

Continuous-Flow Centrifugation Combined With Isopycnic Banding: Rotors B-VIII and B-IX¹

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SUMMARY

Pelleting inactivates some viruses and aggregates other dispersed material. Methods for separation should be developed in which desired particles are always kept in suspension. Rotors that combine continuous-flow centrifugation with isopycnic banding can achieve this effect. In our system a liquid density gradient is set up in a spinning rotor next to the wall in each of 4 sector-shaped compartments. The virus-containing suspension is made

to flow over the centripetal surface of the gradient between the gradient and the tapered core of the rotor. Virus particles sediment out of the stream into the imprisoned gradient. At the completion of a run, components of the gradient are recovered by displacing the gradient centripetally with a dense fluid and channeling the gradient out a center line.—Nat Cancer Inst Monogr 21: 199-216, 1966.

THE A- and B-series zonal centrifuges allow high-resolution separations of particles ranging from whole cells to large protein molecules to be effected on the basis of sedimentation rate (1-4). Since the resolution obtained in rate-zonal centrifugation is a function of the thickness of the sample zone, and hence its volume, it is often necessary to preconcentrate a dilute suspension of particles so that a reasonable mass of material may be introduced into the rotor as a very narrow zone. This is especially true of virus particles and ribosomes, both of which are usually present in very low concentration. During the operation of a zonal centrifuge, a stream of liquid flows through the rotor during part of the loading cycle and gradient recovery. By use of the theoretical approaches of Berman (5), the B-V core, designed for high-performance, continuous-flow centrifugation was developed (6) and it performed as predicted (7). This rotor, however, is usually disassembled to recover the sedimented particles from the rotor wall. If all the sediment is to be recovered (8), use of this rotor is limited to batch operation and to the separation of particles not damaged

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by pelleting. Unfortunately, many viruses, especially the myxoviruses, lose biological activity when pelleted. In addition, sedimented particles often do not resuspend properly, and, in subsequent rate and banding experiments, infectivity may be observed in a spectrum of particles—discrete virus particles and clumped or aggregated particles of many different sizes. It was highly desirable, therefore, to develop a centrifugal method for concentrating and purifying virus particles that would keep them suspended at all times. A specific problem in keeping particles suspended, and a major stimulus to the development of these continuous-flow methods, is the possibility of producing effective vaccines which contain only those antigenic proteins necessary to produce immunity. It is evident that both centrifuge development and biochemical problems must be solved during the course of this work. When the B-II rotor was used as a continuous-flow centrifuge, T3 phage particles were recovered from 20 liters of lysate and then banded in the same rotor in a cesium chloride gradient (9). This suggested that rotors could be designed in which the virus stream flowed over a density gradient stabilized at the rotor wall, and was recovered by displacement through the zonal rotor core (9). A group of rotor cores designed to explore these possibilities is discussed in the present paper and have been previously noted in brief communications (10, 11).

THEORETICAL CONSIDERATIONS

The sequence of steps proposed for the preparation of a vaccine composed only of antigenic viral subunits is as follows:

1. Continuous-flow centrifugation combined with isopycnic banding to concentrate virus particles from up to 100 liters of culture medium using B-VIII or IX rotor cores.
2. Dialysis to lower the density of the virus fraction. (Alternatively the virus may be recovered without banding by use of the B-V core, and then banded in small-volume tubes.)
3. Rate-zonal centrifugation to yield particles having a narrow range of sedimentation coefficients.
4. Dialysis to remove gradient solutes.
5. Dissociation of viral particles to yield suspensions containing antigenic subunits.
6. Rate-zonal centrifugation of dissociated material to yield a purified zone of subunits.
7. Recovery of subunits from the gradient solution.
8. Additional isopycnic banding if required.

It is important to determine at the outset whether the systems have the proper capacities to be used in this sequence, since the particle capacity of a continuous-flow and that of a zonal centrifuge are quite different. The maximum volume of fluid being considered for vaccine production is 100 liters. In practice, the volumes used would probably be

one fourth to one half this amount. The total mass of virus material in a preparation may be predicted from the equation:

$$M_t = TRM_v V \cdot 10^3$$

where M_t equals the total virus mass in grams, T the titer in infectious particles per ml, R the ratio of physical particles to infectious particles, M_v the mass of one virus particle in grams, and V the total volume of virus-containing fluid in liters.

