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Analytical Techniques for Cell Fractions
XV. Rotor B-XXIX—A New High-Resolution Zonal Centrifuge Rotor for Virus Isolation and Cell Fractionation

NORMAN G. ANDERSON, C. E. NUNLEY, AND C. T. RANKIN, JR.

The Molecular Anatomy (MAN) Program,[1] *The Oak Ridge National Laboratory,*[2] *and The Separations Systems Division, The Oak Ridge Gaseous Diffusion Plant,*[2] *Oak Ridge, Tennessee 37830*

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Zonal rotors previously developed in the MAN Program have found wide use in the isolation of subcellular particles, viruses, proteins, and nucleic acids (1,2). Separations may be made on the basis of sedimentation rate or banding density in gradients of large volume on a preparative scale with high resolution. In nearly all rotors previously described the gradients are recovered from the rotor by displacement using a dense solution pumped to the rotor edge (3). For a number of interesting separations, however, it is desirable also to be able to recover gradients or portions of the gradients from the rotor edge (4). Mitochondria, lysosomes, cell membranes, and peroxisomes have very similar banding or isopycnic densities in sucrose gradients but have different sedimentation rates. These particles start together from the sample zone, form rather well-resolved zones during rate sedimentation, and then bunch together in sucrose gradients in the region of 40–45% w/w sucrose. Removal of the denser or edge portion of the gradient and its replacement with fresh solutions at the proper intervals should allow the recovery of particle zones not only having different sedimentation rates but also with a larger range of sedimentation coefficients.

Edge-unloading rotors would also solve an important isolation

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problem in virology, namely, the isolation of virus particles from volumes too small to require use of high-speed continuous-flow rotors and too large for conventional swinging-bucket or angle-head rotors. The Oak Ridge B-series continuous-flow rotors which also employ liquid gradients (these include VIII, IX, XVI, and XXVI) (1,5) allow virus particles to sediment from a moving stream into a short gradient which remains in the rotor. The band of virus is then recovered with the gradient at the end of the experiment. The method has the advantage of not pelleting the virus and thus entrapping it in debris, and of simultaneously achieving purification based on sedimentation rate (more slowly sedimenting particles remaining in stream) and on buoyant density differences. It is presently employed in the commercial purification of influenza vaccine and for purification of virus for research purposes. However, the B-series rotors are most efficiently employed with volumes of 4 to 5 liters or more, while the Oak Ridge K-series rotors (including K–II, III, VI, X, and XI) are designed for volumes in excess of 100 liters (2,6,7). An unfilled requirement therefore exists for rotors which will effectively concentrate and band virus from liter volumes of solution.

Edge-loading rotors also allow sample zones containing particles lighter than the gradient to be introduced at the dense end of the gradient for separation by flotation.

Where very slowly sedimenting materials are to be separated, the sample layer may be introduced through the edge line and followed by a short gradient. At intervals, additional gradient may be added from the edge, moving the initial sample zone toward the axis, but keeping the sedimenting zone near the edge in the region of the highest centrifugal field.

The problems associated with designing surfaces which will channel gradients and the particle zones they contain out of zonal rotors through the center core have been previously considered (3,4,8). But problems of funneling at the edge are somewhat different. During unloading from the center, the zones are being increased in thickness and decreased in area normal relative to the radius, resulting in decreased loss of resolution due to diffusion as the zones move toward the axis. If particles are still sedimenting, they sediment *away* from the core surfaces and do not tend to aggregate. The opposite occurs during unloading from the edge. Zone area increases directly with radius until the funneling surfaces are reached, and sedimenting particles may impact the rotor walls during unloading. A partial solution to the design of edge-unloading rotors has been previously presented (4).

DESIGN OF B-XXIX ROTOR

In a previous study with a rotor constructed of aluminum and designated B–XXIII, tapering of the rotor walls was shown to allow edge unloading, but some loss in resolution was observed (4). Not only has the internal configuration therefore been modified to include tapering to give the maximum radius in an equatorial ring but also the ring has been modified to form a so-called "super circle" with four equal minimum radii and four equal maximum radii. The fluid lines to the rotor edge pass to these maximum radii through small projections on the four septa (Figs. 1 and 2). Clearance between the projections and the rotor wall is 0.008″ but may be increased to 0.032″ with no apparent loss of resolution.

The taper at the edge in the B–XXIII rotor was 20° from vertical. In the B–XXIX rotor, this angle has been reduced to 10°, giving a longer effective sedimentation path in the rotor.

Fluid flowing into the rotor in the form of a gradient of increasing density passes through horizontal lines in the four septa to the rotor edge through small projections which fit into a small

