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Analytical Techniques for Cell Fractions

V. Characteristics of the B-XIV and B-XV Zonal Centrifuge Rotors[1]

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Zonal centrifuges,[3] developed for the mass separation of subcellular particles and viruses on the basis of either sedimentation rate or buoyant density (1–8), have been used to isolate the major subcellular components (7), viruses (4, 5, 9, 10), ribosomal RNA (11), and serum macroglobulins (12). An advantage of the B-IV zonal rotor previously described (6, 13) is that it may be easily converted into a high-speed continuous-flow centrifuge with (14) or without (15) isopycnic banding during operation. A disadvantage of this rotor is that a special centrifuge with extended armor, a cooled upper bearing, and a high-speed seal are required. Where continuous-flow centrifugation capability is not needed, a much simpler rotor with a removable seal which can be spun in un-

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[2] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

[3] Zonal centrifuges developed under the MAN Program are designated by letter to indicate speed range (A for low speed, B for intermediate speed in the range up to circa 60,000 rpm, C for speeds up to circa 160,000 rpm, and D for ultra-high-speed rotors operating up to circa 400,000 rpm) and by Roman numerals which run consecutively and indicate the order in which the rotors have been developed. Where several different materials are used, the predominant one (aluminum, steel, titanium) may also be indicated. For example, B-XV Ti indicates a B series rotor, design XV, constructed of titanium. Zonal rotors of more than 40 different designs have been constructed thus far. B-XIV and XV rotors are available from the International Equipment Co., Needham Heights, Mass.; Measuring and Scientific Equipment Ltd., London; and The Spinco Division of Beckman Instruments, Palo Alto, California.

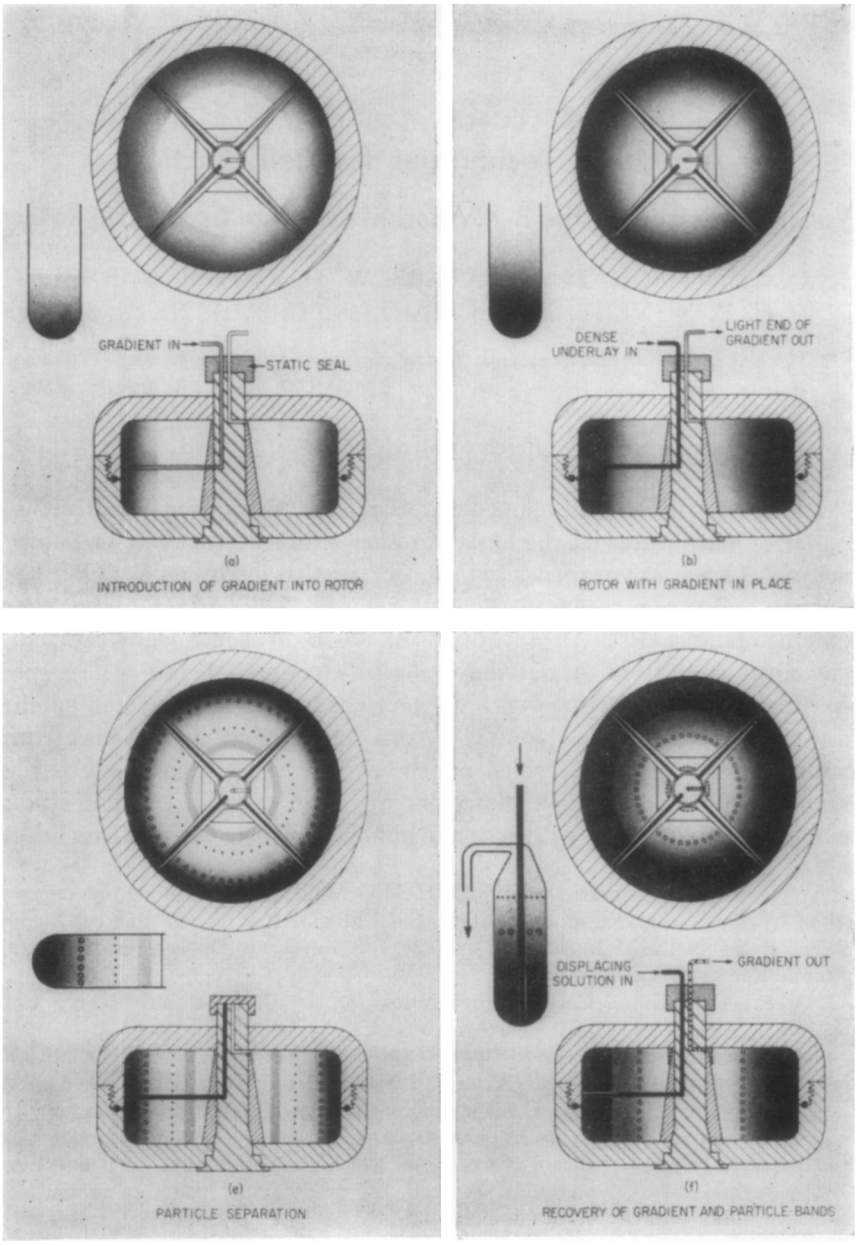
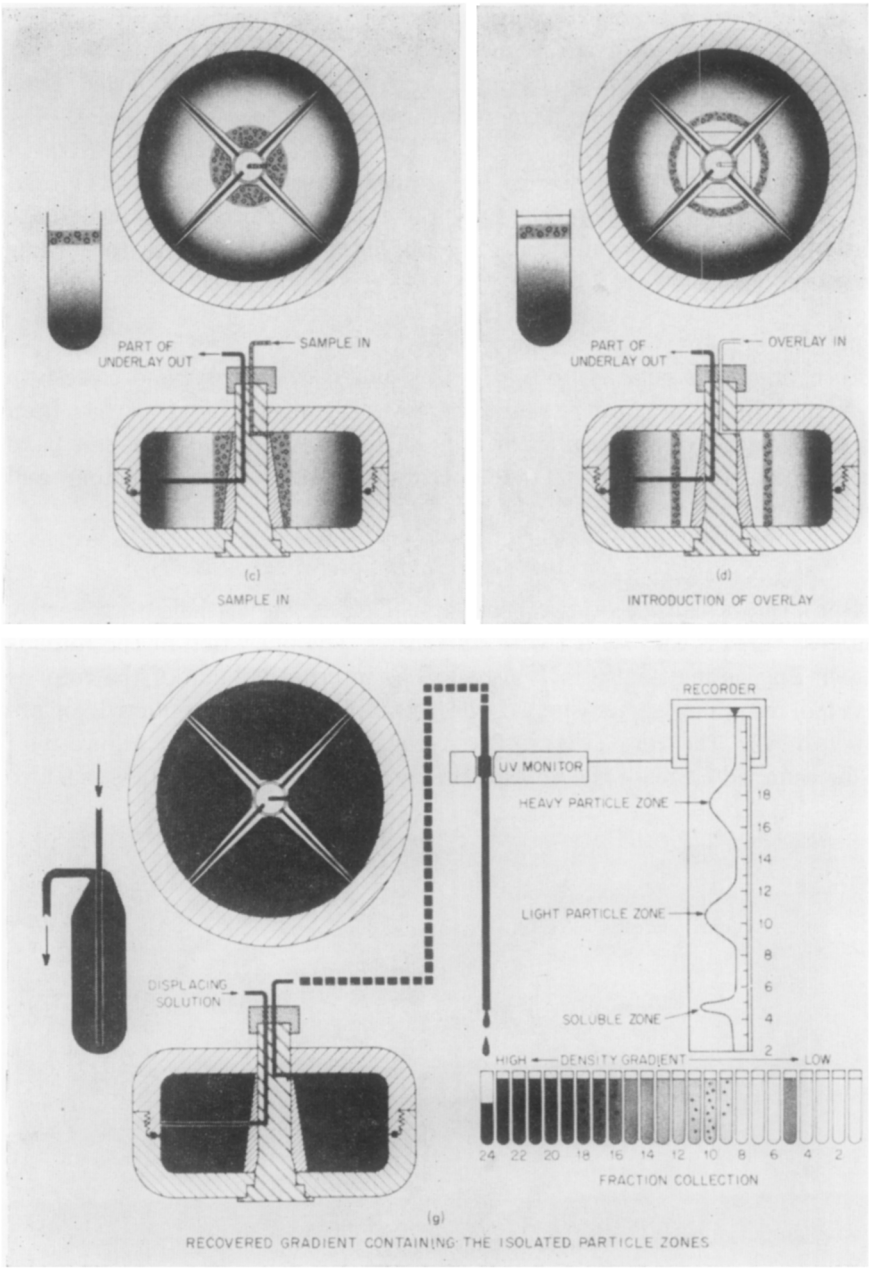


