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Analytical Techniques for Cell Fractions. IV. Reorienting Gradient Rotors for Zonal Centrifugation[1]

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INTRODUCTION

In theory, zonal centrifugation in density gradients is a general method applicable to the separation of particles ranging in size from whole cells to small protein molecules. A series of studies performed in this laboratory (1–4) have culminated in the zonal ultracentrifuge (5, 6) in which the operations of gradient formation, sample layering, particle separation, and gradient recovery are all done during rotation in hollow, sector-compartmented rotors. At speeds in excess of approximately 60,000 rpm, however, fluid-line seals continuously attached to the rotor do not appear practical, although exploratory work in this direction is being continued.

For very high-speed zonal rotors two methods for loading and unloading appear feasible. A removable seal may be attached to the rotor at low speed, removed before acceleration to high speed, and reattached after deceleration (6), or the rotor may be loaded and unloaded at rest and the gradient allowed to reorient itself slowly from a static, or rest, position to a radially oriented configuration in the hollow rotor. The latter approach, using a simple plastic rotor (rotor A-VII), is explored in this paper. The results suggest to us that reorienting gradient rotors are feasible for preparative work at high speeds. Reorienting gradients have been previously employed in the isopycnic separation of serum lipoproteins in single tubes (7).

THEORY

The shearing forces occurring in a liquid confined in a closed cylinder during the transition from rest to a stable orientation in a high centrifugal

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force field may be considered qualitatively by examination of Fig. 1. The horizontal lines indicate levels or surfaces of equal density in a continuous density gradient.

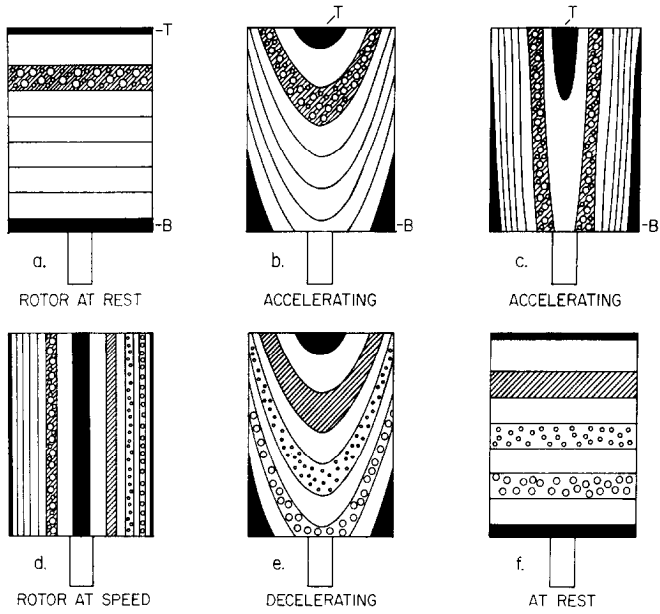

FIG. 1. Schematic diagram of reorienting gradient rotor system. (a) Rotor is filled at rest with density gradient and sample layer. To indicate extremes of zone deformation, a thin upper layer, T, and bottom layer, B, are also indicated. (b) During acceleration, each zone forms a paraboloid of revolution about the axis. Note T and B. (c) Near operating speed, the zones approach a vertical orientation. (d) At a sufficiently high speed, the zones become nearly vertical. Separation of particle zones is shown at right. (e) During deceleration, zones again form paraboloids of revolution. (f) At rest, various zones may be recovered by draining rotor contents out the bottom, or displacing the gradient out through the top.

At all speeds each isodense surface becomes part of a paraboloid of revolution whose intersection with an axial plane is described by the formula:

$$L = \frac{r^2\omega^2}{2 \times 980}$$

where L is the height from the apex of the paraboloid of a given point on the isodensity surface, r is the radius at that point, and ω is the angular velocity in radians per second (6). At a given rotational speed, ω, all isodensity curves are identical in shape, being merely transposed vertically. Acceleration results in a series of configurations shown diagrammatically

in Figs. 1b and 1c. At high speed, where the ratio between the centrifugal force and the acceleration due to gravity is very high, the isodensity surfaces will approach verticality, as is shown in Fig. 1d.