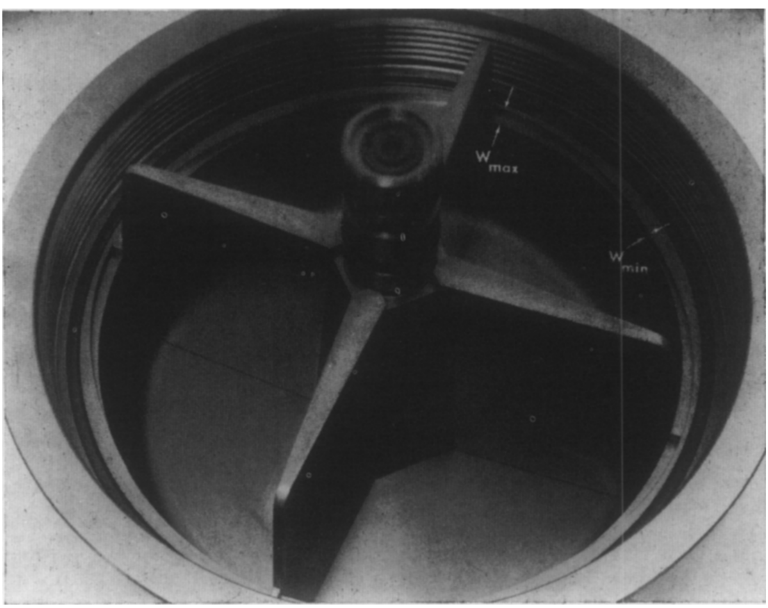

FIG. 1. Partially assembled B–XXIX rotor showing variation in width (W) of equatorial collecting ring. Maximum widths (W_{max}) occur at the four positions where septal projections approach the outer edge. Midway between these points the minimum width (W_{min}) occurs. Note also sloping inner surfaces of the rotor wall.

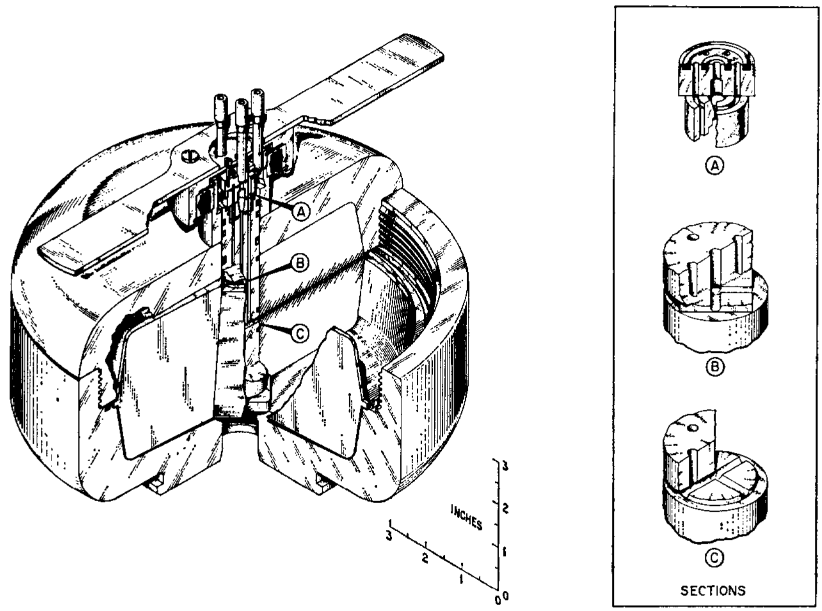

FIG. 2. Cutaway drawing of B–XXIX zonal rotor and seal.

groove running equatorially around the inner rotor wall. In each quadrant this groove is 1/8″ high and varies in radius by 1/8″ according to the equation:

$$X^{2.25} + Y^{2.25} = 3.368^{2.25}$$

Fluid flowing into the rotor at the greatest radius of this groove may flow circumferentially a maximum of approximately 45° in each direction from each septum. A portion of a gradient or a sample zone thus increases to a surface area of 2.65 in.^{2} during this horizontal flow. Further movement toward the axis results in further increase in zone surface area along the 10° tapered walls. While passing these tapered wall surfaces, the zone moves centripetally a distance of 0.461 in. and reaches a maximum surface area of 55.2 in.^{2} It then decreases in area almost directly as the radius. In a centrifugal field, tapering of this steepness can be effective; in contrast at 1*g* considerable mixing would occur.

The curve for volume as a function of radius is shown in Figure 3A, starting with zero volume at the center. This curve is useful for calculating zone positions using center loading and unloading. For edge loading the curve shown in Figure 3B is preferable where volume is calculated as a function of distance from the greatest radius.

