The Development of Low-Speed "A" Series Zonal Rotors

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SUMMARY

The evolution of a series of low-speed zonal centrifuge rotors for the separation of cells, bacteria, and subcellular particles visible in the light microscope (Rotors A-V, VI, IX, and XII) is described. All are dynamically loaded and unloaded and may be used for either rate-zonal or isopycnic-zonal separations. Transparent plastic end caps allow direct observation of the separations. The last rotor of the series (A-XII) has a total rotor volume of 1400 ml and a maximum radius of 17.78 cm. The sample zone volume can be as large as 50 ml, and the density gradient volume is usually one liter. Experimental studies with model particles demonstrate that narrow starting zones and useful separations can be obtained.—Nat Cancer Inst Monogr 21: 113-136, 1966.

In ZONAL centrifugation (1-9) density gradients stabilize the liquid milieu through which particles sediment to allow particles having different sedimentation rates to be separated into discrete zones or bands. Early studies with swinging-bucket rotors demonstrated the feasibility of separation of cell components and viruses in density gradients, but only very small amounts of material could be separated, and considerable time and effort were required to form and recover the gradients.

Since high gravitational fields have a marked stabilizing effect on liquid density gradients, the possibility of forming and recovering gradients during rotation was investigated, and a simple system for distributing a gradient stream into tubes in swinging-bucket rotor was developed (10). These gradients, however, were recovered at rest. The possibility of eliminating centrifuge tubes was next studied, and a hollow-bowl rotor and simple methods for introducing and recovering density gradients were tested (11). The success of this early work prompted the development of rotor systems covering a wide range of speeds and capacities (9). These include the Series A rotors, suitable for the separation of particles ranging from whole cells to mitochondria. In this paper the design and operation of rotors A-V, A-VI, A-IX, and A-XII are described.

1 This research performed under the Joint National Institutes of Health-Atomic Energy Commission Zonal Centrifuge Development Program which is supported by the National Cancer Institute, the National Institute of Allergy and Infectious Diseases, and the U.S. Atomic Energy Commission.

1 Operated for the U.S. Atomic Energy Commission by the Nuclear Division of Union Carbide Corporation.
PRINCIPLES OF OPERATION

Two methods of operation have been explored with the “A” or low-speed rotor series. In the first method the rotor is loaded and unloaded while spinning (fig. 1) at a speed sufficient to stabilize the gradient against the rotor wall (6, 9). Although this method of dynamic loading appears to give very high resolution, it requires precision seal systems. In the second method the gradients are loaded and unloaded at rest, the gradient being reoriented from a horizontal to a vertical configuration during rotation (12, 13) in a hollow rotor containing fixed septa.

In a reorienting gradient rotor the gradient is introduced to the bottom of the rotor during rest. The sample is introduced by reverse flow through the top and is followed by a light overlay. During gradual acceleration the axial gradient is reoriented to a radial gradient by centrifugal force. After particle separation has been effected, the gradient reorients to the original configuration as it is brought to rest. Gradient recovery is by displacement out the upper fluid line or by drainage out the bottom (12).

The choice between dynamically loaded and reorienting gradient rotors for specific applications cannot be made solely on the basis of theoretical studies (13). It is important to compare experimentally the resolution obtained in the two rotor systems. It should be emphasized that the “A” series includes rotors of both types, but that this paper is limited to dynamically loaded designs.

ROTOR A-V

Work on rotors A-III and A-IV (9) demonstrated the feasibility of dynamically loaded and unloaded zonal centrifuges. It appeared desirable to (a) observe the entire procedure, (b) detect anomalous sedimentation at various times during the centrifugation, and (c) determine directly the optimal time to unload the gradient. For this reason it was considered desirable to construct the end caps of the more advanced rotors from a transparent plastic.