Deformations occurring at the various levels may be best understood by describing the changes occurring in layers originally at the top, middle, and bottom of the rotor. The fluid originally against the upper rotor cap becomes squeezed into a small paraboloid of revolution during acceleration (Fig. 1b, T) and then occupies the center of the rotor at high speed (Fig. 1d). A zone in the middle of the rotor (Fig. 1a) at rest increases in area during acceleration, and then decreases in area slightly as an approximately vertical position is approached. The zone at the very bottom of the rotor at rest (Fig. 1a, B) decreases markedly in surface area during acceleration (Fig. 1b, B) but covers the entire surface of the rotor wall at high speed (Fig. 1d). The greatest area changes, therefore, occur in those zones near the top and bottom at rest, which zones are near the center and the edge at high speed.

A mathematical analysis of the areas of isodensity surfaces (Fisher, Price, and Anderson, in preparation) shows that very little shearing occurs in the center of the gradient in a sector-compartmented rotor. While increases and decreases in area occur, the differences in rate of increase or decrease in the areas of adjacent zones is rather small. By placing a dense "cushion" in the bottom, and an overlay of light fluid above the sample layer at the top, the sample layer and the density gradient may be restricted to that part of the rotor where least shearing occurs. As the fluid layers change position during acceleration and deceleration, their tangential velocity will change, since the velocity at any point in the rotor is a function of both the rotational speed and the radius of the point. Fluid in the upper layer, originally near the edge of the rotor, decreases in tangential velocity relative to previously underlying fluid during acceleration, for example. Vertical septa are, therefore, considered necessary to prevent swirling during reorientation of the gradient.

EXPERIMENTAL

A small methacrylate rotor shown diagrammatically in Fig. 2 was constructed and mounted in a brass holder attached to a heavy flywheel rotor as shown in Fig. 3. The large mass allowed smooth controlled acceleration and deceleration in the International PR-2 centrifuge. This rotor, designated rotor A-VII, has a capacity of 127 ml. The gradient, when reoriented vertically during rotation, is 1.27 cm thick.

To test the rotor, 27 ml of a pH 7.5 sodium phosphate-NaCl buffer, μ = 0.1 (ref. 8), was pipetted to the bottom of the rotor at rest. An 80-ml linaer density gradient was formed using a two-cylinder gradient apparatus, and