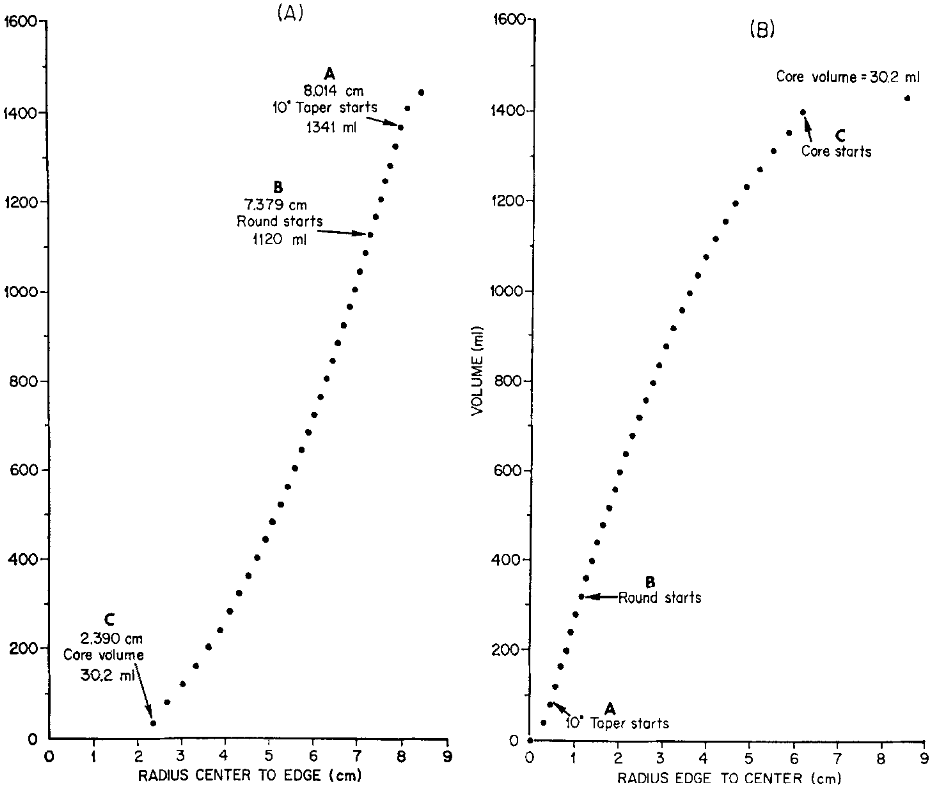

FIG. 3. Volume as a function of radius for the B–XXIX rotor (A) Calculated from the rotor *center*; for use when loading and unloading rotor from the center. (B) Calculated from the rotor *edge;* for use when loading and unloading from the rotor edge.

The stages in loading and unloading the B–XXIX rotor from the *edge* are shown in Figure 4. The steps in *center* loading and unloading are identical to those illustrated in a previous paper (3). The characteristics of the B–XXIX rotor are listed in Table 1.

EXPERIMENTAL STUDIES

Comparison of center and edge unloading with isopycnic bands.[3] The initial experiments were to compare center and edge unloading in the B–XXIX rotor using a nonsedimenting (isopycnic) parti-

[3] The following conventions are adopted in presenting data from zonal centrifuge experiments. Fractions are numbered and data plotted from left to right in the order collected, regardless of whether the rotor is unloaded from the center or from the edge of the rotor. Since a plot of the gradient is always included, reference to it will indicate at once whether unloading was done from the edge or center.

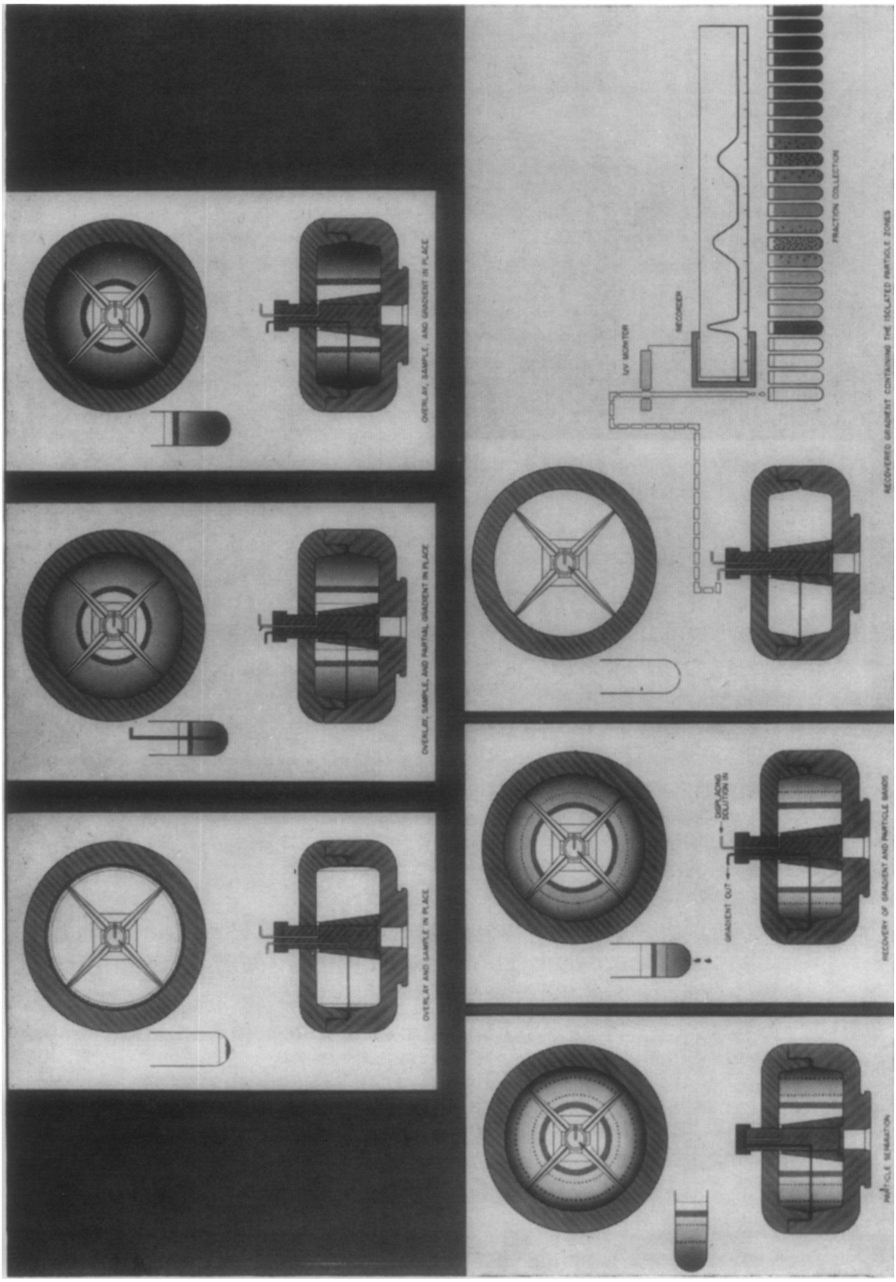

FIG. 4. Sequence of loading and unloading B–XXIX zonal rotor from the edge. Comparable steps in the loading and unloading of a swinging-bucket tube are included.

