choice of techniques that separate cells mainly on the basis of size. With respect to primitive CFU-e that cannot be identified morphologically, there is no a priori basis for judging which approach will be more rewarding. In view of the tendency for mammalian hematopoietic cells to have a restricted range of densities, velocity sedimentation has been favored over isopycnic sedimentation and has proved to be a fruitful approach. Using velocity sedimentation at unit gravity, it has been possible to separate age-related cohorts of CFU-e from each other and from CFU-s and to segregate cohorts of cells according to their phase in the cell cycle. Granulocytic progenitor cells (CFU-c) appear to migrate in the same fashion as one class of CFU-e.

In spite of its usefulness, velocity sedimentation at unit gravity is a cumbersome and time-consuming procedure. Therefore, in this study we investigated the use of an alternative technique, isokinetic-gradient sedimentation. Although there has been considerable experience with this technique, it has not been applied to the study of erythropoiesis. In comparison with velocity sedimentation at unit gravity, isokinetic-gradient sedimentation requires no specialized apparatus, the gradients may be prepared well in advance of the procedure, and the actual performance time is much shorter. It is difficult to compare the two techniques with respect to resolution of CFU-e populations, cell recovery, and degree of cell purification, since relatively few data are available for velocity sedimentation at unit gravity. On a qualitative basis, the separation of CFU-e into populations differing with respect to their requirements for in vitro erythropoietin by means of isokinetic-gradient sedimentation is quite similar to that observed with velocity sedimentation at unit gravity. The slowly migrating cohort of CFU-e that required no exogenous erythropoietin for growth is probably identical with the erythropoietin-independent fraction of erythroid precursors (fraction II) described by McCool et al.

Two modal populations of CFU-e that required exogenous erythropoietin in vitro were obtained with isokinetic-gradient sedimentation. Sedimentation profiles for CFU-e following velocity sedimentation at unit gravity usually reveal one modal population, occasionally with a trailing shoulder. Kinetic studies have indicated that within this modal population there is heterogeneity with respect to which phase of the cell cycle the cells are in. Whether or not differences in cell cycle phase are responsible for the two modal populations of erythropoietin-dependent CFU-e that were obtained with isokinetic-gradient sedimentation has not been established. In contrast to the CFU-e, the 8–9-day BFU-e migrated as a single population during isokinetic-gradient sedimentation and during velocity sedimentation at unit gravity.

Quantitatively, we recovered 34% of the total nucleated cells applied to the gradient and 48% of the CFU-e; no recovery studies for BFU-e were performed. A twofold to threefold enrichment of the CFU-e was obtained, and they were separated from 85% of the marrow cells. Although few quantitative data have been published for velocity sedimentation at unit gravity, recalculation of published data indicates that the recovery of total nucleated cells with that technique averages 40%. In the one instance in which recovery of CFU-e was examined, the yield was 57%, with a 4.7-fold enrichment. No quantitative data for the recovery and enrichment of marrow BFU-e have been reported with unit-gravity sedimentation.
The loss of CFU-e during velocity sedimentation at unit gravity may be due to the long period of time during which the cells are manipulated in the absence of erythropoietin. This is less of a problem with isokinetic-gradient sedimentation. Ficoll itself is not toxic to CFU-e growing in culture, and part of the loss of CFU-e is probably due to the "wall effect" associated with centrifugation. The fact that we obtained a twofold to threefold increase in CFU-e proliferation by mixing unseparated marrow cells with the fractionated marrow cells suggests that some CFU-e were indeed not "lost" but rather were unable to proliferate owing to the absence of helper or regulatory cells.

In comparison with the two other cell separation techniques commonly employed (discontinuous and continuous albumin density-gradient sedimentation), isokinetic-gradient sedimentation provided better overall separation of CFU-e from the bulk of the marrow and a higher recovery. Although on practical grounds isokinetic-gradient sedimentation is a preferable technique, continuous density-gradient (isopycnic) sedimentation is still a valuable adjunctive technique, since it can be used to determine the buoyant densities of the cells in the cell population under study.

ACKNOWLEDGMENT

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