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Improved Nonsynchronous Flow-Through Coil Planet Centrifuge without Rotating Seals: Principle and Application

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Abstract

The nonsynchronous flow-through coil planet centrifuge holds a coiled column assembly which is rotated at a freely adjustable rate in a given centrifugal force field. Flow is introduced through the running column without the use of rotating seals. The

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apparatus is capable of performing efficient countercurrent chromatography with polymer phase systems and elutriation of cells with physiological solutions. The versatility of the present scheme has been demonstrated by the partition of *E. coli* plasmid DNA and *Salmonella typhimurium* strains and by elutriation of mammalian cells.

INTRODUCTION

The nonsynchronous flow-through coil planet centrifuge is a particular type of planetary centrifuge which allows a freely adjustable rotational rate of the coiled separation column at a given revolutional speed. In the past the apparatus equipped with a dual rotating seal has been successfully built and used in our laboratory to demonstrate its capability in cell separations with both physiological solutions (*1*) and Albertsson's polymer phase system (*2*). Recently, the nonsynchronous flow-through coil planet centrifuge has been improved so that the system requires no rotating seals and, therefore, the separations can be performed without risk of leakage or cross contamination (*3*). However, the design of the apparatus caused considerable noise and vibration which limited its practical use.

This paper describes a newly designed nonsynchronous flow-through coil planet centrifuge without rotating seals and the preliminary results in separating cells and macromolecules.

APPARATUS

Principle of the Seal-Free Flow-Through Centrifuge System (*4*)

Figure 1 shows various types of seal-free flow-through centrifuge schemes and their mutual relationship. Each diagram illustrates the orientation and motion of the cylindrical coil holder attached to the flow tubes. The other end of the flow tube is fixed stationary on the central axis at the top of the centrifuge.

These schemes are classified into three categories according to the mode of the planetary motion as indicated at the top of the figure. They are also numbered I through VII in the order of their complexity where the simplest form of Scheme I in the synchronous series evolves toward the most complex nonsynchronous Schemes VI and VII through the nonplanetary Scheme V. In Scheme I the holder revolves around the central axis of the centrifuge and simultaneously counterrotates about its own axis at the same angular

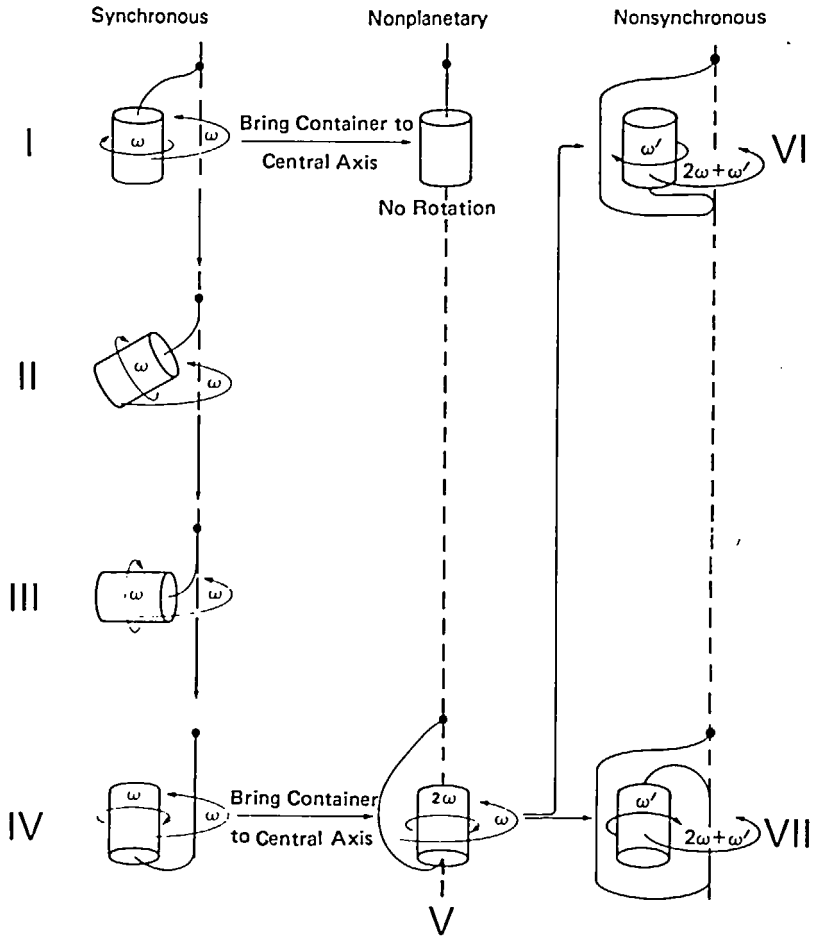


FIG. 1. Principle of seal-free flow-through centrifuge schemes. Each diagram indicates the orientation and motion of a cylindrical column holder with a bundle of flow tubes. They are classified into three categories as indicated on top of the figure and also numbered from I to VII according to the order of complexity. The simplest form of Scheme I in the synchronous evolves toward the most complex nonsynchronous through nonplanetary Scheme V. A sudden strange transition from the synchronous to the nonplanetary occurs at IV–V. The nonplanetary serves as a base for the nonsynchronous. The present apparatus was designed according to Scheme VI.