For polio, with a mass of 12×10^{-18} g per particle, a titer of 10^8 , an infectivity ratio of 100 (12), and a volume of 100 liters, the mass of viral particles would be 12 mg. In a 100 liter volume of influenza culture fluid, having a titer of 10^8 , an infectivity ratio of 10 (12), and containing large amounts of other particulate material with the same density as the desired virus, the masses of material expected are well within the limits of the B-IV zonal centrifuge for rate and isopycnic zonal separations. If a continuous-flow centrifuge having sufficiently high flow rates that will also band particles is feasible, then experimental studies on subunit vaccine preparation can be initiated.

In order to design a continuous-flow rotor that will allow viruses to band in a density gradient, we sought to (a) set up the gradient in a spinning rotor, (b) flow large volumes of fluid over the gradient without disturbing it at high speeds, and (c) recover the gradient and suspended particles without loss of banding resolution. Two cores (B-VI and B-VII), consisting of tapered cylinders, were constructed to determine whether stabilizing vanes or septa were necessary. Experimental studies with these cores indicated that excessive mixing occurred between the continuous-flow stream and the gradient. A core having 4 radially arranged vanes (B-VIII) was therefore constructed and tested.

THE B-VIII CORE

Description

The B-VIII core shown in figure 1 consists of a solid, tapered conic section and 4 vertical, radially oriented fins. The principles of operation are best understood by the schematic diagrams shown in figure 2. All fluid lines were arranged so that, as far as possible, air bubbles were swept out of the system during operation. The line extending through the annular ring of the seal is connected directly with the top and the outer edge of the fluid compartment. The center seal line extends straight to the bottom of the core and enters the rotor chamber at the lower or smaller diameter end of the core.

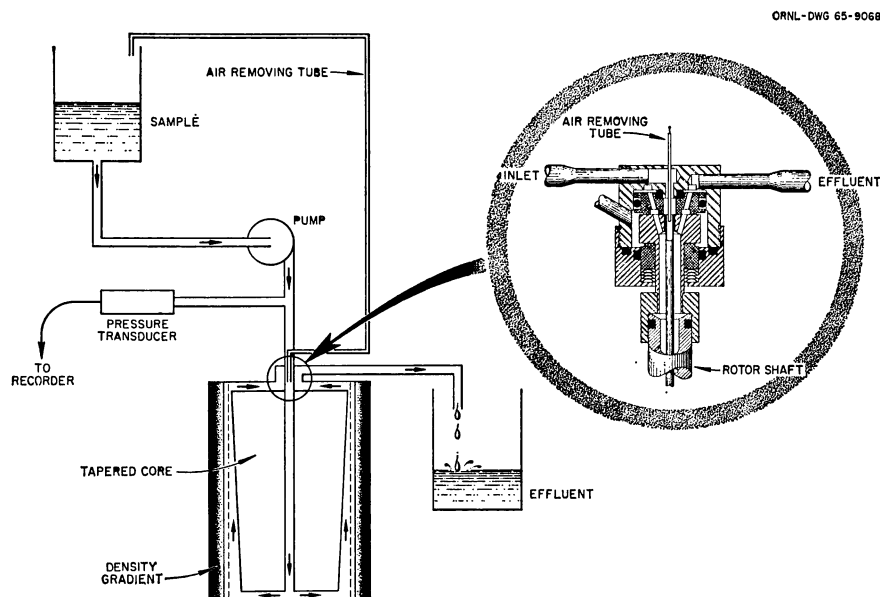
Operation

Initially, all fluid lines and the rotor chamber are filled with a sterile solution of low density through the center line, and all air in the rotor and lines is expelled. After acceleration to low speed (3000 rpm) the

gradient solution is pumped into the rotor edge (fig. 2b). This solution may be introduced as a gradient or as a volume of fluid of high density. In the latter instance the gradient is formed by diffusion. As the solution moves into the rotor it is held against the rotor wall by centrifugal force, and an equal volume of the fluid is displaced out the center line. The total internal volume of an unmodified B-VIII rotor core is 930 ml; the fluid volume centripetal to the maximum radius of the tapering core is 813 ml.