FIG. 1. Schematic diagrams of operation that apply to both B-XIV and B-XV zonal centrifuge rotors. Rotor shown at various stages of loading and unloading in top and side view. (a) Start of gradient introduction into rotor spinning at low speed, (b) completion of loading of gradient into rotor, (c) movement of sample layer into the rotor through the center line, (d) introduction of overlay into rotor



to move the starting zone away from the core faces, (e) separation of particles at high speed, (f) displacement of separated zones out of rotor at low speed, and (g) completion of unloading and collection of sample tubes. Note that the swinging-bucket equivalent of each step is also included.

modified preparative centrifuges appears to offer several advantages. However, when a rotor is designed to operate without an upper bearing, the configuration must be changed to be stable when supported from below by a flexible shaft. Two prototype steel rotors (B-X and B-XI) were developed during the course of this work (8) which demonstrated the feasibility of the concept. The rotors described here (B-XIV and B-XV) represent an advanced version of the earlier prototypes which are suitable for routine separations. A brief description of these rotors has appeared (16).

DESIGN

In over-all dimensions, rotors B-XIV and B-XV correspond closely to B-X and B-XI previously described (8). However, the rotor has been greatly simplified, the number of components reduced, and measures have been taken to insure better temperature control during loading and unloading.

Operating Principles

Both rotors consist of two semihemispherical halves held together by buttress threads and sealed with an O-ring. The operation of the rotor is shown diagrammatically in Figure 1. The internal volume of the rotor is divided into four sector-shaped compartments by four septa which are integral with the core (Figure 2). A coaxial seal (1, 6, 13) allows fluid to be pumped through the core to the rotor edge or to the inmost surface

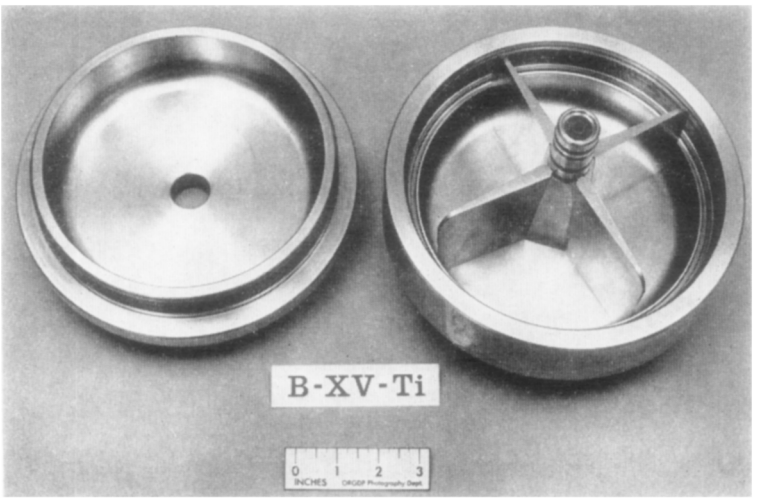


FIG. 2. Partially assembled B-XV rotor. Upper section of rotor (shown at left) screws into lower section at right.

of the core. The seal is attached to the rotor during loading and unloading at low speed (circa 3000 rpm) and is removed and replaced with a small cap for high-speed operation.