TABLE 1

Characteristics of the Titanium B-XXIX Rotor

Material	Titanium alloy 6A14V
Weight	13.063 kg
Total volume	1430 ml
Core taper volume	30.2 ml
Edge taper volume	310 ml
Provisional maximum speed	28,000 rpm
Provisional maximum centrifugal force	74,874 *g*
Minimum radius	1.45 cm
Radius at edge of core taper	2.39 cm
Radius to beginning of edge taper	7.348 cm
Maximum radius	8.552 cm

cle band. The rotor was initially filled with 40% (w/w) sucrose (1050 ml) followed by 380 ml of 55% (w/w) sucrose. The sample consisting of prewashed and prebanded ragweed pollen was introduced from the center in a volume of 1 ml and was followed by 50 ml of distilled water. The rotor was run 10 min at 2500 rpm and unloaded through the center line by displacement with 55% (w/w) sucrose pumped into the rotor edge (Fig. 5A). In a comparable experiment, the rotor was unloaded from the edge by displacement with water pumped into the rotor center (Fig. 5B). The peak width at half-height was identical in both experiments, showing that the tapering surfaces at the edge were effective.

Comparison of the B–XV and B–XXIX rotors. In these experiments both rotors were loaded and unloaded from the edge. Initially the rotors were filled with water. A mixture of 19 ml of 0.02% (w/v) of polystyrene (Dow Uniform Latex particles; ρ 1.05, average diameter 0.091 μ; standard deviation 0.0058 μ) and 1 ml of 66% w/w sucrose was them pumped in to the edge, followed by a 500 ml gradient extending linearly from 5 to 10% (w/w) sucrose. The rotor was operated at 2500 rpm and was unloaded as soon as loading was complete. The particles are isopycnic at 1.05 gm/cc (13% w/w sucrose) and were still sedimenting during unloading. The results with the B–XV and B–XXIX are shown in Figures 6A and B, respectively. Considerable decrement in resolution is seen in the B–XV compared with the B–XXIX, but no loss of beads on the B–XV wall was apparent, either by comparing areas under the curves or on examining the rotor walls at the end of the experiment. However, this experiment does not distinguish between resolution loss at the edge during loading and during unloading.

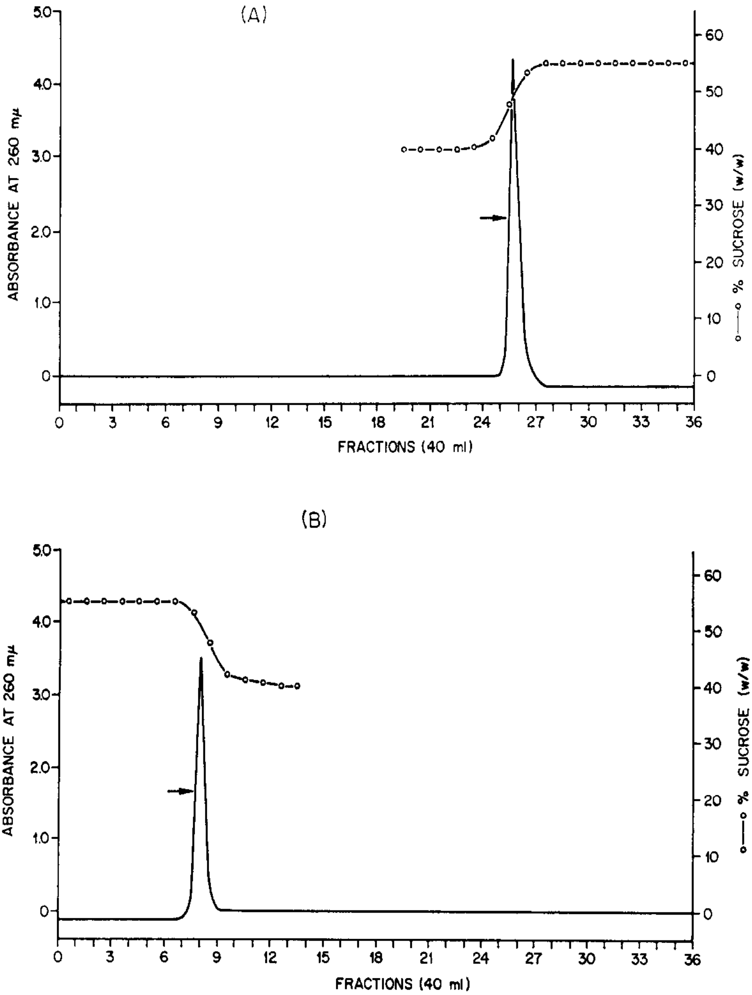

FIG. 5. (A) Isopycnic banding of alcohol-washed and prebanded ragweed pollen in sucrose gradient. Sample loaded through center flow line, recovered by displacing with 55% (w/w) sucrose to edge, and collected at the center flow line. (B) Gradient, sample, and run conditions were the same as in A. Unloading was accomplished by introducing water through the center flow line and collecting at the edge flow line.

Since both rotors have identical core surfaces, this problem can be examined by loading at the center and unloading from the edge. One liter linear 5–16% (w/w) sucrose gradients were introduced from the edge, and additional 16% sucrose was pumped in to fill the rotors. The samples (3 ml, 3% w/v bovine serum albumin plus 7 ml 4% sucrose) were then introduced through the center line followed by 50 ml of 3% w/w sucrose as the overlay.