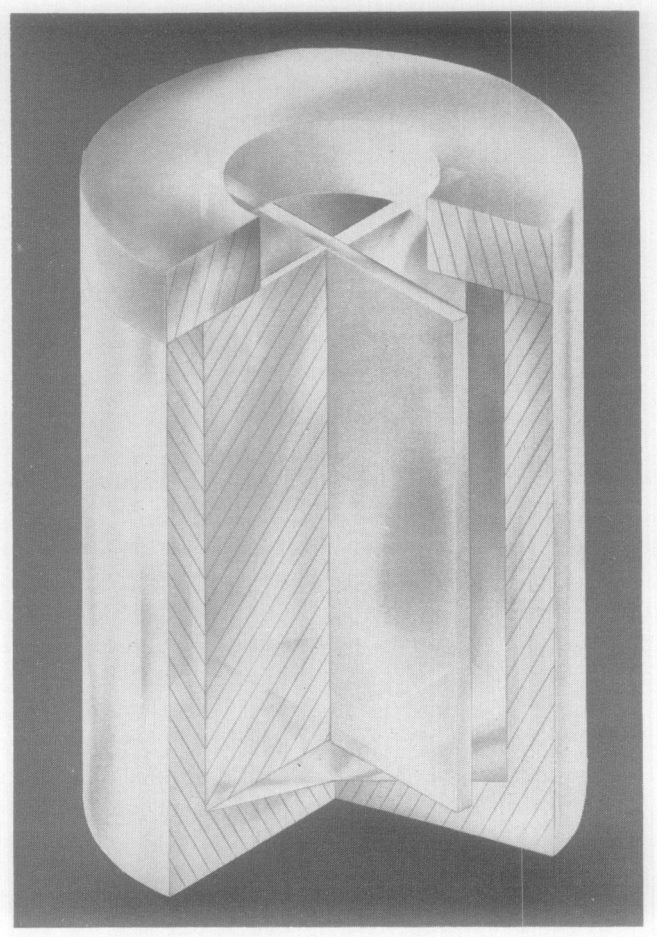

FIG. 2. Drawing of rotor A-VII. The hollow interior is divided into four sector-shaped compartments by vertical septa. The shallow conical bottom extends below the septa. The rotor is filled and emptied through a narrow tube inserted through the top opening to the bottom. At 3,000 rpm the gradient extends from the edge of the rotor only as far in as the edge of the center upper hole. The gradient does not, therefore, completely fill the rotor at rest. A rubber stopper is inserted to minimize air friction effects during operation.

was pumped to the bottom of the rotor using a Technicon proportioning pump and a flow rate of 1.89 ml/min. The gradient extended from 17 to 60% (w/w) sucrose. A 20-ml underlay of 60% sucrose was pumped in after the gradient. After an interval of 15 min the gradient was pumped out of the rotor from the bottom using the same pump and flow rate, and was collected in 5-ml volumes. The same experiment was performed with the

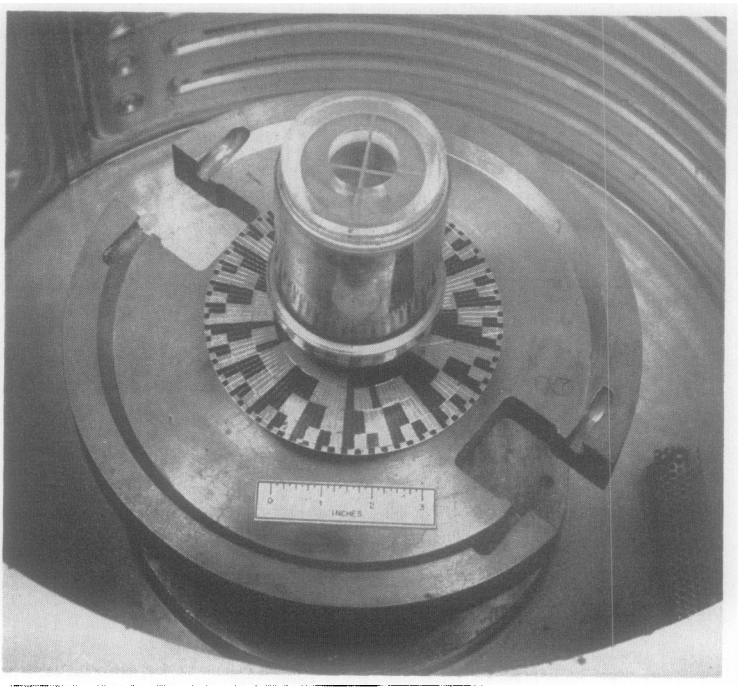

FIG. 3. Complete A-VII rotor assembly.

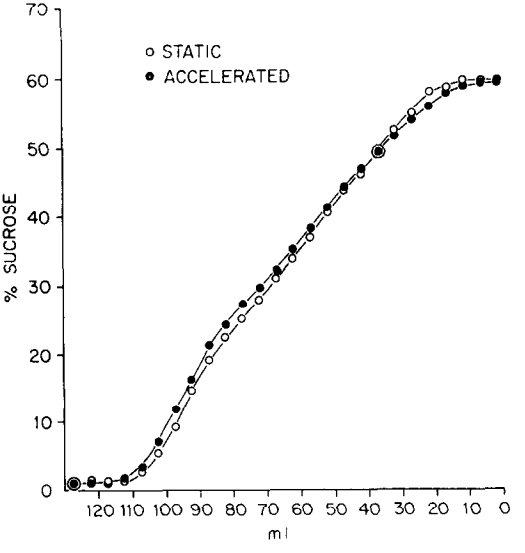

FIG. 4. Analysis of density gradients recovered from rotor filled and emptied at rest, and rotor accelerated to 3,000 rpm between filling and emptying.