After a gradient has been set up in the rotor, the direction of fluid flow through the rotor is reversed, and a fluid having approximately the same density as the sample is pumped in through the center (lower) line. Any portion of the density gradient centripetal to the maximum core diameter is displaced out of the rotor through the inner portion of the upper line. While this light fluid is being pumped, the rotor is accelerated to operating speed, and the sample is pumped through the rotor in the same direction (in by the center line, out by the edge line; *see* fig. 2c).

Two points concerning operation of this rotor system should be noted. Any air trapped in the fluid flowing through the rotor is trapped at the narrow (lower) end of the core and rapidly raises the back pressure. To prevent this situation, a bleed line was incorporated in the seal to divert part of the feed stream and any air back into a reservoir (text-fig. 1).



TEXT-FIGURE 1.—Schematic diagram of B-VIII-B-IX rotor systems and seal. As the sample stream is pumped into the rotor, hydrostatic pressure is monitored at the entrance of the center line of the seal. A small part of this stream and trapped air bubbles are allowed to flow back into the sample reservoir. The remaining particle-rich stream passes to the lower part of the rotor, back through the tapered region of the core, and out the edge line.

The second point concerns the effect of diffusion of gradient solutes into the fluid stream. Centrifugal force produces a large hydraulic pressure in the rotor chamber. This pressure has a negligible effect on the flow of fluid through the rotor because the pressure gradients in the entrance and exit lines cancel each other out as they are brought back toward the axis of rotation, where the pressure due to centrifugal force is small. In practice the exit line leaves the rotating seal face on a small radius, a situation sufficient to make the rotor a centrifugal pump. This effect is countered by any increase in density of the exit stream because of inward diffusion of the gradient solute. Considerable back pressure is produced by a very small amount of solute during high-speed operation. Since diffusion is a time-dependent process, it is essential to have *a constant flow of fluid through the rotor at all times*. When flow during high-speed operation is interrupted briefly, enough back pressure develops to stop flow, and the rotor speed must be reduced to start flow again.

When the continuous-flow portion of an experiment was completed, it was generally desirable to continue centrifugation for 30 minutes to 1 hour to insure that all particles were banded at their proper levels. When there was any danger that enough of the gradient solute had diffused out of the rotor via the exit stream to lower the maximum gradient density below the banding density of the virus, 50 ml of dense gradient solution was pumped to the rotor edge before the final banding period.

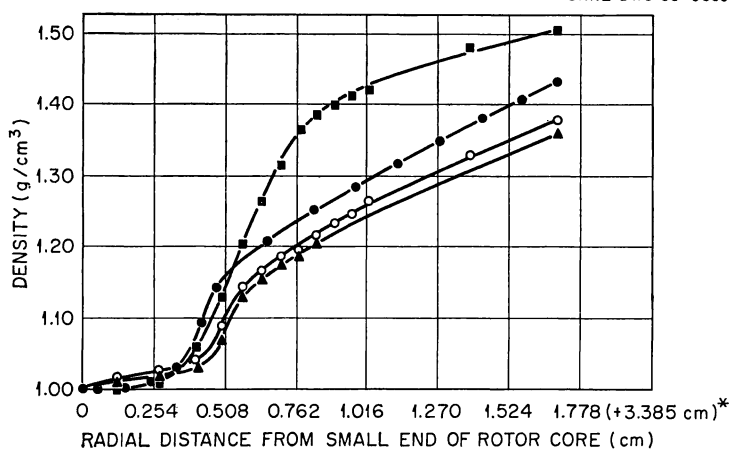
The gradient is recovered (fig. 2d) at a speed between 3000 to 5000 rpm. A solution having a density equal to or greater than the maximal gradient density is pumped to the rotor edge, displacing the contents toward the rotor axis. As the banded zones approach the tapered core, they are funneled down to the lower exit line and out through an ultraviolet absorbance monitor. Since a portion of the culture fluid is left in the rotor close to the core, the absorbance initially will be high and will grade out into the separated zones.