velocity (ω). This synchronous planetary motion of the holder is quite comparable to the circular motion of the beaker when a chemist gently mixes its contents with his hand. This simple twist-free mechanism works equally well in all other types of the synchronous schemes with tilted (Scheme II), horizontal (Scheme III), and even inverted (Scheme IV) orientations of the holder. The holder of Scheme IV is then moved to the central axis of the centrifuge to form Scheme V in which the holder simply rotates around the central axis of the apparatus at the doubled rate (2ω). Then the holder of this nonplanetary scheme is again moved away from the central axis to undergo a synchronous planetary motion identical to Scheme I or IV to produce, respectively, the nonsynchronous Scheme VI or VII. Because the rate of the top planetary motion (ω') is entirely independent of the rotational rate of the base (2ω), the rotational rate of the holder becomes freely adjustable at a given revolutional rate. The nonsynchronous flow-through coil planet centrifuge described in this article has a basic design of Scheme VI, Fig. 1.

Design of the Apparatus

Figure 2 shows a cross-sectional view through the central axis of the apparatus. The rotor consists of two major rotary structures, i.e., (rotary) Frames I and II which are coaxially bridged together with the center piece (dark shade).

Frame I consists of 3 plates rigidly linked together and directly driven by Motor I. It holds 3 rotary elements, the centerpiece (center), Countershaft I (bottom), and Countershaft II (top), all embedded in ball bearings. A pair of long arms extending symmetrically and perpendicularly from the middle plate forms the tube supporting frame which clears over Frame II to reach the central shaft on the right side of Frame II.

Frame II (light shade) consists of three pairs of arms linked together to rotate around the central shaft. It supports a pair of rotary shafts, one holding a coil holder assembly and the other the counterweight.

There are two motors, Motors I and II, to drive the rotor. When Motor I drives Frame I, the stationary pulley 1 introduces counterrotation of Pulley 2 through a toothed belt and, therefore, Countershaft I rotates at $-\omega_I$ with respect to rotating Frame I. This motion is further conveyed to the centerpiece by 1:1 gearing between gears 1 and 2. Thus the centerpiece rotates at $2\omega_I$ or at ω_I with respect to the rotating Frame I. The motion of Frame II, however, also depends upon the motion of Motor II.