Over-all Configuration

The component parts of a B-XV rotor are shown partially assembled in Figure 2, and completely assembled in Figure 3. Two versions have been made, one with the center piece extending through the lower

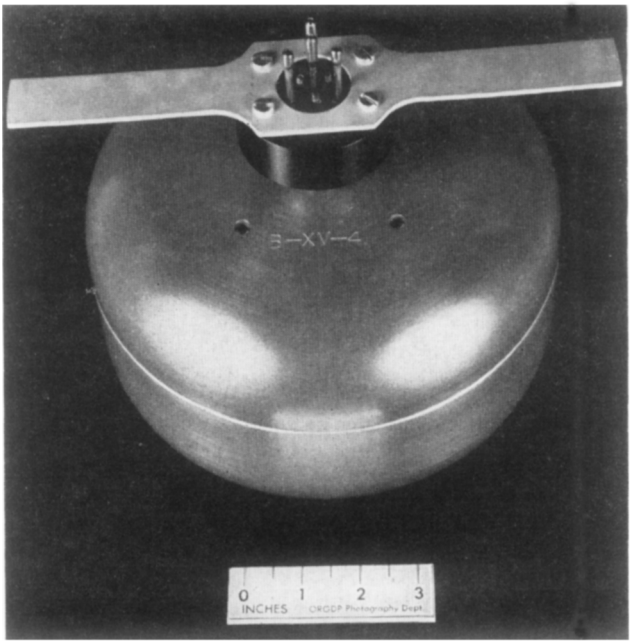


FIG. 3. Completely assembled B-XV rotor with removable upper seal in place.

hemisphere (not shown), the other with the center piece protruding only through the upper hemisphere. The ratio of the moments of inertia of the rotors is chosen to ensure stability when supported and driven from below with no upper bearing (16, 17).

Construction of the Seal

The removable static seal is attached to the rotor through a stainless-steel sealed bearing which centers the static seal and maintains its alignment against the flat rotating seal surface. Two flexible arms attach to a ring in the Spinco model L centrifuge chamber to prevent rotation of the static seal and, in addition, to serve as handles for the insertion and removal of the seal.

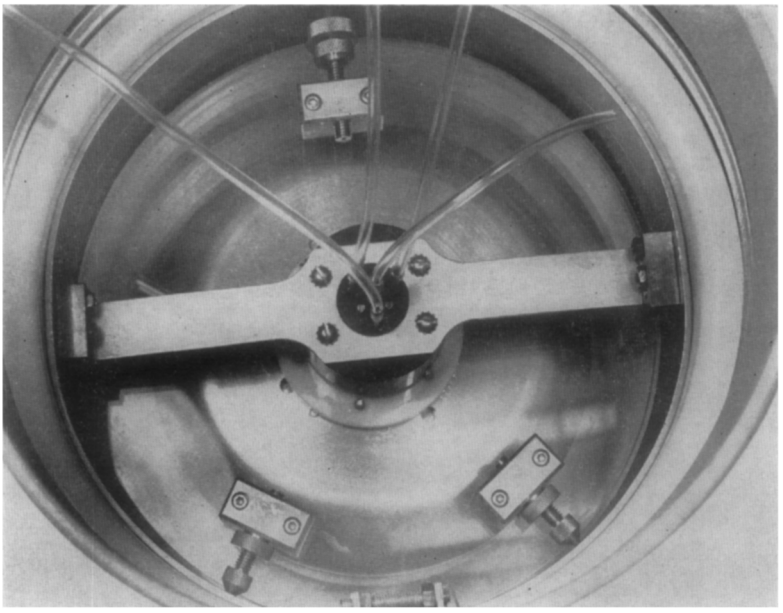


FIG. 4. Rotor and seal in place in Spinco model L preparative ultracentrifuge.

When the rotor is in place in the centrifuge, a Lucite cover is positioned immediately over the rotor to reduce air flow and to assist in the maintenance of low temperature during loading and unloading. The seal assembly is mounted above the Lucite plate (Fig. 4). An additional two-piece Lucite closure is used in place of the metal lid during loading and unloading further to diminish the flow of warm air through the centrifuge. The rotor, seal, and Lucite closures are shown in side view in Figure 5.

For high-speed operation, the static seal is manually removed and replaced with a closure having a rotatable upper section which allows it to be grasped safely when it is placed on or removed from the rotor. A Teflon ring on the underside of the lower Lucite shield prevents the rotor from being lifted off the drive spindle when the closure is removed.

The Rotor Core

The rotor core is similar to that previously described for B-X and B-XI (8), with a few small modifications. The septa, which are integral with the core, divide the internal rotor space into four sector-shaped compartments, and serve to prevent mixing and swirling due to Coriolis forces during loading and unloading, and to accelerate and decelerate the entire rotor contents uniformly. The edge lines, which are drilled through the septa, can be seen in Figure 2.