In dynamically loaded series A rotors, length has been minimized to give a ratio of \( I_{\text{spin}} / I_{\text{transverse}} > 1 \) for the moments of inertia (14), which thus provides a configuration spinning about its most stable axis. Rotor A-V was tested at very low speeds (500–1000 rpm). Loading and unloading of rotors at much higher speeds are feasible (6, 15); however, the stabilizing effect of centrifugal force is much less at very low speeds, and the possibility of mixing during introduction of the sample layer is correspondingly increased. Since pressures and stresses are relatively small, large-diameter methacrylate resin end plates could be used. Text-figure 1 is a schematic diagram of the A-V rotor. The interior is divided into 8 sector-shaped compartments, each 2.72 cm deep, with maximum and minimum radii of 13.60 and 3.49 cm. The upper and lower end plates are of 3.26 cm-thick clear plastic, and the internal volume is 1300 ml.
All parts of a zone of one density in a zonal centrifuge constitute a paraboloid of revolution about the axis of rotation (6, 13). The single conical-section core, therefore, allows the sample layer to slope 45° or more as it approaches the rotor center. The rotating seal was constructed of filled Teflon (Rulon 3), while the static fluid-line seal was constructed of stainless steel and flexibly mounted to allow alignment to the plane of the rotating seal during operation. Chilled water was circulated through the upper seal to remove frictional heat.

Water was added to the rotor in 100 ml increments, and the position of the meniscus was determined at 1000 rpm. From these data a chart of volume as a function of radius was drawn. The position of any portion of the gradient recovered during unloading could then be related to its original position in the rotor. All work was performed in an International Equipment Company Model PR-2 refrigerated centrifuge with a transparent plastic lid.

1 Available from the Dixon Corporation, Bristol, R.I.
Performance

To determine whether density gradients could be introduced into the rotor and recovered without extensive mixing, a density gradient was analyzed refractometrically both before introduction and after spinning in the rotor for 15 minutes at 1000 rpm. Differences between the two gradients were negligible.

A sample zone introduced into the rotor is widened by laminar flow through the tubing and rotor core, by any turbulence or convection in the rotor, and by diffusion. To determine how much widening would occur in practice, a sample zone containing 4 percent bovine serum albumin having a total volume of 20 ml was introduced over a sucrose gradient. When moved to a position just external to the core, the sample had a calculated width of 0.1 cm. The rotor was spun at 1000 rpm at 5° C (152 X g at R_{max} and 39 X g at R_{min}) for 15 minutes. When the gradient was recovered, the absorbance recording of the sample zone showed a peak whose width at half height was equivalent to a zone width of 0.3 cm in the centrifuge. This amount of broadening is not considered excessive.

Three types of experiments were performed to determine the resolution obtainable with this rotor. In the first, ragweed pollen grains were observed during sedimentation. In the second, rat red cells were centrifuged to their isopycnic position, while in a third series of experiments a method for isolating calf thymus nuclei that retained their ability to incorporate amino acids was developed (16). Preliminary experiments with ragweed pollen suggested that more uniform behavior could be obtained if the grains were first fixed in alcohol. Two hundred mg of pollen was suspended in 10 ml of 95 percent ethanol, centrifuged briefly, and the pollen resuspended in 10 ml of 8.5 percent sucrose (w/w). This volume of material was used as the sample layer and was introduced centripetal to a 1200 ml gradient extending from 17 to 55 percent (w/w) sucrose. Fifty-five percent sucrose was also used as the underlay for the gradient. After 10 minutes at 1000 rpm, the gradient was recovered by displacement. Photographs of the rotor during the run are shown in figure 2. When the fractions were examined by dark field phase-contrast microscopy, no particles were observed through the gradient except at the level of the zone observed visually. In experiments with rat red cells, two isopycnic bands were observed repeatedly. The basis for this fractionation has not been determined. These results indicate that the principles previously employed in the Series B zonal centrifuge (6, 15) can be applied to large-particle separations in a low-speed rotor.