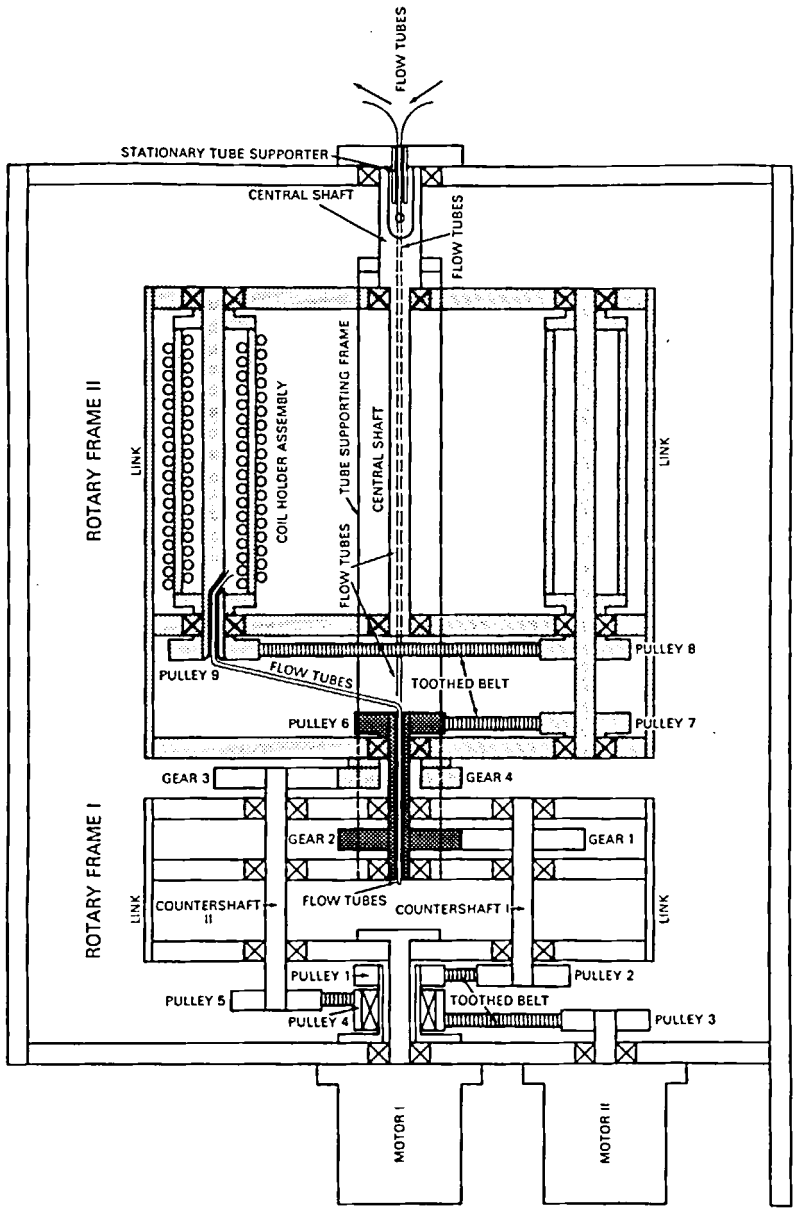


FIG. 2. The cross-sectional view of the prototype. Motor I produces a high speed revolution while Motor II gives a slow rotation of the coil assembly around its own axis. This design is based on Scheme VI in the nonsynchronous shown in Fig. 1.

If Motor II is at rest, Pulley 5 becomes stationary as is Pulley 1 so that Countershaft II counterrotates at ω_I as does Countershaft I. This motion is similarly conveyed to the rotary arms of Frame II by 1:1 gearing between Gears 3 and 4, resulting in rotation of Frame II at $2\omega_I$ or the same angular velocity as that of the centerpiece. Consequently, coupling of Pulleys 6 to 7 and 8 to 9 with toothed belts produces no additional motion to the rotary shaft which simply revolves with Frame II at $2\omega_I$ around the central axis of the apparatus.

When Motor II rotates at ω_{II}, idler Pulley 4 coupled to Pulley 3 on the motor shaft rotates at the same rate, which in turn modifies the rotational rate of Pulley 5 on Countershaft II. Thus Countershaft II now counterrotates at $\omega_I - \omega_{II}$ on Frame I. This motion further alters the rotational rate of Frame II through 1:1 gear coupling between Gears 3 and 4. Subsequently, Frame II rotates at $2\omega_I - \omega_{II}$ with respect to the earth or at $-\omega_{II}$ relative to the centerpiece which always rotates at $2\omega_I$. The difference in rotational rate between Frame II and the center piece is conveyed to the rotary shafts through coupling of Pulleys 6 to 7 and 8 to 9. Consequently, both rotary shaft rotate at ω_{II} about their own axes while revolving around the central axis of the apparatus at $2\omega_I - \omega_{II}$. This gives the rotation/revolution ratio of the rotary shaft

$$r/R = -\omega_{II}/(2\omega_I - \omega_{II}) \quad (1)$$

Therefore, any combination of revolutional and rotational speeds of the coil holder assembly can be achieved by selecting the proper values for ω_I and ω_{II}. The coiled column was prepared by winding 1 mm i.d. PTFE tubing continually onto six units of 0.68 cm o.d. stainless steel pipe to make about 600 helical turns with a total capacity of 15 mL. The column units were symmetrically mounted around the rotary shaft with screws through the hole at each end of the units. The flow tubes connected to the coiled column are first led through the hole of the rotary shaft and then pass through the opening of the centerpiece to exit at the middle portion of Frame I. The flow tubes are then led along the tube support frame to clear Frame II and then reach the side hole of the central shaft near the right wall of the centrifuge where they are tightly held by the stationary tube supporter. These tubes are lubricated with grease and protected with a piece of flexible plastic tubing at each supported portion to prevent direct contact with the metal parts.

Figure 3 shows the overall picture of the apparatus. The revolutional speed of the coil holder assembly is continuously adjustable up to 1000 rpm combined with any given rotational rate between 0 and 50 rpm in either direction.