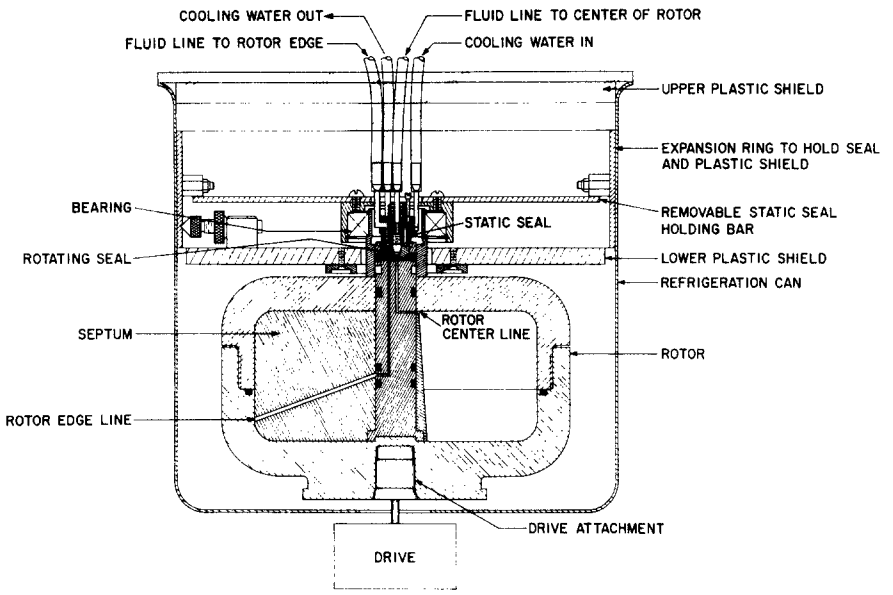


FIG. 5. Side view of assembled rotor in position in centrifuge during loading.

The center seal line connects to the rotor center, and the edge seal line to the rotor edge. To equalize the gradients in each sector, a small clearance is provided between the septa and the rotor wall. The flat center core faces serve to funnel the density and particle zones toward a point on their upper center surfaces where connection is made to the center fluid line. The edge lines through the septa may be either at right angles to the axis (Fig. 1) or tapered downward (Fig. 5) to allow fluid to be withdrawn by suction before disassembly.

Rotor Data

The pertinent data on the B-XIV and B-XV rotors are shown in Table 1. In addition to the speed advantages gained by titanium construction, it should be noted that such rotors may be steam-sterilized *in toto,* and are also free from corrosion by most salt solutions at pH's used in biological studies (pH 2–12).

The suggested operating speeds and maximum safe speeds for the B-XIV and B-XV rotors as a function of the specific gravity of the fluid used are shown in Figure 6. Note that, when a dense homogeneous fluid is centrifuged for a long time, a gradient is gradually formed which will markedly increase the wall pressure in the rotor. For safe operation the density of a gradient is considered as being that of its densest portion.

Analytical data can be obtained from the B-XIV and B-XV rotors if

TABLE 1
Characteristics of B-XIV and B-XV Zonal Rotors

Condition	B-XIV, aluminum	B-XV	
		Aluminum	Titanium
Weight (empty)	3.571 kg	7.439 kg	12.7 kg
Volume	649 cc	1666 cc	1666 cc
Speed	30,000 rpm	21,000 rpm	26,000 rpm
Maximum g	60,000 at 29,400 rpm	45,000 at 21,500 rpm	60,000 at 24,000 rpm
Maximum radius	6.62 cm	8.79 cm	8.79 cm
Maximum stress (tensile)	50,000 psi	50,000 psi	82,000 psi
Maximum radial growth	0.013 in.	0.0175 in.	—
Maximum pressure	5000 psi	5400 psi	8000 psi
Maximum end load	75,000 lb	125,000 lb	175,000 lb

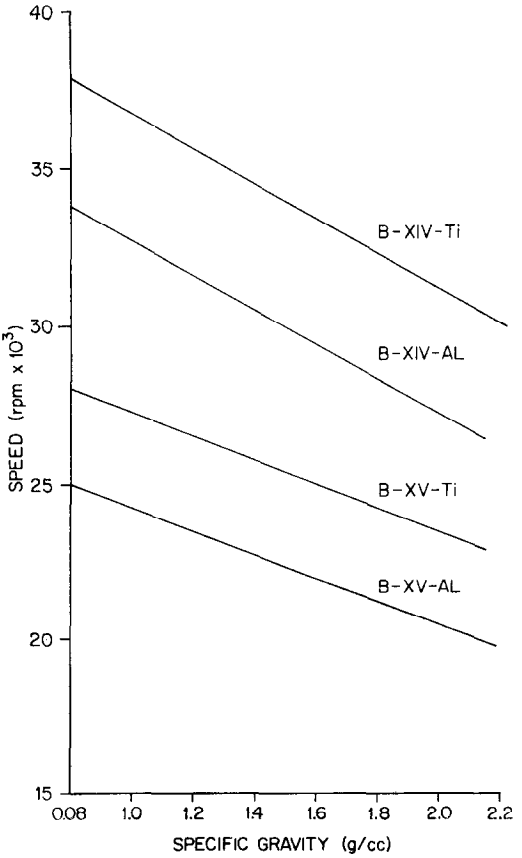


FIG. 6. Operating speeds of aluminum and titanium B-XIV and B-XV zonal rotors as function of density of fluids in rotor. The values given are quite conservative.

the starting position of a particle, its position at the end of the run, the temperature, the shape of the gradient, and the integral of $\omega^2 dt$ for the entire experiment are known (18). The position of the starting zone and of recovered zones in the rotor can be calculated, providing that the volume-position of these zones among the recovered fractions is accurately known, and providing that equations are available which relate recovered volume to radius in the rotor.