The taper of the core, therefore, serves to define the maximum volume of the virus-containing fluid in the rotor (the stream volume) and to guide the gradient out of the rotor at the end of the run (fig. 2e).

EXPERIMENTAL STUDIES WITH THE B-VIII CORE

To determine whether gradients could be set up and recovered from the B-VIII rotor, solutions of cesium chloride, cesium tartrate, and potassium citrate were introduced into the rotor during rotation. Results are described in text-figure 2. Diffusion produced suitable gradients in a very short space of time. The maximum slope, as expected, occurs close to the interface between the stream and gradient.

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TEXT-FIGURE 2.—Density gradients recovered from B-VIII rotor after continuous-flow centrifugation.

Symbol	Solution	Gradient		Maximum operating speed (rpm)	Effluent collected (liters)
		Volume used (ml)	Density (g/cm ³)		
■	Cesium chloride	500	1.62	28,000	~3.5
●	Cesium chloride	300	1.62	28,000	~8.0
▲	Potassium citrate	250	1.54	30,000	~4.5
○	Cesium tartrate	300	1.51	36,000	~4.0

In these experiments relatively small volumes of fluid were run through the rotor. The results show that gradients can be set up and recovered from the B-VIII rotor. However, since a continual removal of gradient material takes place, it may be necessary to add small amounts of concentrated gradient solution at experimentally determined intervals.

Four experimental runs, in which virus-containing media were used, were made with the unmodified B-VIII rotor (table 1). Gradient solutions were established by diffusion of the dense gradient solution into water. The results obtained with adenovirus 2 are shown in text-figure 3. An electron micrograph of a dialyzed sample from the virus peak is shown in figure 3.

The most serious problem with the B-VIII rotor was the high pressure necessary to force fluid through the rotor.

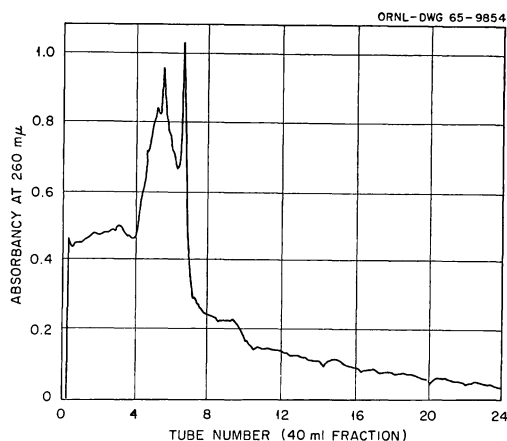
TABLE 1.—B-VIII separation runs

Top operating speed (rpm)	Gradient		Virus particle	Sample volume (liters)	Gradient unloading speed (rpm)	Average flow (liters/hr)
	Material	Loading speed (rpm)				
28,000	Cesium chloride	Statically	Adenovirus 2	3	8000	1.7
36,000	Cesium chloride	2000	Respiratory syncytial	5	8000	1.5
30,000	Potassium citrate	8000	Moloney	3	8000	2.0
30,000	Cesium tartrate	8000	Moloney	2	8000	1.9