ROTOR A-VI

Rotor A-VI is the largest low-speed zonal rotor thus far constructed. It is designed to explore large-scale nuclear isolation and the separation of different cell types in a mixture such as occurs, for example, in bone marrow suspensions.
The rotor has a 3 liter capacity and is designed for operation at 6000 rpm and a maximal centrifugal force of $7100 \times g$. The design specifications were fixed by the speed, permissible diameter, and thrust-bearing capacity of the PR-2 centrifuge. The design does not approach the stress limits of the 7075-T6 aluminum used for the rotor wall. The partially disassembled rotor is shown in figure 3, and the rotor and seal system assembled for use are shown in figure 4. A fairly uniform centrifugal field is achieved with a large-diameter central core to keep the entire gradient close to the rotor edge. The central core diameter is 22.86 cm and the inside rotor diameter is 35.56 cm, which gives a gradient chamber length of 6.35 cm. At the design speed of 6000 rpm with a sucrose density gradient in position, a rather large radial expansion (approximately 0.04 cm) occurs. Since the end caps increase in diameter by only a few thousandths of a centimeter, a mechanism was needed to center the rotor chamber wall with respect to the end caps, and to maintain concentricity to the drive spindle. Plated brass plates (one in each end cap), which expanded during centrifugation at a rate intermediate between that of the rotor wall and the end plates, were used. Without the brass plates, departure from circularity increased from the normal 10 to 25 μ.

When the rotor was properly constructed, it was in balance. The only metals in contact with the fluid were anodized aluminum, nickel, monel, or stainless steel. “O”-rings were used to seal the rotor chamber and all components in the liquid lines.

Three methods have been investigated for obtaining flow into and out of the centrifuge at operating speed. The use of skimmers as previously suggested (6) was not successful at the relatively low peripheral speed obtained at the edge of the skimmer disks. As a second approach, a self-aligning ball-and-socket rotating seal was tested but was ineffective because of distortion of the ball during attachment to the drive and because of cold-flowing of the reinforced Teflon of the static seal when under pressure. The last design, a flat, modified face seal supported by the centrifuge chamber lid proved effective when the centrifuge was in near-perfect balance. Its alignment with the rotating seal was critical. A self-aligning seal was not designed until the A-IX rotor was developed.

One top end cap for rotor A-VI has been fabricated from Lucite to permit viewing of the rotor contents completely across the rotor diameter. The rotor contents were observed in detail by use of a stroboscopic light. At the center of the rotor, a large nut limits deflection of the cap and reduces the stress. Power limitations of the PR-2 drive motor have restricted the top speed in practice to 5160 rpm. Critical frequencies of the spindle system occur at 700 and 900 rpm, but these have been passed with no difficulty.

The A-V rotor showed that successful separations could be achieved at low speed and demonstrated the utility of a completely transparent system, whereas A-VI showed that plastic end caps could be used at speeds up to 5000 rpm. Our attention was therefore directed toward the de-
development of a general purpose rotor with both upper and lower end caps of plastic transparent materials, and with a self-aligning seal that could achieve sufficient speed to separate subcellular particles as small as mitochondria. In addition, an attempt was made to simplify the rotor as much as possible and to provide for its convenient disassembly. [Rotor A-VII has been described (12) and rotor A-VIII, which also utilizes the reorienting gradient principle, is described elsewhere.]

**ROTOR A-IX**

Rotor A-IX, shown in figure 5 and text-figure 2, has been designed to replace rotor A-V, but with a higher maximum operating speed and greater stability. The rotor volume of 1300 ml is divided into 8 sector-shaped compartments, each having a depth of 1.49 cm and a maximum radius of 17.78 cm. The central core is a single 45° cone with a maximum radius of 4.79 cm. Flow lines to the rotor edge pass over the septa, which thus allows an unobstructed view of the entire compartment. The rotor weighs approximately 22.68 kg full and is held on the centrifuge drive spindle by its own weight.

**Text-figure 2.**—Diagrammatic representation of rotor A-IX. (1) Upper end plate; (2) nut; (3) spindle; (4) rotating face seal (Rulon); (5) flow channel; (6) "O"-ring; (7) "O"-ring; (8) "O"-ring; (9) "O"-ring; (10) "O"-ring; (11) tapered core; (12) septa; (13) septa holder; (14) spacer ring; (15) lower retaining ring; (16) retaining bolts; (17) upper retaining ring.
The rotor has been spun to 4000 rpm, both empty and filled with water. Design speed of 6000 rpm has not been reached due to the limited power of the drive system. Departure from concentricity was measured by use of an inductance proximity probe placed in the centrifuge chamber close to the edge of the rotor. The resulting signal was amplified and displayed on a calibrated oscilloscope. Rotor runout or wobble when empty and when filled with water is shown in text-figure 3. These rotors have not required balancing to achieve satisfactory performance. The large runouts observed at 400 and 900 rpm when the rotor is filled are rigid-body critical frequencies of the centrifuge system. Flow measurements with water suggested that the centrifuge will serve as a satisfactory continuous flow device for large particles, as well as a rate-zonal centrifuge. Flow rates of 100 ml per minute were easily achieved.