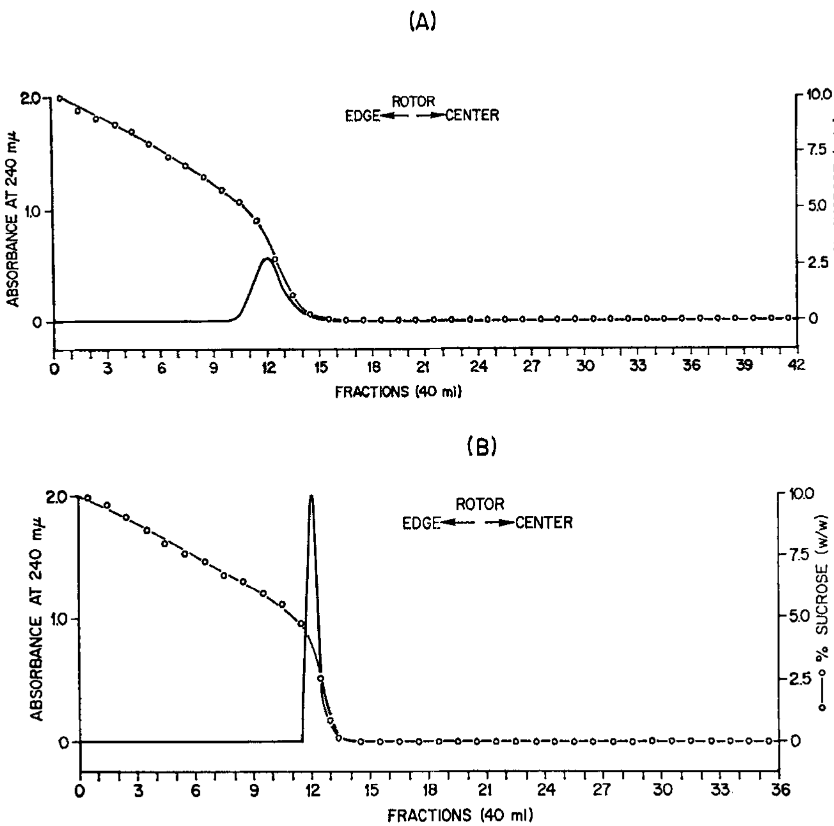

FIG. 6. (A) Resolution of B–XV rotor with edge loading of the overlay, latex bead sample, and gradient. The displacement fluid (water) in center line; collection from edge flow line. (B) B–XXIX rotor—run and unloading conditions the same as for A.

The rotors were unloaded from the edge by pumping distilled water in through the center line. The results with the B–XV rotor are shown in Figure 7A, and the B–XXIX results are shown in Figure 7B. The marked difference in peak width and in gradient shape emphasizes that the vertical surface of the B–XV rotor wall produces considerable loss in resolution during edge unloading.

Width of samples introduced at the rotor edge. The experiments described above and illustrated in Figures 7A and 7B were repeated, pumping the overlay, sample and gradient directly through the rotor from edge to center. The results are shown in Figures 8A and B for the B–XV and B–XXIX, respectively. This is a severe test of resolution since the 50 ml overlay would form a layer only 1.14 mm thick at a radius of 7.4–7.5 cm in both rotors. The sucrose from the gradient and part of the sample would tend to diffuse into the over-

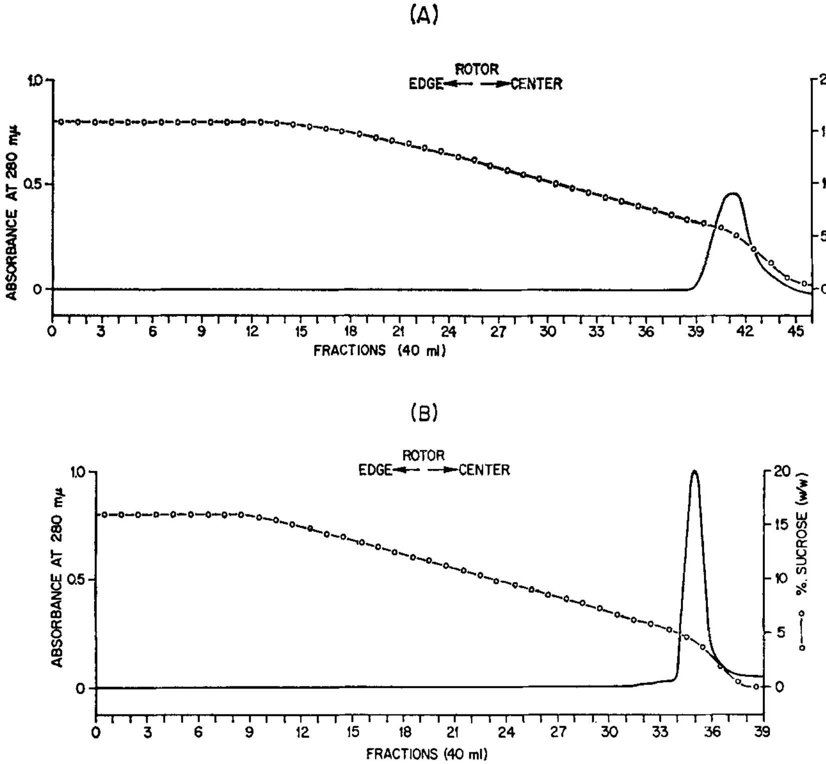

FIG. 7. (A) B-XV rotor—gradient loaded through edge flow line, bovine serum albumin sample and overlay loaded through the center flow line. Unloaded by introducing water to center flow line and collected from edge flow line. (B) B-XXIX rotor—same loading and unloading conditions as for A.

lay, and does, as is indicated in Figure 8. However, the sample mixed entirely through the overlay in the B-XV and spread much less in the B-XXIX. These results suggest that extensive loss in resolution occurs in the B-XV during both loading and unloading from the edge, with the greatest decrement occurring during loading when the bands are edge-loaded.