rotor slowly accelerated to 3,000 rpm, and decelerated to rest over a 15-min interval. The gradients were analyzed for sucrose content refractometrically, and the results are recorded in Fig. 4. It is evident that very little disturbance in the gradient has occurred during the transition from rest to 3,000 rpm and back to rest.

To determine whether a sample zone could be maintained in position during the rest-to-rotation transition, 1 ml of an 8% solution of bovine serum albumin was introduced to the bottom of the rotor between the overlay and the sucrose gradient. After acceleration to 3,000 rpm and deceleration to rest over a 15-min period, the rotor contents were pumped out through the ultraviolet absorption analyzer, which was set at 280 mμ with a 0.2-cm light-path flow cell (9). The results, replotted in terms of rotor radius with the rotor volume also included, are shown in Fig. 5. The

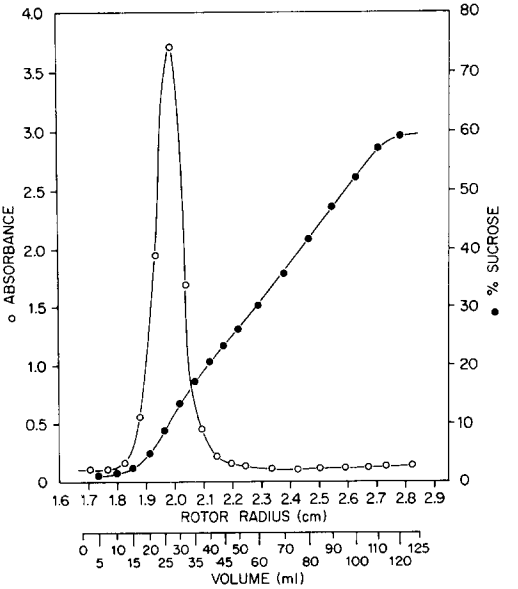

FIG. 5. Stability of sample layer during acceleration to 3,000 rpm and deceleration to rest. The sample layer was 1.0 ml of 8% bovine serum albumin layered over a sucrose gradient.

sample as introduced had a calculated width of approximately 0.1 mm. After acceleration to 3,000 rpm and deceleration to rest, the width of the sample peak at half-height as observed by the monitoring system was 1.0 mm. This amount of broadening, which is probably due to both diffusion and laminar mixing in the fluid lines, is not prohibitively large for the purposes described in the discussion.

In the absence of septa, circular flow in the rotor during acceleration and deceleration produce marked boundary spreading.

To see whether a zone of material which had been banded sharply at a point further down the gradient could be recovered with good resolution, 40 mg of ragweed pollen in 2 ml of 8% sucrose was used as the sample layer. The experimental conditions were identical with those described for the bovine serum albumin experiment. After deceleration from 3,000 rpm to rest the absorbance at 280 mμ and the sucrose concentration were determined, as shown in Fig. 6. A clear yellow pigment released from the pollen