Text-figure 3.—Runout of rotor A-IX-b on International Model PR-2 centrifuge drive, expressed in mils (10⁻³ inches).

The seal is shown in text-figure 4 and figure 6. A collar attached to the rotor is connected to the static seal through a sealed, stainless steel bearing. Lateral movement of the rotor and the rotating seal is transmitted directly to the static seal. Rotation of the static seal is prevented by a removable arm attached to the centrifuge cooling chamber. The complete A-XII system is shown in figure 7.

Operation

The rotor is accelerated to approximately 1000 rpm, and the density gradient is pumped in at the rate of approximately 25 ml per minute with a modified Spinco zonal centrifuge gradient pump. For most purposes, gradients ranging from 17 to 55 percent or from 10 to 30 percent (w/w) sucrose are useful. Both the rotor and the solutions used to prepare the gradient are prechilled to 5° C. When the gradient has filled the rotor and begins to emerge through the core line, the gradient pump

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4 Bentley Proximity Probe, Bentley-Nevada Corp., Minden, Nev.
5 Spinco Division, Beckman Instruments, Palo Alto, Calif.
is stopped and a drain tube, connected to the line between the pump and the seal, is opened. This allows fluid to escape from the edge of the rotor as the sample layer and the overlay are introduced.

The sample layer (usually 5–20 ml) may be introduced through the rotor center or core line with a large syringe, a small vessel pressurized with air, or with a small peristaltic pump. The sample should have a density less than that of the light end of the gradient, or excessive boundary widening occurs. As soon as the sample layer is in, 100 ml or more of a light fluid, such as a physiological salt solution or a dilute buffer, is pumped in, the drain line between the pump and the rotor edge line closed, and the gradient pump started with the pump set to pump only dense fluid. The excess overlay is then pumped out through the rotor center line into a reservoir containing 75 ml of water. The rotor edge line is then clamped off. As the rotor accelerates, expansion occurs, and fluid to compensate for this increase in volume is drawn from the reservoir. This mechanism assures that the seal does not dry out and that the rotor does not become unbalanced by the introduction of air. The rotor is then accelerated to operating speed.
After the desired separation has been made, the rotor is decelerated to between 200 and 1000 rpm, and the gradient is recovered by pumping dense sucrose into the rotor through the rotor edge line.

Ultraviolet absorbance of the gradient is monitored by a two-wavelength system previously described (17). Forty-milliliter fractions were generally collected. The \( r_i \) of the outer edge of a fraction in the rotor can be deduced from the equation:

\[
V_i = h\pi (r_i^2 - r_c^2) + V_{rc} - Nh w (r_i - r_c)
\]

where

- \( V_i \) = volume collected to reach end of zone or sample \( i \),
- \( V_{rc} \) = volume from point of collection to outer edge of core,
- \( r_i \) = radius of zone \( i \) in rotor before unloading,
- \( r_c \) = radius to edge of core,
- \( N \) = number of septa,
- \( h \) = height of sector-shaped compartments, and
- \( w \) = width of septa.

For rotor A-IX, \( V_{rc} = 25 \text{ ml}, \) \( r_c = 4.79 \text{ cm}, \) \( N = 8, \) \( h = 1.49 \text{ cm}, \) and \( w = 0.64 \text{ cm}. \)

To observe the sedimentation of particles, a disk of Lucite having the same outside diameter as the inside of the refrigerated chamber of the centrifuge was mounted under the rotor and painted half white and half black. When the black background was used, particles could be observed by reflected light, whereas particles having appreciable color could best be observed against the white background.