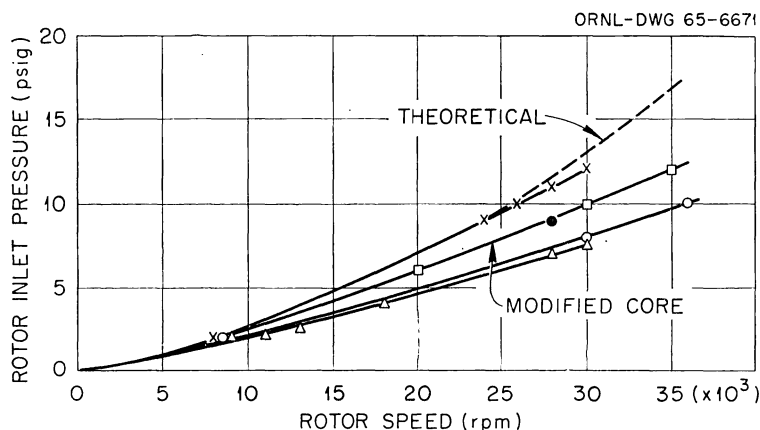
Text-figure 4 shows the pressure of the fluid stream entering the rotor as a function of rotor speed for different gradient solutions of selected volumes and densities. These pressure measurements are reproducible as long as no air is entrained in either of the flow channels. The inlet pressure can also be approximated by comparison of the flow path of the fluid stream through the rotor to a U-tube manometer with a fluid of different density in each of the legs, as illustrated in text-figure 5. Diffusion coefficients are ignored. The calculations are handled as follows:

$$(P_o - P_s)_{\text{atm}} = 0.873 \times 10^{-8} (\text{rpm})^2 [D_2^2 (\bar{\rho}_{2S} - \bar{\rho}_{21}) + D_s^2 (\bar{\rho}_{21} - \bar{\rho}_{10}) - D_s^2 (\rho_{2S})] \quad (1)$$

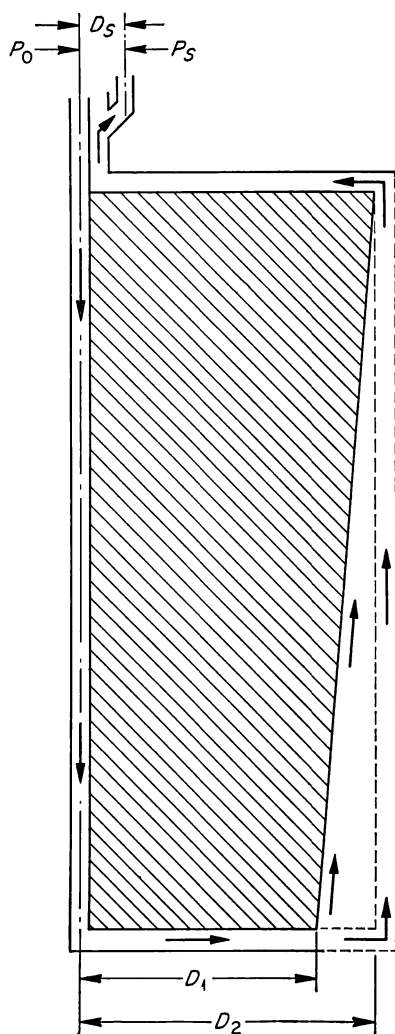
where D_1 = diameter at small end of core taper, inches; D_2 = diameter at large end of core taper, inches; D_s = smallest diameter at which effluent leaves the rotating system, inches; P_o = pressure at the axis, atm; P_s = pressure at D_s , atm; $\bar{\rho}_{2S}$ = average density between D_s and D_2 at the top (measured), g/cm³; $\bar{\rho}_{21}$ = average density between D_1 and D_2 at the



TEXT-FIGURE 3.—Isolation of adeno-virus 2 in B-VIII centrifuge. The virus was found in the second (highest) peak.



TEXT-FIGURE 4.—B-VIII rotor system inlet pressure. Average flow, about 3 liters per hour. X: 500 ml cesium chloride, density 1.62 g/cm³, theoretical; ●: 300 ml cesium chloride, 1.62; △: 250 ml potassium citrate, 1.54; ○: 300 ml cesium tartrate, 1.51; □: 500 ml cesium chloride, 1.62, modified core.



TEXT-FIGURE 5.—Flow path diagram for the B-VIII rotor system. $D_s = 0.635$ cm; $D_1 = 6.769$ cm; and $D_2 = 7.620$ cm or 6.960 cm in revised version.

bottom (estimated), g/cm³; $\bar{\rho}_{10}$ = average density between axis and D_1 at the bottom (measured), g/cm³.