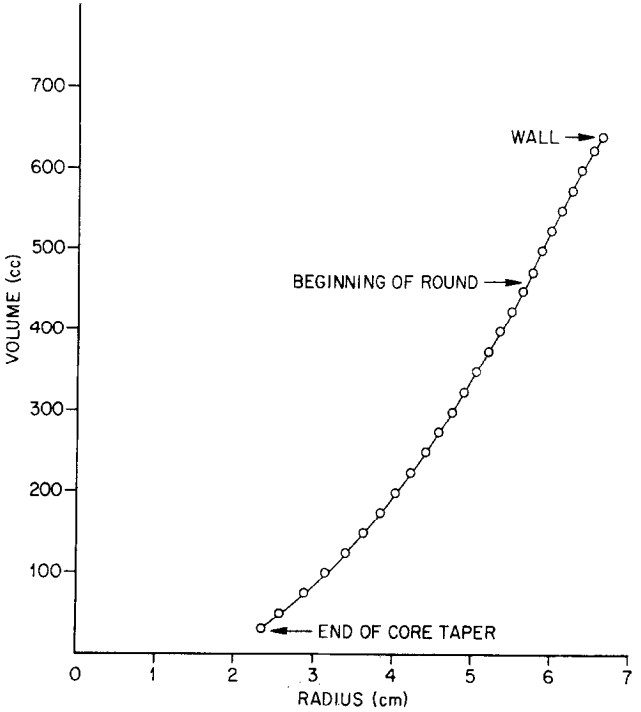


FIG. 7. Rotor volume as function of radius for B-XIV rotor.

The plots of rotor volume vs. radius are given in Figures 7 and 8 for rotors B-XIV and B-XV, respectively. These may be used to locate the position of a fraction in the rotor from the effluent analysis and to determine the width in the rotor of each fraction or peak obtained.

Rotor Performance

The first test of any new rotor system is to determine how closely the gradient recovered at the end of the run resembles the gradient introduced into the rotor. By using a cam in a piston gradient pump which provides a gradient which is linear with volume,[4] 500 ml gradients rang-

[4] Gradient pump model 131 obtained from Spinco Division of Beckman Instruments, Inc.

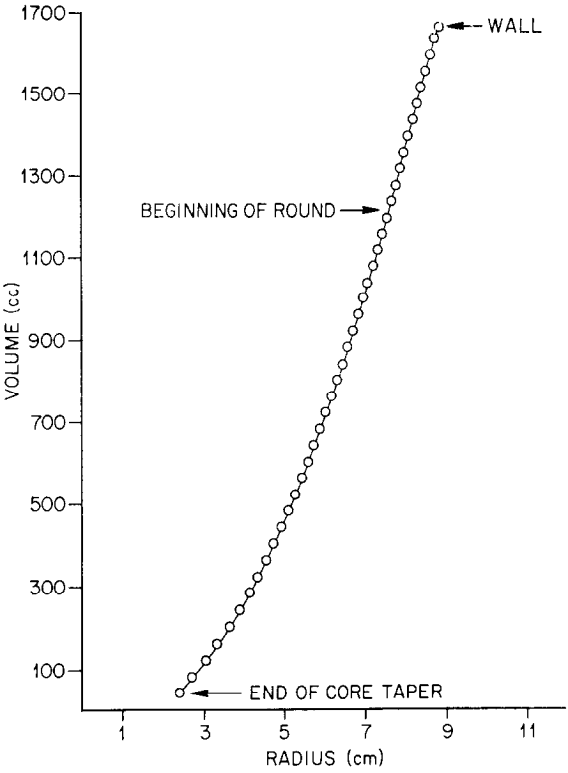


FIG. 8. Rotor volume as function of radius for B-XV rotor.

ing from 17 to 55% sucrose (w/w) were: (*a*) collected in 40 ml fractions straight out of the pump, (*b*) pumped into the B-XIV rotor at 3500 rpm and promptly displaced with additional 55% sucrose, (*c*) introduced into the rotor at 3500 rpm, accelerated to 30,000 rpm and run for 30 min, and then decelerated to 3500 rpm for unloading. The results are shown in Figure 9. Similar studies with a 1200 ml gradient in rotor B-XV are shown in Figure 10. In the latter instance the third experiment (*c*) was run at 20,000 rpm for 30 min. It is evident that little disturbance of the gradient occurs during passage through the rotor.

The boundary spreading which occurs when a sample is introduced into the B-XV rotor and then recovered, with and without acceleration to high speed, was next examined. Samples (20 ml) containing 3% bovine serum albumin stained with bromphenol blue in 5% sucrose were layered over 1200 ml gradients extending from 17 to 55% sucrose (w/w), followed by an overlay of 200 ml distilled water. When the sample was loaded in and unloaded at 3500 rpm, the results shown in Figure 11A were obtained. The width at half-peak height equaled the sample vol-

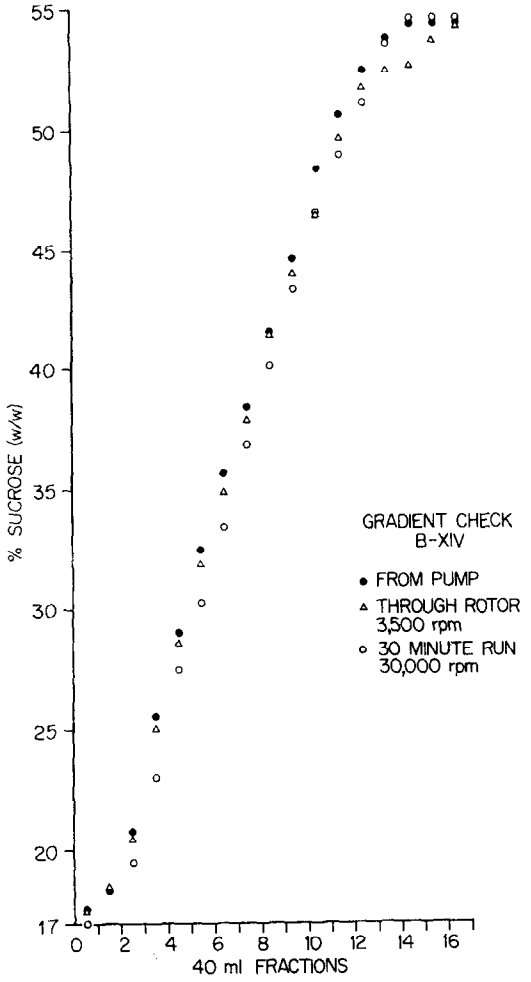


FIG. 9. Demonstration that gradients can be introduced into and recovered from the B-XIV rotor with little change: (●) gradient as produced by pump; (△) gradient introduced into rotor at 3500 rpm and recovered at once; (○) gradient introduced at 3500 rpm, accelerated to 30,000 rpm for 30 min, and then unloaded at 3500 rpm.