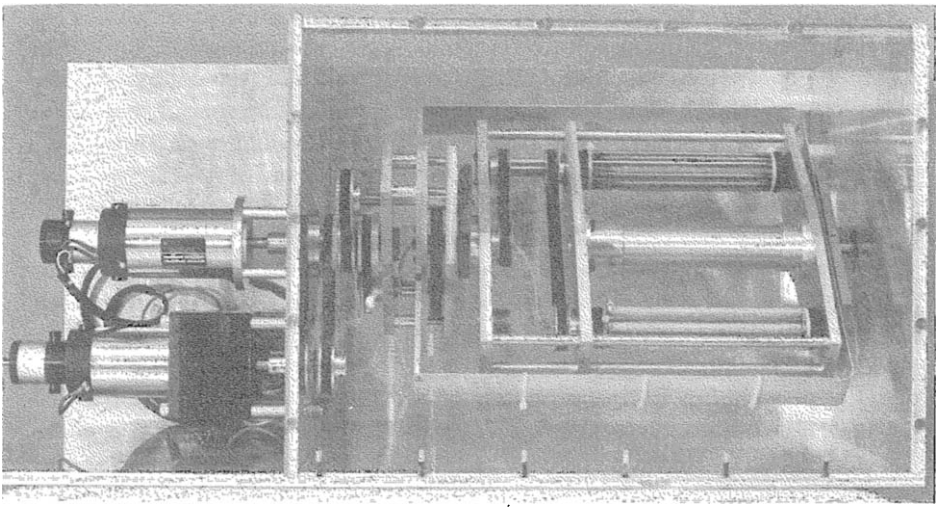


FIG. 3. Overall view of the prototype.

PRINCIPLES AND METHODS

Countercurrent Chromatography with Polymer Phase Systems

When a water-filled coiled tube is rotated slowly in the gravitational field, any object either heavier (glass bead) or lighter (air bubble) present in the coil tends to move toward one end of the coil. This end is called the head and the other end the tail of the coil. When such a coil contains two immiscible solvent phases, the rotation soon establishes a hydrodynamic equilibrium where each helical turn is occupied by nearly equal volumes of the two phases and any excess of either phase remains at the tail end of the coil. Under this hydrodynamic equilibrium condition, continuous elution of either phase through the head of the coil permits retention of the other phase in each helical turn while the two phases are constantly mixed by rotation of the coil. Consequently, solutes or particles introduced locally at the head of the coil are subjected to an efficient partition process resulting in chromatographic separation according to their partition coefficients. This countercurrent chromatographic scheme has been successfully applied with the conventional two-phase solvent systems in both unit gravity field (*5–7*) and

centrifugal force field provided by the synchronous coil planet centrifuge (*8, 9*). Because of its freely adjustable rotational rate under a given centrifugal force field, the nonsynchronous coil planet centrifuge further expands the application of the countercurrent chromatography to separate cells and macromolecules with polymer phase systems. The polymer phase systems are characterized by extremely low interfacial tension with high viscosity, and therefore have a high tendency of emulsification.

Preliminary experiments were performed to demonstrate the versatility of the present method which can separate a broad spectrum of samples. For solute separation, plasmid DNA was separated from RNA, and for cell separation, *Salmonella typhimurium* G30 cells were partitioned according to their membrane characteristics.

For plasmid DNA separation, crude preparation of the plasmid pBR 322 was obtained from a lysate of *E. coli* C600 r^-m^- according to the procedure used by Falkow et al. (*10*). The polymer phase system composed of 5% (w/w) dextran 500, 4% (w/w) polyethylene glycol 6000, and 10 m*M* sodium phosphate (pH 6.8) was equilibrated in a separatory funnel and separated before use. In each separation the coiled column was first filled with the stationary lower phase, and the sample solution containing 100 μg DNA was injected through the sample port located on the flow line between the inlet of the column and the outlet of the pump. Then the mobile upper phase was pumped through the head of the column at 8.5 mL/h (FMI Lab Pump, Fluid Metering, Inc.), while the apparatus was run at 1000 rpm combined with 5 rpm coil rotation. The effluent from the outlet of the column was continuously monitored with an LKB Uvicord S at 256 nm and fractionated with an LKB fraction collector. Aliquots of the fractions were then electrophoresed on 1% agarose gels run at 5 V/cm and, after completion, the gel was stained with ethidium bromide to visualize the DNA and RNA bands.

Samples of *Salmonella typhimurium* differring in cell surface lipopolysaccharide were prepared by growing *S. typhimurium* strain G30 (the gift of Dr P. Rick) which lacks UDP glucose-4-epimerase in proteose peptone–beef extract medium, either with galactose (0.01 to 0.1 *M*) or in its absence. In the presence of galactose, this strain makes a normal lipopolysaccharide; in its absence, it makes a short chain terminated at the point of addition of the first galactose in the molecule (*11*). Bacteria with these different lipopolysaccharides have previously been shown to differ in partition in countercurrent distribution (*12*).