Concentration of small particles in the B-XXIX rotor. The B-XXIX rotor may be used to concentrate particles from approximately 1 liter of solution and band the particles in a small volume gradient at the edge. By using the equation:

$$t = \frac{1}{S}\,\frac{900}{(\text{rpm})^2}\left[\frac{\log_e r_{\max} - \log_e r_{\min}}{\pi^2 \times 10^{-13}}\right]$$

and maximum and minimum radii of 7.38 and 2.39 cm, the graph in

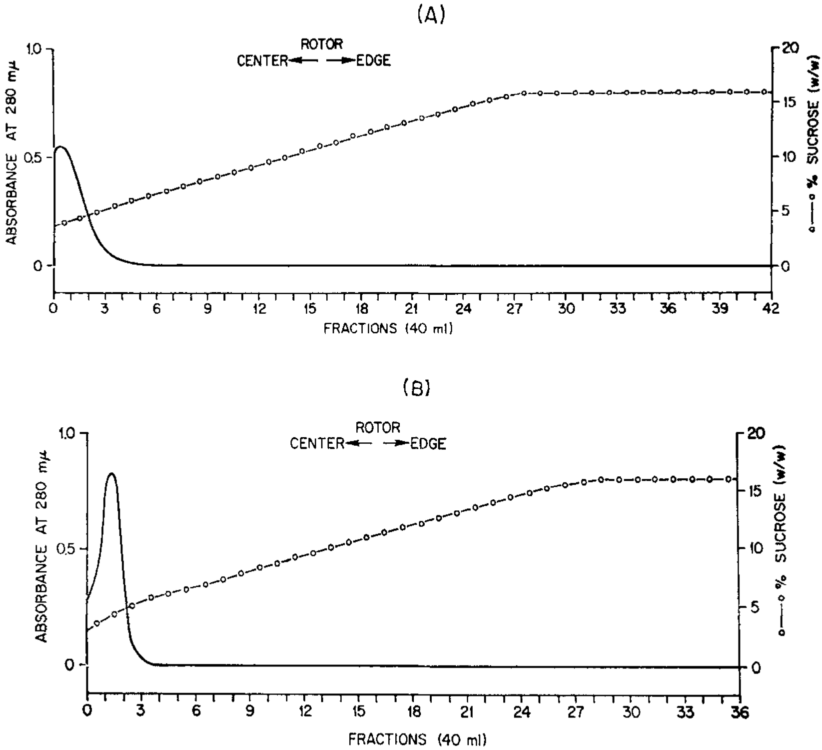

FIG. 8. (A) B–XV rotor—overlay, bovine serum albumin sample, gradient, and displacement fluid introduced to edge flow line and collected from center flow line. (B) B–XXIX rotor—same loading and unloading conditions as A.

Figure 9 was prepared. The results apply for any sedimentation coefficient for a particle in a medium, *m*, and temperature, T.

Since the solutions in the center and edge lines differ appreciably in density under these conditions, the question arises whether a prohibitively high center line pressure will be required to displace the gradient out of the rotor. To examine this question, polystyrene latex beads similar to those previously used were suspended in water. The particles had an average diameter of 1 mμ and a density of 1.05 gm/cc. One liter of water containing 0.002% w/v latex was allowed to flow into the rotor at rest. The rotor was then accelerated to 2500 rpm, and a cushion of 380 ml of 20% w/w sucrose syphoned in through the rotor edge line. The rotor was run 2 hr at 25,000 rpm, which is sufficient time to allow complete sedimentation of the particles. The rotor was unloaded from the center by pumping 20% w/w sucrose to the rotor edge (Fig. 10A). In a comparable experiment, the rotor was unloaded from the edge by displace-

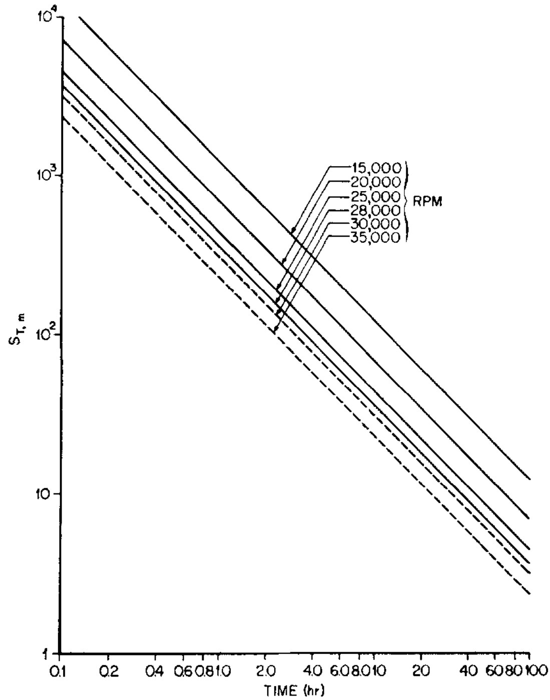

FIG. 9. Time required to sediment various particles from R_{min} 2.39 cm to R_{max} 7.38 cm in medium, *m*, and temperature, T, and rotor speed from 15,000 rpm to 35,000 rpm. Dashed lines for 30,000 rpm and 35,000 rpm indicate that these speeds are above the present recommended maximum.

ment with distilled water pumped in through the center line (Fig. 10B). No difficulty due to back-pressure was observed.