ume (20 ml) and was equal to a band width of 0.12 cm in the rotor. Some widening was seen (Fig. 11B) when the rotor was accelerated to 20,000 rpm for 30 min followed by deceleration to 3500 rpm for unloading. The width at half-peak height in the latter instance is 30 ml or 0.19 cm in the rotor. Similar studies with the B-XIV rotor also showed that very little boundary widening occurs with this rotor. A chart recording of one such experiment is shown in Figure 12.

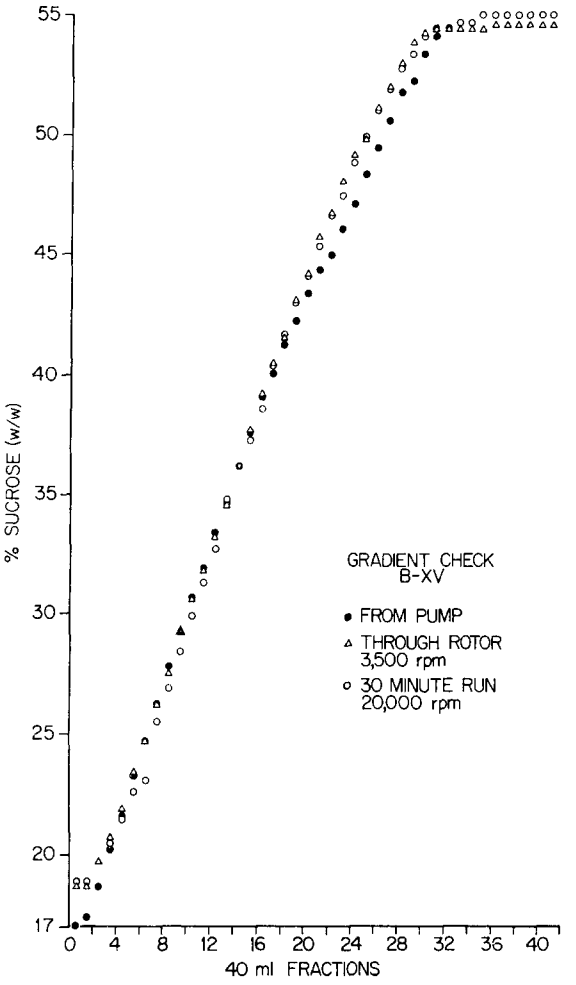


FIG. 10. Gradient recovery from B-XV rotor. Gradients recovered directly from pump, from rotor immediately after loading, and from rotor run 30 min at 20,000 rpm are nearly identical.

These studies demonstrate that zones can be introduced into and recovered from the upper or less dense portion of the gradient with little loss in resolution. It does not, however, prove that sharp zones can be formed and recovered from the area close to the rotor wall. To examine this point, alcohol-extracted ragweed pollen grains (1 ml packed cells in 20 ml 5% sucrose) were placed on a 17–55% sucrose gradient in the B-XIV rotor followed by 50 ml of water as an overlay. Since the cells band very quickly, it was not necessary to accelerate the rotor past 3500 rpm. The results are shown in Figure 13 and indicate that sharp

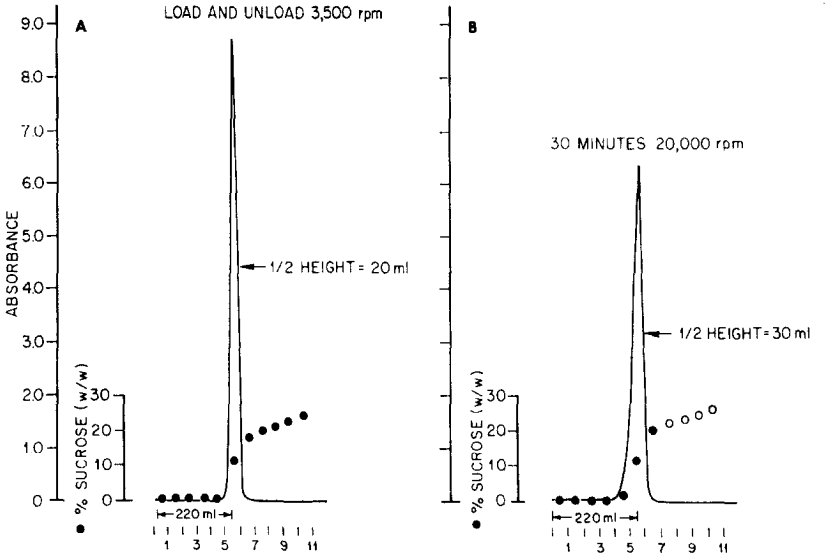


FIG. 11. Degree of widening of sample boundary in B-XV rotor. Details in text 40 ml samples collected.