The polymer phase system used for separation was composed of 6.2% (w/w) dextran 500 (Sigma), 4.4% (w/w) polyethylene glycol 6000 (Union Carbide Corp.), 0.05 *M* Tris-Cl (pH 7.0), 10 m*M* potassium phosphate (pH 7.0), and 0.01% sodium azide. In each separation the column was initially

filled with the polymer phase system consisting of about equal amounts of the upper and lower phases, and galactose(+) and galactose(−) cell mixture suspended in 1 mL of the upper phase was injected through the sample port. Then the upper mobile phase was pumped through the head of the column at 8.5 mL/h while the apparatus was run at 1000 rpm with the optimum coil rotation of 5 rpm. The eluates from the tail of the column were continuously monitored with an LKB Uvicord S at 280 nm and fractionated into test tubes. An aliquot of each fraction was diluted with distilled water and the elution profile of cells was determined by measuring the turbidity at 280 nm with a Beckman DU spectrophotometer.

Cell Elutriation with Physiological Solution

Figure 4 illustrates the principle of particle elutriation in a rotating coil (*4*). The coil is initially filled with water and then sample particles are introduced at the tail (top), followed by the rotation of the coil. Under a slow rotation (left), all particles always stay together at the bottom of the coil and move toward the head of the coil at a uniform rate of one helical turn per one rotation of the coil. In this case no separation is observed. When the rotational speed is increased (right), the particles fail to remain at the bottom of the coil and they are consequently retarded in their movement toward the head of the coil. Because the degree of this retardation depends on the sedimentation rate of the particles, the particles are chromatographically separated along the length of the coil according to their relative size and density. The fractionation of the particles becomes possible by introducing continuous flow through the column during separation.

This method can be applied to the separation of small particles with the nonsynchronous flow-through coil planet centrifuge. Preliminary experiments have been performed to demonstrate the potential capability of the present apparatus by the separation of human and sheep erythrocytes according to their size difference. Isotonic buffered saline solution (pH 7.4) was prepared by dissolving NaCl, 90 g; Na_2HPO_4, 13.65 g; and $NaH_2PO_4 \cdot H_2O$, 2.15 g in 1 L of distilled water and diluting 85 mL of this stock solution with distilled water to bring the final volume to 1 L. The human erythrocyte suspension was prepared from EDTA-treated, fresh adult blood by washing with buffered saline solution three times, repeating centrifugation, and decanting the supernatant. One part of the loosely packed cells obtained was finally mixed with 4 parts of the same solution. The sheep erythrocyte suspension was similarly prepared from ACD-treated blood stored at 4°C. Equal volumes of human and sheep erythrocyte suspension were combined and 0.2 mL of this mixture was used for each separation.

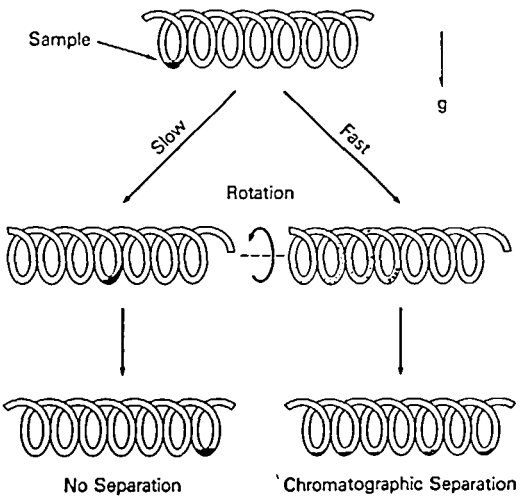
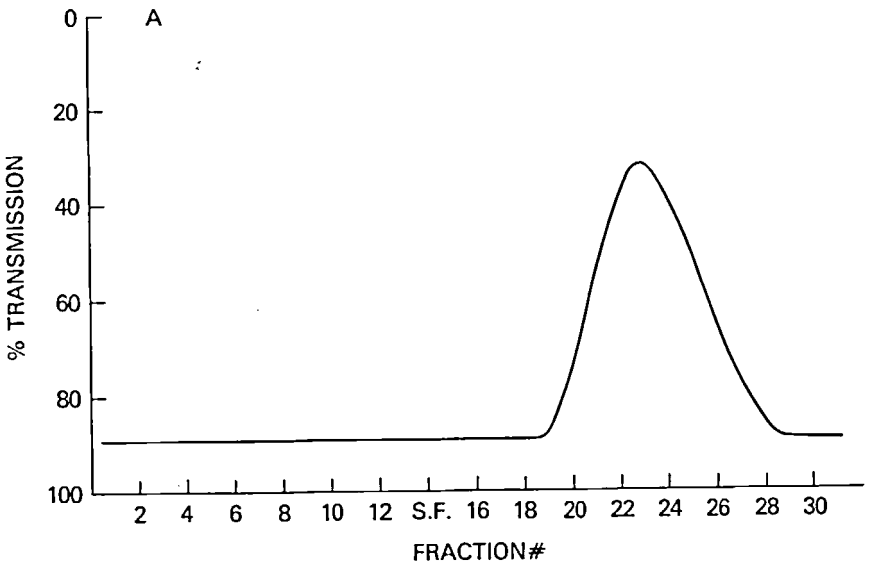


FIG. 4. Principle of particle separation with a rotating coiled tube. The coil is filled with water and the particle mixture is introduced in the tail end, both ends of the coil then being closed (top). Slow rotation of the coil carries all particles together toward the head without separation (left). As the rotational rate is increased, the larger particles move faster than the smaller particles and the particles are chromatographically separated along the length of the coil according to their sedimentation rates.