Concentration of influenza virus. To test the procedure used in the experiment illustrated in Figures 9 and 10B with a biological particle, a sample of 10 ml of flu virus (influenza virus vaccine polyvalent killed virus, lot no. 66B1298)[4] was diluted to 1 liter with phosphate-buffered saline and allowed to flow into the rotor at rest. The rotor was accelerated to 2500 rpm, and 380 ml 55% (w/w) sucrose was added through the edge flow line; 50 ml of water was introduced through the center flow line as overlay; the rotor was accelerated to 25,000 rpm, and run at that speed for 2 hr. The rotor was decelerated and unloaded by pumping water to the rotor center and collecting from the edge flow line (Fig. 11). Electron microscopy indicated that the peaks contain the expected components: the peak nearest the rotor edge contained intact virus, and the one nearest the center contained ghosts and other contaminants.

[4] Kindly supplied by Eli Lilly & Company, Indianapolis, Indiana.

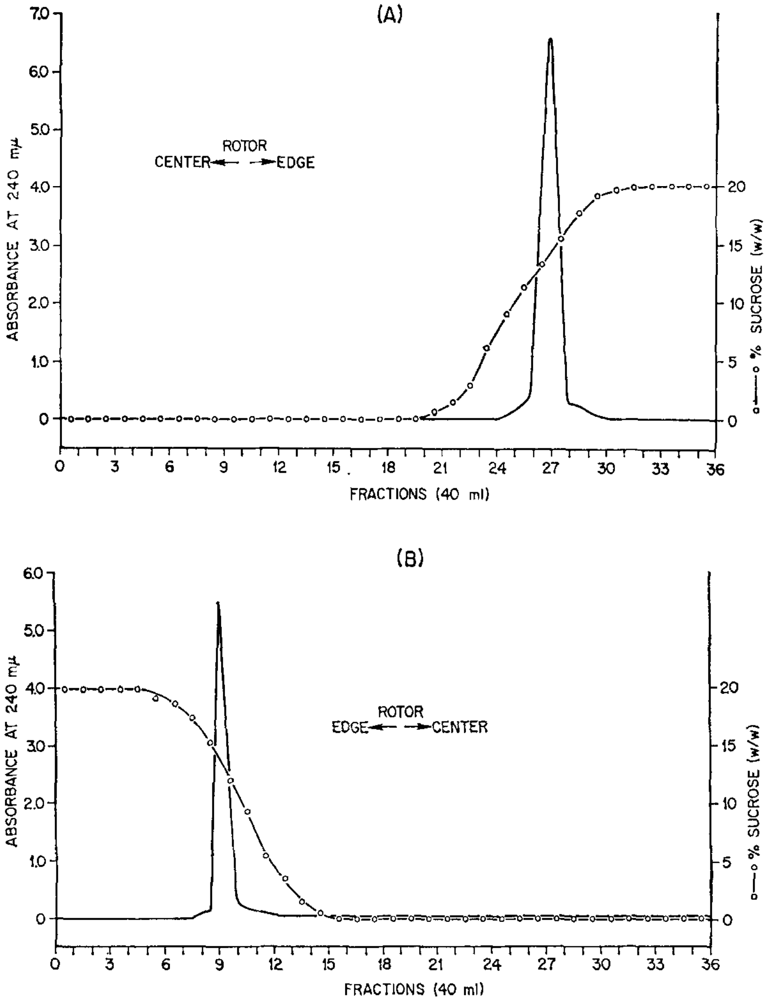

FIG. 10. (A) Demonstration of complete sedimentation of particles from a large sample into a dense cushion in a B–XXIX rotor. Latex beads from 1 liter sample sedimented and band collected by displacing contents of rotor by introducing displacement solution to edge flow line and collecting from the center flow line after running to 100% sedimentation time. (B) Similar to A B–XXIX rotor was unloaded by introducing water to center flow line and collected at edge flow line.

Concentration of T4 bacteriophage. Since some virus cannot be banded in sucrose, denser gradient materials must be used. Among the densest known viruses are certain of the DNA-containing bacteriophages which band at a density of approximately 1.5 gm/cc in CsCl. If CsCl gradients dense enough to band particles of this density can be recovered from the B–XXIX rotor, then the rotor is

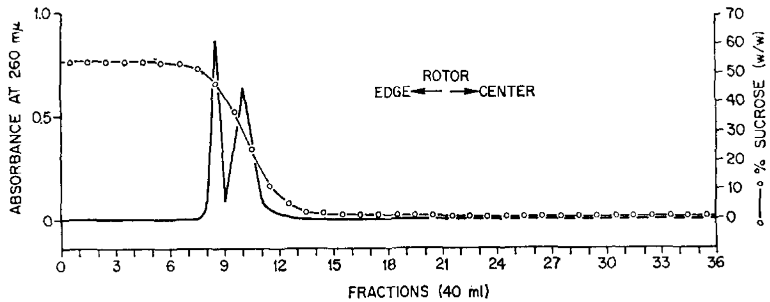

FIG. 11. Influenza virus concentration in B–XXIX rotor on sucrose cushion. By using data from Figure 9 and 55% (w/w) sucrose cushion, a 1 liter sample of influenza virus was sedimented, banded, and collected by introducing water to the center flow line and collecting from the edge flow line. The virus was concentrated in the left peak.