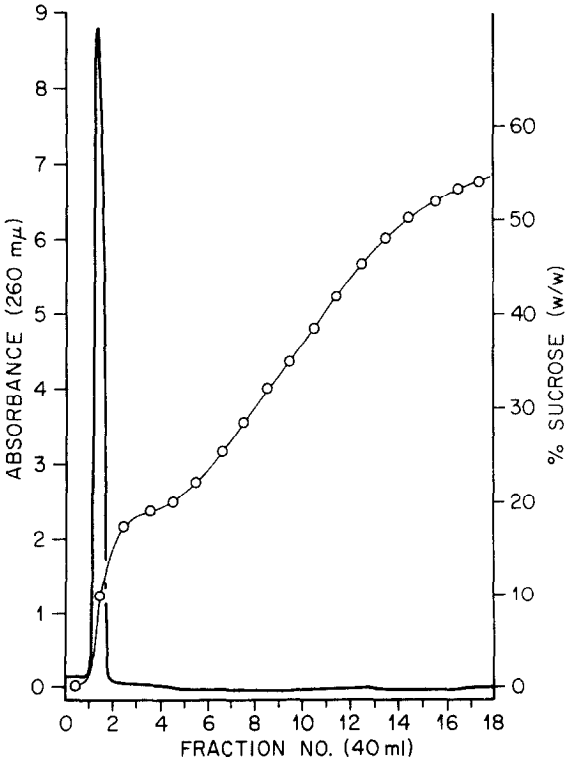


FIG. 12. Recovered sample zone from B-XIV rotor. Details in text.

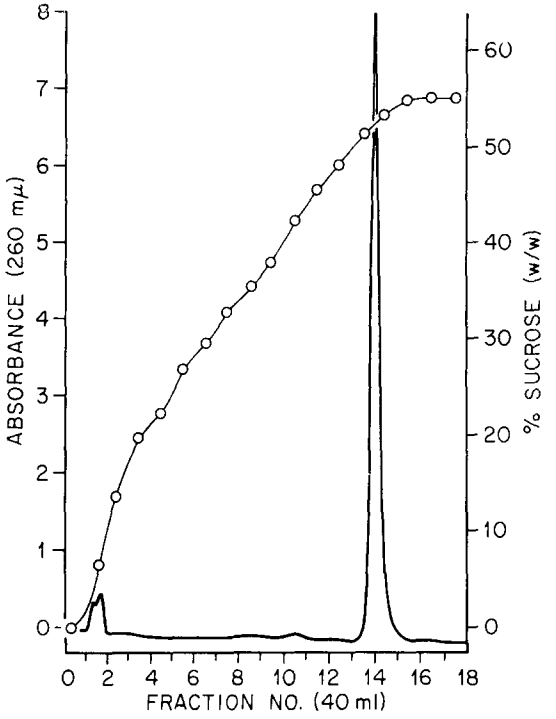


FIG. 13. Recovery of banded ragweed pollen in B-XV rotor. Details in text.

zones can be recovered far down the gradient. Similar studies at both low and high speeds have been done in B-XV with good results. In some instances, however, multiple sharp peaks were observed, suggesting that there were small differences in rate at which different sectors of this rotor were unloaded. (This difficulty has been corrected by increasing the distance between the septa and the rotor wall at the point where the edge line opens at the septum edge.) These results show that the rotor is operating satisfactorily from a purely physical viewpoint.

Evaluation for Molecular Separations

The separation of macroglobulin from plasma illustrates the lower limits of the separations thus far attained with the B-IV zonal rotor (12). Similar separations have been explored with the B-XV titanium rotor. Since the latter does not attain the speed of the B-IV, the sample must be moved out in the rotor a considerable distance to achieve a sufficiently high centrifugal force. In the experiment, shown in Figure 14, 25 ml of undiluted rat plasma was layered over a 1000 ml gradient extending from 10 to 22% (w/w) sucrose which was linear with radius. The solutions used to make the gradient were made by dissolving sucrose in Miller-

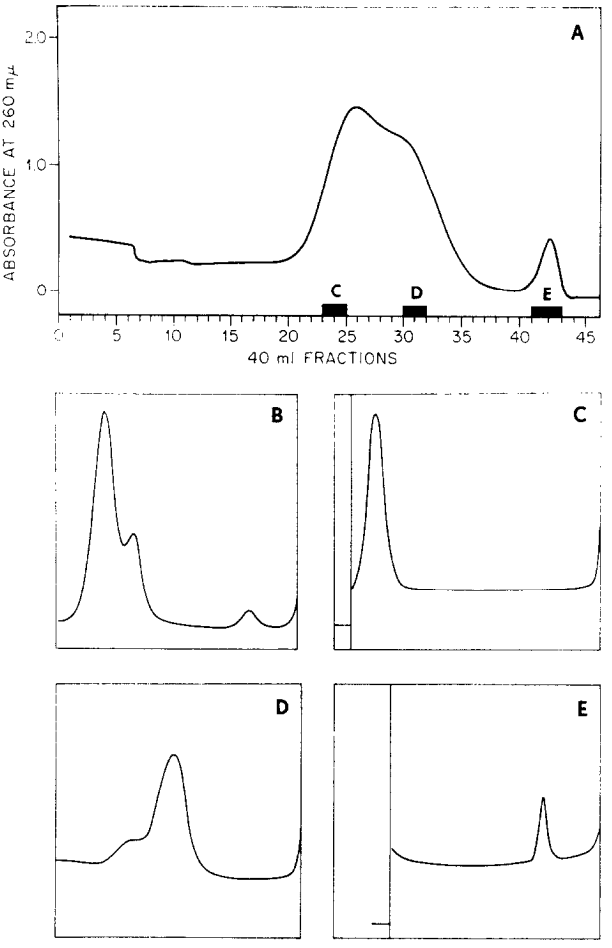


FIG. 14. Fractionation of rat serum proteins in a titanium B-XV zonal rotor: 25 ml of undialyzed rat serum was layered over a 1 liter gradient (linear with radius) extending from 10 to 22% sucrose (w/w) made up in Miller-Golder buffer (pH 7.5, $\mu = 0.2$); sufficient overlay was added to move the sample more than half way out to the rotor edge; the rotor was centrifuged at 27,000 rpm until $\omega^2 t = 46.8 \times 10^{10}$. Chart A shows absorbance of recovered gradient with light end of the gradient to the left. Fractions C, D, and E shown on Chart A were dialyzed, concentrated by pressure ultrafiltration, and analyzed in the Spinco model E analytical ultracentrifuge at 59,780 rpm. In B is shown the original serum after 40 min at speed. C shows an essentially pure albumin peak (28 min), D a partial separation of the globulin peak (60 min), and E an essentially pure macroglobulin peak (32 min).