The separation was performed as follows: The column was first filled with the buffered saline solution and the sample mixture was injected through the sample port. Then the column was eluted with the buffered saline solution while the apparatus was run at 1000 rpm combined with a slow column rotation in either direction. The effluent from the outlet of the column was continuously monitored with an LKB Uvicord S at 280 nm and fractionated in the test tubes. The original sample mixture and some fractions were analyzed with a Coulter analyzer to obtain size distribution curves.

RESULTS AND DISCUSSION

Figure 5 illustrates the typical result obtained in separation of *E. coli* plasmid DNA with the polymer phase system. Figure 5(A) is an elution profile of the sample monitored with the Uvicord S at 256 nm. A broad peak recorded on the chart represents various RNA components which are partitioned rather evenly between the mobile and stationary phases. However, this monitoring system failed to detect a small amount of plasmid



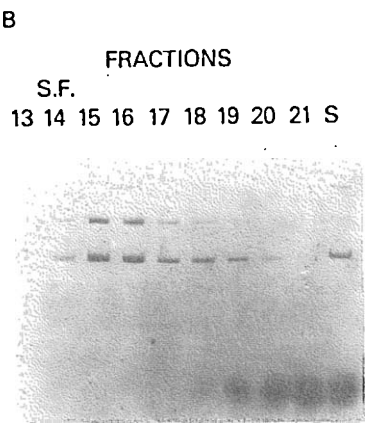


FIG. 5. Separation of *E. coli* plasmid DNA with the nonsynchronous flow-through coil planet centrifuge. (A) Countercurrent chromatogram of the crude sample on the polymer phase system. The chart shows a broad peak of RNAs. The plasmid DNA eluted immediately after the solvent front is not detected. (B) Agarose gel electrophoresis of the fractions. Fractions obtained with countercurrent chromatography above are further analyzed with gel electrophoresis. The plasmid DNA is detected in the early fractions and fairly well separated from the RNAs.

DNA in the sample which is almost entirely partitioned in the upper mobile phase and, therefore, eluted immediately after the solvent front (Fraction 14) marked on the chart. In order to analyze plasmid DNA, aliquots of early fractions, 14–21, were electrophoresed on 1% agarose gels run at 5 V/cm and, after completion, gels were stained with ethidium bromide to visualize the DNA and RNA bands. The plasmid DNA (sharp bands) is seen to run at the solvent front whereas the RNA (diffuse band near bottom of gel) first appears 3 to 4 fractions later. Although there is some degree of overlap, pooling of fractions containing DNA but not RNA, as determined on agarose gels, yielded recoveries of 74%. Boiling of the sample for 2 min followed by quick cooling in a Dry Ice–ethanol bath, prior to loading, resulted in the separation of closed circular plasmid DNA from denatured linear DNA, of which greater than 90% is retained in the stationary phase. The largest sample loading attempted was 400 μg of DNA, with equal resolution obtained.

Figure 6 shows three partition chromatograms of *Salmonella typhimurium* obtained with the present method. The cells grown without galactose are mostly partitioned either at the interface or in the lower stationary phase and, therefore, retained in the column for a long period of time, these cells being recovered by eluting the column contents after stopping the centrifuge run (top). The cells grown with galactose develop the outer layer with long polysaccharide which alters their partition behavior. As a result, they are mostly distributed in the upper mobile phase and, therefore, eluted from the column much earlier (middle). The chromatogram obtained from the mixture of these two types of cells reproduces the above two peaks at the respective locations (bottom).

The experiments have been continued with radioisotope labeling techniques which facilitate the analysis of the cell fractions with a high specificity. The recovery rate of the cells are also accurately measured in this way. It has been found that the column cleaned with 1*N* NaOH solution overnight gives near 100% recovery of the activity and the column can be reused without the risk of contamination.

Figure 7(A: top) shows the typical results of cell elutriation with a physiological solution. The preliminary experiments for separation of sheep and human erythrocytes were performed in two different ways. In the first method the coiled column was slowly rotated (12.5 rpm) in such a direction that the cells were held back toward the inlet of the column against the flowing stream (22 mL/h) (head-tail elution). Under this mode of elution the small sheep cells were eluted with a sharp peak which was followed by a broader peak of the large human cells as shown in Fig. 7(A: top). This elution profile closely resembles the size distribution curve of the original sample mixture obtained with a Coulter Analyzer (Fig. 7B).