A camera was mounted above the centrifuge, and lights mounted to illuminate the rotor so that photographs could be taken at intervals. An over-all view of the system is shown in figure 7.

**Performance**

The A-IX rotor may be used for a variety of purposes (18), and the results obtained will depend largely on the specific conditions employed.

To illustrate the separations that may be obtained, a 1 liter gradient ranging linearly with radius from 10 to 42 percent (w/w) sucrose was introduced into the rotor, and the remainder of the rotor volume was filled with 55 percent sucrose. Twenty ml of a 10 percent brei was prepared from unperfused rat liver and introduced into the rotor at 400 rpm. The sample was moved out beyond the core with 150 ml of pH 7.5 Miller-Golder buffer, \( \mu = 0.1. \) The sedimentation of nuclei, red cells, and mitochondria is shown in figure 8. Phase contrast microscopy was used to identify the various fractions. No cross-contamination between nuclei and mitochondria was observed.
ROTOR A-XII

After several months of successful use, the acrylic end cap of an A-IX failed. The damage, shown in figure 9, occurred along the outer periphery at low speed (1000 rpm) with a 17 to 55 percent sucrose gradient in the rotor. A complete stress analysis, including studies of the effects of thermal stress, was made and checked in a series of studies on plastic models. To insure as far as possible that further failures did not occur, the rotor was redesigned as the A-XII rotor. In the A-XII rotor, the thickness of the acrylic resin in the area where failure occurred was increased, and a combination of a gasket and a metal-to-metal stop was added to distribute and control the clamping pressure.

Design Considerations

The A-XII rotor was designed to give the same separation performance as the A-IX, but with improved physical properties. The maximum stress, in terms of the yield values of the materials of construction, the diameter-to-height ratio, and the speed range employed, is in the rotor end caps, and is produced by fluid pressure generated by centrifugal force. The fluid pressure load acting on the end cap is parabolic, increasing toward the periphery of the rotor. The magnitude is governed by the density of the fluid and speed of the rotor. The hyperbolic pressure load, acting upon a round, thick plate, clamped at the periphery, produces the maximum stress at the periphery. In A-IX, the weakest sectional strength was at this point. Since the rotor end plates are clamped together by the 12 flange screws, the stress caused by the screws also acts on the periphery of the end caps. The clamping stress adds geometrically to the stress caused by the fluid pressure. An uneven clamping pressure applied to the clamping screws tends to concentrate the clamping load in a neighborhood of one or a few screws. To help distribute the clamping pressure evenly, a metal-to-metal stop was used at the periphery, and a gasket added between the end cap and the flange to distribute the load. The gasket also helps to eliminate the possibility of application of a point concentrated load on the plate by dried sucrose droplets or other particles.

The cast thermoplastic acrylic resin used as the end cap is a plastic glass, and like other glasses, its strength is markedly affected by scratches or chips.

The plastic has the maximum stress limitation of about 8000 psi in shear at room temperature with decreasing strength as the temperature decreases. The coefficient of thermal expansion of the material is higher than the aluminum used to clamp the two end caps, whereas the thermal conductivity of the plastic is much lower than aluminum. Generally, the rotor is used at 4° C. If the rotor is assembled at room temperature and then cooled slowly to 4° C, the clamping pressure would tend to decrease. On the other hand, if the rotor is subjected to a rapid temperature drop as well as the hyperbolic fluid load by centrifugation, the net
clamping pressure will increase during the temperature change, because the aluminum clamping flange will react to the temperature change more quickly than the plastic. In addition, if the rotor is left filled with an aqueous solution for a long period, increases in the clamping pressure due to the plate growth caused by the water absorption will be observed. A weight gain of 0.2 percent per day is observed for thin plastic pieces. If the rotor is subjected to a higher temperature after assembly, as may occur if the rotor is left in direct sunlight, increased clamping pressure results. The A-XII rotor should be assembled and stored in a 5°C cold room and placed in a chilled centrifuge immediately before use.

Experiments have also been performed with the A-XII to determine the effect of changing the number of septa. The banding performance obtained with ragweed pollen was examined with 2, 4, and 8 septa. The results with 4 or 8 were indistinguishable, whereas considerable loss in resolution was observed with 2. The number of septa has therefore been reduced to 4 in the A-XII rotor, which results in a small increase in rotor volume.