In this experiment the macroglobulin peak was run to the rotor wall in an attempt to achieve maximal separation of the albumin and globulin peaks without pelleting the macroglobulin. For routine preparation of macroglobulin from plasma, a somewhat shorter centrifugation time is used.

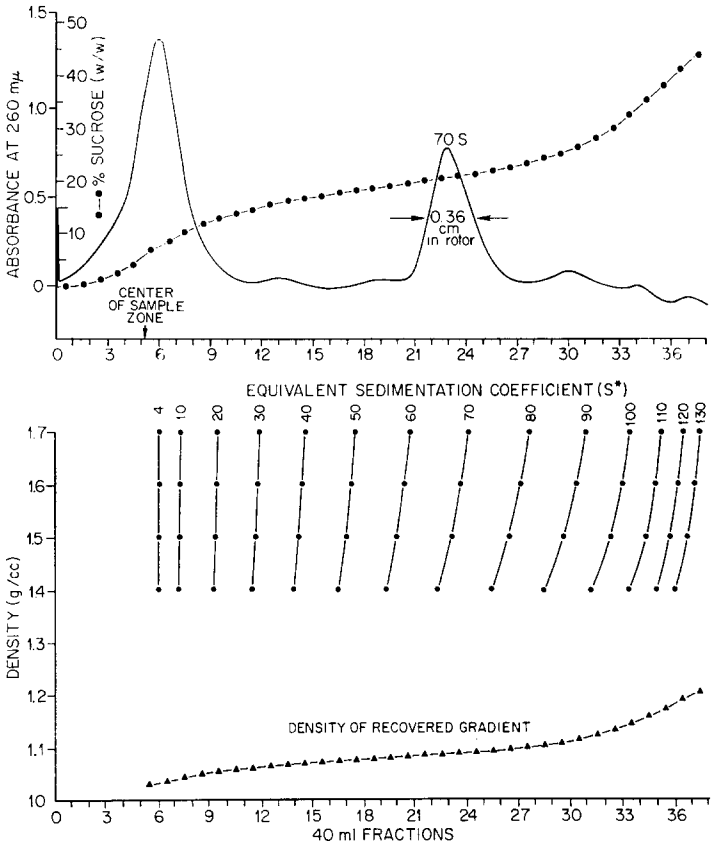


FIG. 15. Separation of rat spleen ribosomes. Four spleens (3.5 gm) homogenized in 20 ml of 8.5% (w/w) sucrose, centrifuged to remove cells, nuclei, and mitochondria (5 min at 10,000 rpm in Spinco No. 30 rotor) and 5 ml of 1% deoxycholate added to supernatant solution. The gradient volume was 1200 ml and extended from 10 to 25% sucrose with an underlay of 55% sucrose. $\omega^2 t = 6 \times 10^{10}$. Gradient solutions in B-XV contained 0.005 M Tris Cl, 0.0015 M $MgCl_2$, and 0.001 M KCl at pH 7.6. Temperature 0°C. The lower portion of the figure shows the positions ideal particles having different sedimentation coefficients would have as a function of their density. The volume vs. density curve for the recovered gradient is also given (18).

Golder buffer (19), $\mu = 0.2$ at pH 7.5. Examination of three fractions in the analytical ultracentrifuge showed complete separation of the macroglobulin, but only incomplete separation of the albumin and globulin peaks (Fig. 14).

The separation of ribosomes from a spleen homogenate treated with deoxycholate is shown in Figure 15. Small amounts of subunits are seen, and, in addition, indications of three polysome peaks appear.

Detailed studies on the isolation of subcellular particles including nuclei, mitochondria, lysosomes (20), microsomes, glycogen, polysomes, and viruses in the B-XIV and B-XV rotors will be recorded elsewhere.

DISCUSSION

Zonal centrifugation in liquid density gradients may be considered the preparative counterpart of analytical ultracentrifugation as conventionally done in initially homogeneous solutions. However, when carried out in swinging buckets it suffers from two disadvantages as a preparative method. The first disadvantage is the small capacity of the thin starting zones used to achieve good resolution; the second is that as faster rotors are designed, the volume of the tubes is decreased (except when new and stronger rotor material is employed).

The solution to the problem of high-speed preparative zonal centrifugation has been to arrange to do it inside the largest pressure vessel that can be spun at a given speed. The speed chosen and the materials of construction used dictate the diameter of the vessel or rotor. The total internal volume is then dependent upon the rotor length. As discussed previously by Barringer (17), the configurations in which the length and the diameter of the rotor are equal are inherently unstable, and the rotor must be designed so that the ratio between length and diameter is either greater or less than one, i.e., the rotor must be either an elongated cylinder or disc-like. The former configuration was used in the B-IV rotor, and the latter was chosen for the B-XIV and B-XV rotors described here. The advantage of disc configuration is that the rotor may be spun with no upper bearing; the disadvantage is that the rotor volume is less than that in a tall cylindrical rotor capable of operation at the same maximum speed. In this instance, the objective has been to combine both simplicity of operation and adaptability to widely available centrifugal equipment with preparative-scale capacity at speeds capable of separating particles down to the level of large macromolecules.

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