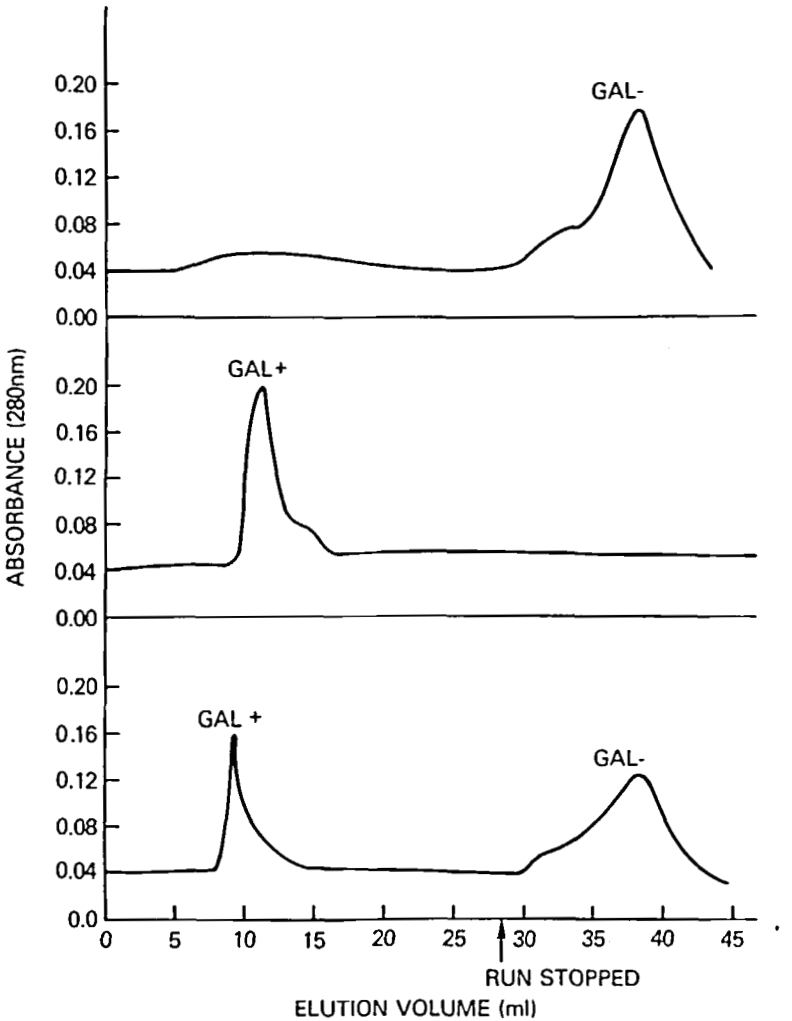


FIG. 6. Separation of *Salmonella typhimurium* strain on the polymer phase system with the nonsynchronous flow-through coil planet centrifuge. *S. typhimurium* G30 strain cells cultured on the galactose deficient media (gal −) are mostly distributed either at the interface or in the lower stationary phase and, therefore, retained in the column (top). The same strain of cells grown with galactose (gal +) develops lipopolysaccharide outer layer which alters their partition behavior. These cells are mostly distributed in the upper mobile phase and eluted out much earlier (middle). Countercurrent chromatography applied to the sample mixture containing these two types of cells produces two peaks each at the identical location (bottom).