The A-XII rotors have accumulated several hundreds of hours in operation over a 9-month period without failure. The maximum speed attained was 3900 rpm, a limitation imposed by the PR-2 drive. The rotor is capable of 6000 rpm with 60 percent sucrose in the chamber.

SCANNER FOR A SERIES ROTORS

The A-XII rotor is capable of yielding precise data on the sedimentation rates of large particles if measurements are made during centrifugation. A simple way of obtaining these measurements is with a photoelectric scanner, as shown in figure 10.

The scanner uses a standard 6-volt lamp and a cadmium selenide photocell. Since most of the decrease in transmission of a particle zone is due to scattered light, the photocell is arranged to scan the rotor with a very narrow beam. The output of the photocell is fed to the Y axis of an X-Y recorder. The position of the photocell on the rotor radius with respect to the rotor axis is measured by a potentiometer, which controls the voltage to the X axis. While the scanner swings through an arc as it traverses the cell, the position-measuring potentiometer is driven through a cam system to produce a voltage output proportional to the true radius. The X-Y recording is thus linear with rotor radius. Scans are repeated at preset intervals, but the recorder pen does not record on the return cycle of the scanner. Normally, the X axis of the recorder is set to give a plot in which the radius is enlarged by a factor of 3 for clarity. The location of sedimenting bands or zones can thus be precisely determined.

The optimum interval between repetitive scans varies markedly with particle sedimentation rate and rotor speed. A timer is incorporated in the scanner to provide a preselected scanning interval of 1, 5, 10, 15, 20, or 25 minutes per cycle. After the completion of 10 scans, the scanner
is automatically shut off. An example of a scan made with this instrument is shown in text-figure 5. Additional scanner features under consideration include measurement of either backward or forward scattered light. For improved visual observation of sedimenting bands, a uniform light source below the rotor is required. A source consisting of three concentric fluorescent lamps with suitable reflectors and diffusers that fit in the International PR-2 centrifuge has therefore been developed.

Text-figure 5.—Sequential scan of ragweed pollen particles in 17 to 55 percent sucrose gradient sedimented in the A-XII rotor with a photoelectric scanning device. Changes in transmitted light were recorded in the visible light range spectrum. International PR-2 centrifuge was used at 950 rpm with scanning at 60 seconds per cycle. Numbers denote the scanning order.

REFERENCES

Figure 1.—Schematic diagrams of operation of Series A dynamically loaded rotors. Hollow cavity in the rotor is divided by septa into sector-shaped compartments. Two fluid lines are connected through a flat face seal to the rotor core and the rotor edge in such a manner that fluid may be pumped in either direction through the rotor during rotation. At low speed the rotor is filled by pumping the gradient in through the edge line (A) until the rotor is filled (B). The direction of fluid flow is then reversed, the sample layer pumped in through the core line (C) and then pushed out by a fluid less dense than the sample until the sample layer is free of the core (D). Rotor is accelerated to operating speed to achieve the desired separations (E). Rotor is then decelerated to unloading speed and the gradient displaced out through center core by pumping a dense fluid to the rotor edge (F).
Figure 2.—Sedimentation of ragweed pollen in rotor A-V.
Figure 3.—Rotor A-VI partially disassembled. Core with septa shown at left, main body of rotor in center, with transparent upper end plate at right.
Figure 4.—Rotor A-VI and seal assembly mounted in PR-2 centrifuge. The rotor is visible during operation through the plastic top of the centrifuge.
Figure 5.—Rotor A-IX; 6000 rpm, 7150 × g, 1.3 liters.
**Figure 6.**—Components of type “A” seal.
Figure 7.—Complete A-XII low-speed zonal centrifuge system. Centrifuge and rotor shown in left foreground, with gradient pump at extreme right. Between are absorbance monitor and recorder.
Figure 8.—Separation of rat liver subcellular components in a sucrose gradient in the A-IX rotor.
Figure 9.—A-IX rotor after failure at low speed.
Figure 10.—Photoelectric scanner for A series rotors.