suitable for concentrating and banding any virus. A 6 ml sample of a crude T4 bacteriophage suspension containing ~ 10^{14} particles/ml was diluted to 1 liter with water and was placed in the rotor at rest. The rotor was then accelerated to 1500 rpm, and 430 ml of CsCl (density 1.6709 gm/cc) was introduced to the edge of the rotor. An overlay of 50 ml of water was then added through the center line, thus displacing 50 ml of CsCl from the edge, leaving 380 ml of the salt solution in the rotor. The rotor was then accelerated to 25,000 rpm and run for 2 hr. It was decelerated to 2000 rpm and the gradient recovered from the edge by pumping distilled water in through the center line (Fig. 12). The peak at density 1.525 gm/cc was almost pure virus, whereas the lighter peak 1.495 was cellular debris. The virus was recovered in less than 40 ml and may be further concentrated by banding in angle-head centrifuge tubes as previously described (9,10).

With CsCl the back-pressure due to differences in density between the solution in the rotor and in the edge lines was sufficient to prevent unloading at 3000 rpm. At 2000 rpm, as soon as flow started, the pressure was initially 15 lb/in.^{2} in the center line and rapidly fell to 5 lb/in.^{2}.

DISCUSSION

The titanium B–XXIX rotor employs a double tapering scheme to allow gradients to be recovered from the rotor edge with resolution which approaches that observed during center unloading. This makes possible a variety of new centrifugal procedures which were

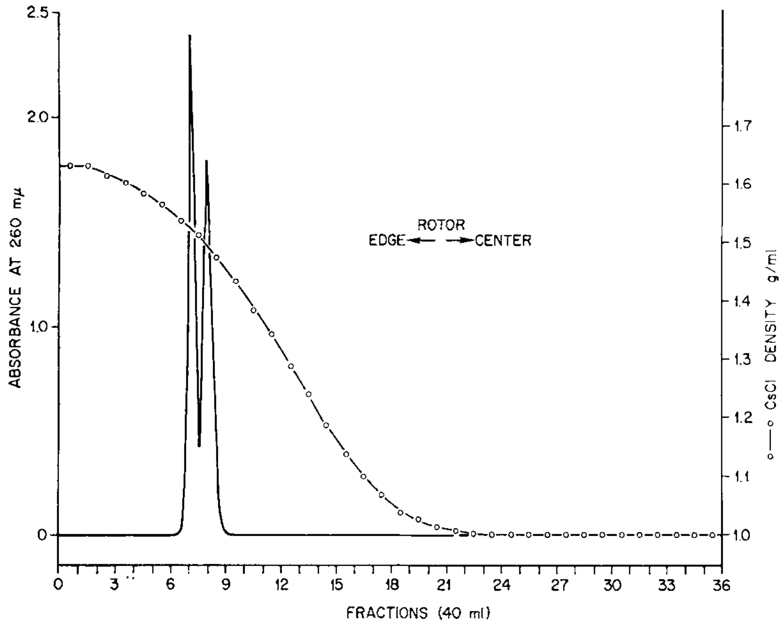

FIG. 12. T4 bacteriophage concentration in B–XXIX rotor. CsCl 1.6709 gm/cc was used as cushion and 1 liter sample of T4 bacteriophage sedimented, banded, and collected by introducing water to the center flow line and collecting from the edge flow line. Virus was concentrated in the left-hand peak.

difficult to do or could not be done effectively with previous rotors, excepting only the aluminum B–XXIII previously described. These include:

1. Edge loading of particles which float up in the gradient.
2. Sequential recovery of sedimenting particles before they reach their isopycnic points.
3. Concentration of particles such as viruses from liter volumes into a short, steep, edge gradient with recovery without use of large volumes of dense displacing fluid.

The seal used in these studies is removed for high-speed operation and is only attached at speeds below 5000 rpm. With higher speed seals the possibility exists of continuously moving the gradient radially at a slow rate while the rotor is at speed, thus collecting sequentially at the edge a spectrum of particles whose sedimentation coefficients vary over a wider range than can ordinarily be accommodated in a single gradient.

Some loss in resolution probably occurs in the lines through the septa where a reverse gradient occurs. However, the total volume in these lines is approximately 0.994 ml, while the density differences through these lines does not exceed 0.00003 gm/cc/cm during recovery of the steepest portions of the gradient. The average flow rate during unloading is ~ 40 ml/min, giving an average velocity in the edge lines of 500 cm/min. This flow rate appears to be sufficient to prevent excessive mixing due to the slight density inversion.

In several of the commercially available versions of the Oak Ridge B–XV rotor, additional peripheral metal has been added at the rotor edge to increase the radius at the top and bottom of the rotor, but not as its equator. This metal is in a higher centrifugal field and is therefore of less value than the same amount of metal distributed similarly but at a smaller radius. A dividend of the B–XXIX design is that precisely the distribution of material desired for reasons of physical strength is obtained for quite another reason, namely, improvement of resolution.

The maximum operating speed of the B–XXIX rotor is conservatively set at 28,000 rpm pending the outcome of destructive tests. The application of the B–XXIX to the fractionation of tissue homogenates is described in subsequent papers. The smaller version of the B–XXIX, analogous to the B–XIV in size, is numbered B–XXX. We are at present unaware of any use of the B–XV rotor, excepting certain rate flotation experiments, which is not done equally well or much better by the B–XXIX. The latter rotor clearly supersedes the former.

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