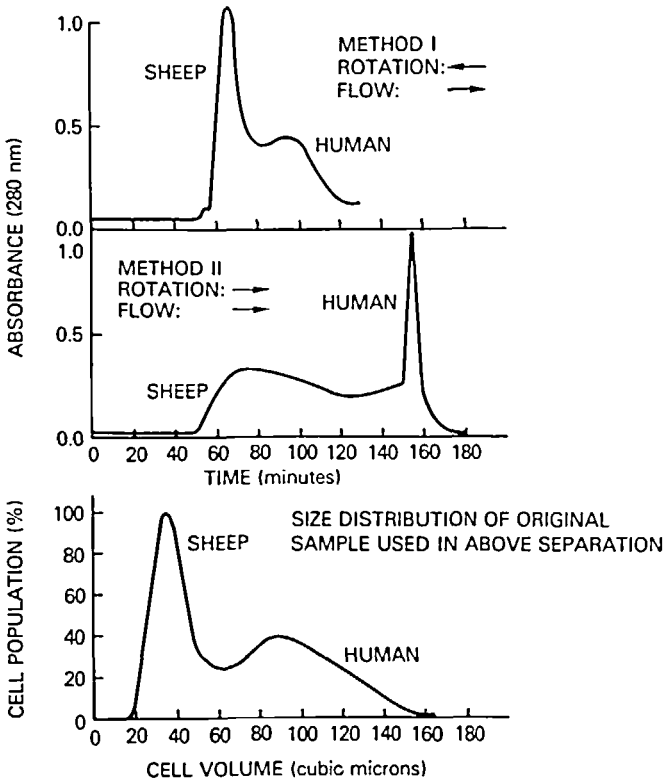


FIG. 7. Separation of human and sheep erythrocytes by elutriation with the nonsynchronous flow-through coil planet centrifuge. In the top figure (A), a mixture of human and sheep cells was eluted with the physiological solution in two different ways. In Method I (A: top), cells were held by coil rotation against the flowing stream, which produced an elution profile of cells quite similar to the size distribution curve of the original sample obtained with a Coulter Analyzer (bottom figure: B). In Method II (A: bottom), cells were slowly carried toward the outlet of the column by rotation while the flowing stream promotes the traveling rate of the smaller cells. This method produced a broad peak of the sheep cells and a sharp peak of the human cells.

In the second method the rotational direction of the coiled column was reversed so that all cells are slowly carried toward the outlet of the column by rotation (4 rpm) while the flowing stream (45 mL/h) further promotes the traveling rate of the smaller cells (tail-head elution). This mode of elution yielded a different elution curve characterized by a broad peak of the sheep cells and a sharp peak of the human cells as shown in Fig. 7(A: bottom). This method favors the recovery of the sample and also facilitates the programming of the separation times.

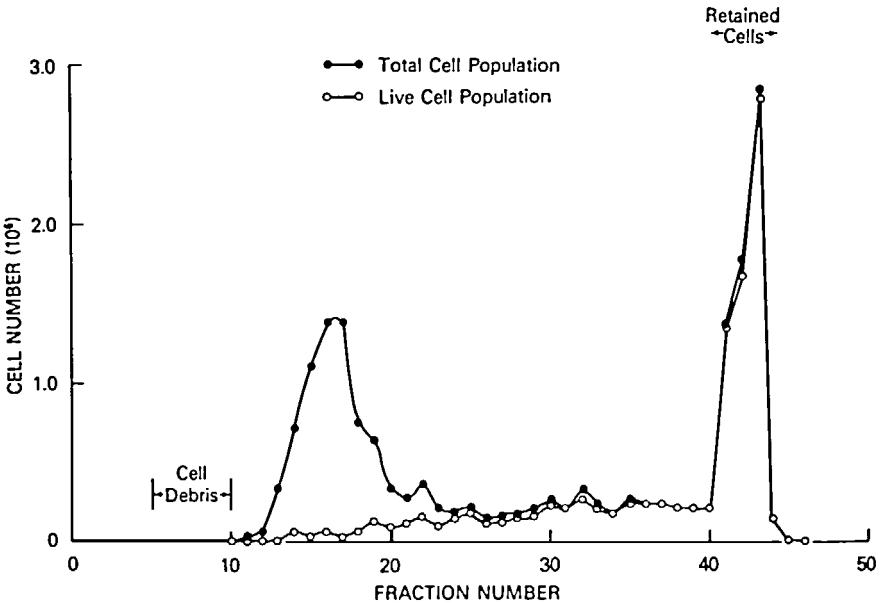


FIG. 8. Elutriation profile of rat liver cells with the nonsynchronous flow-through coil planet centrifuge. Damaged cells were eluted first as a sharp peak while most of the viable cells were retained in the coiled column.

The present method is now being applied to the separation of rat liver cells in collaboration with Dr Richard J. Chenery, Laboratory of Chemical Pharmacology, NHLBI. Sample cell suspension is prepared by means of intravenous collagenase infusion through the fresh rat liver. Figure 8 shows preliminary results of rat liver cell elutriation with a culture medium obtained by the head-tail elution. The elution profile of the total cell population revealed a large early peak followed by a low broad peak while a large population of the cells was still retained in the column. Dye exclusion microscopic viability test revealed that almost all cells in the first peak were damaged cells. The rest of the eluted cells were mostly viable and increased in size with the fraction number. The retained cells were the largest in size and the last few fractions consisted of aggregated cells probably resulting from incomplete digestion of the hepatic cords.

The fact that the nonviable cells were eluted early as a sharp peak strongly suggests that the cells were already damaged before being subjected to centrifugation; this was confirmed by a viability test of the original sample. The PTFE coiled column used for separation is easily sterilized by treating

with either acid or alkaline solution and the fractions thus obtained may be further used for cell culture or other experiments without risk of contamination.

The versatility of the nonsynchronous flow-through coil planet centrifuge has been demonstrated on separations of macromolecules and cells in both partition and elutriation techniques. The present method yields an efficient separation of a wide spectrum of materials and permits the use of physiological solutions which preserve the biological activity of samples. We believe that this separation technique will be very useful in biological and biochemical researches.

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