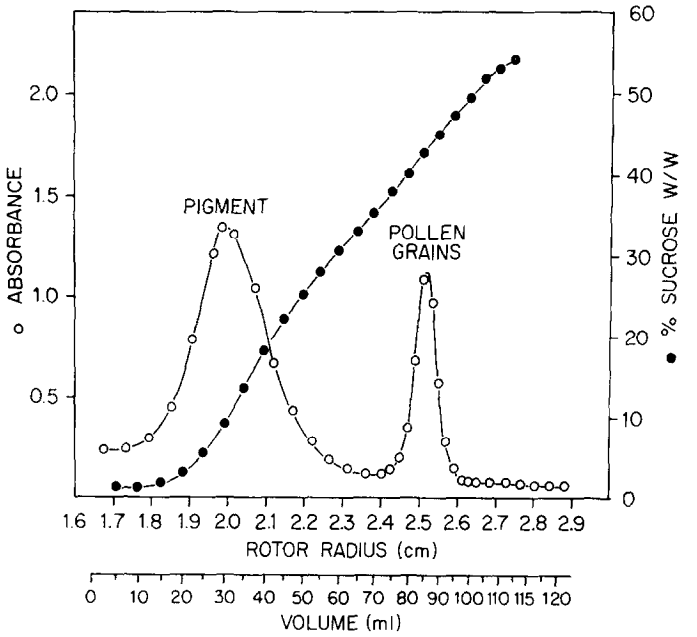

FIG. 6. Banding of ragweed pollen grains in sucrose gradient.

grains accounted for nearly all of the absorbance in the region of the starting zone. Extraction of the pigment from the pollen grains as they sedimented through the gradient probably accounts for the skewed tail observed in the starting zone curve. A small number of pollen grains were found through the starting zone. These may have been cells which had begun to swell in the sucrose solution. The vast majority of the cells banded in a narrow zone at the 43% w/w sucrose level. The width of the peak at half-height corresponds to a zone thickness of 0.87 mm in the rotor during rotation. In these experiments the pollen was not treated with alcohol, as is generally done when the grains are used as standards for particle counters.

DISCUSSION

The present experiments provide empirical verification of the feasibility of reorienting gradient rotors by showing that, in a low-speed rotor, liquid density gradients can be recovered virtually unaltered after transition from rest to speed and back to rest. Tests using bovine serum albumin and ragweed pollen showed further that sample zones are not excessively widened during the transition.

The basic advantage of the reorienting gradient rotor is its application to the problem of large-capacity rotors operating at very high speeds where rotating fluid-line seals may not be feasible.

We are now considering a specific problem to which reorienting gradient rotors can be adapted, involving the banding of milligram quantities of nucleic acid or protein in large-volume gradients. Banding in salt gradients has been severely restricted by the small amount of solution which can be centrifuged at high speed, and by the length of time required to form the gradient in the centrifugal field. The zonal ultracentrifuge recently described (6), which employs a rotating fluid-line seal, provides both rapidly made gradients and large capacity. However, such rotors are restricted in their present state of development to speeds below 40,000 rpm. In principle, the reorienting gradient rotor provides large gradients formed in a reasonably short time and the possibility of very high-speed operation, thus permitting the separation of large quantities of material with high resolution.

Since the diameter of a high-speed rotor is determined and limited by the strength of available materials, large rotor capacity can be obtained only by using elongated, cylindrical configurations. (The length, in turn, is limited by vibrational problems.) A zone occupying a narrow band in the density gradient during rotation will, when the rotor is returned to rest, be physically thicker as its cross-sectional area is decreased. This is true even though the zone occupies the same volume of fluid during rotation and at rest. Band widening by diffusion and by laminar mixing along the wall during unloading is therefore minimized.

In these experiments, the density gradients have been preformed. It is evident that reorienting gradient rotors may be used with preformed gradients for separations based either on sedimentation rate or on density alone by isopycnic banding, and that the gradients for the latter type of separation may be formed in the rotor by the centrifugal field.

Rotors using the reorienting gradient principle for operation at speeds above 141,000 rpm are now undergoing preliminary testing.

SUMMARY

Liquid density gradients and experimental sample bands have been successfully recovered from a hollow-bowl centrifuge rotor in which a static

gradient is reoriented into a radial configuration during acceleration and is subsequently recovered in its original orientation at rest. Analysis of fluid movements in the rotor indicate that very little shearing occurs during gradient reorientation. The reorienting gradient rotor principle makes feasible the construction of high-speed, large-volume rotors suitable for banding nucleic acids and proteins in salt gradients, and for the separation of macromolecules on the basis of their sedimentation rate through density gradients.

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