Two alterations were made in the B-VIII rotor in an attempt to lower the back pressure. First, the free space in the lower end of the core was increased to allow a small amount of mixing between fluid flowing into the rotor and the fluid already in the rotor. A small increase in the density was expected as a result of this mixing. Secondly, the core taper was reduced from 1° to $\frac{1}{4}^\circ$. The volume of culture fluid in the rotor was thereby decreased from 117 ml to 30 ml and the dwell time for a given aliquot of fluid proportionately reduced.

The characteristics of the original and modified designs of the B-VIII core are listed in table 2.

TABLE 2.—Characteristics of B-VIII rotor core

Dimension	Original	Modified
Effective core length	24.4 cm	23.5 cm
Total chamber volume	930 ml	1020 ml
Fluid stream volume in chamber	117 ml	30 ml
Gradient volume in chamber	813 ml	990 ml
Maximum core diameter (excluding fins)	7.62 cm	6.96 cm
Minimum core diameter (excluding fins)	6.77 cm	6.77 cm
Core taper	1°	¼°
Unloading speed (minimum)	1178 rpm	2556 rpm

Experimental studies showed that the back pressure was reduced appreciably by these modifications (text-fig. 4). The core angle could not be reduced to such a small angle that particle zones were not funneled out properly. As previously discussed, these zones are sections of a paraboloid of rotation (2, 13). It is important to know whether, at the rotational speeds used for gradient recovery, a zone will contact the core initially at the top and then move down as expected. By use of the equation:

$$L = \frac{r^2 \omega^2}{2g} \quad (2)$$

where L is the distance from the apex along the axis of a paraboloid of rotation, r is the radius from the axis of rotation, g is the acceleration due to gravity, and ω is the angular velocity in radians per second. The critical unloading speed, in revolutions per minute, defined as the speed at which a zone will touch the upper and lower ends of the core simultaneously during unloading, may be derived from equation 2 as follows:

$$L_1 - L_2 = \frac{r_1^2 \omega^2}{2g} - \frac{r_2^2 \omega^2}{2g} = \frac{\omega^2}{2g} (r_1^2 - r_2^2) \quad (3)$$

$$\omega = \sqrt{\frac{(L_1 - L_2)2g}{r_1^2 - r_2^2}} \quad (4)$$

and

$$\text{RPM} = \frac{60}{2\pi} \sqrt{\frac{(L_1 - L_2)2g}{r_1^2 - r_2^2}} \quad (5)$$

The critical unloading speeds for the original B-VIII and modified B-VIII cores are 1178 and 2556 rpm, respectively. Even at this speed the flow of fluid across the core face would tend to maintain the resolution of zones.

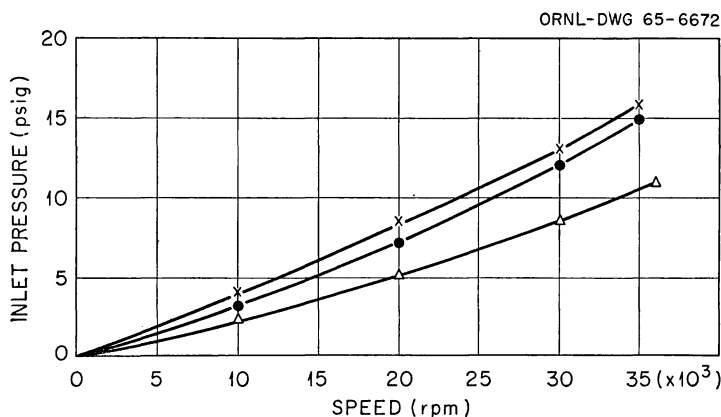
THE B-IX CORE

The B-IX core was designed to minimize the pressure required to force fluid through the rotor and to reduce the core weight. The rotor characteristics are given in table 3. To reduce the inlet pressure, the core taper was held to the minimum commensurate with a low critical unloading speed and reasonable resolution during unloading. The flow channels through the core were kept as small as possible without restricting flow. The core diameter has been enlarged to increase the centrifugal force across the flowing stream and to reduce the volume of gradient material in the rotor. The weight of the core has been decreased approximately 40 percent by being made hollow.

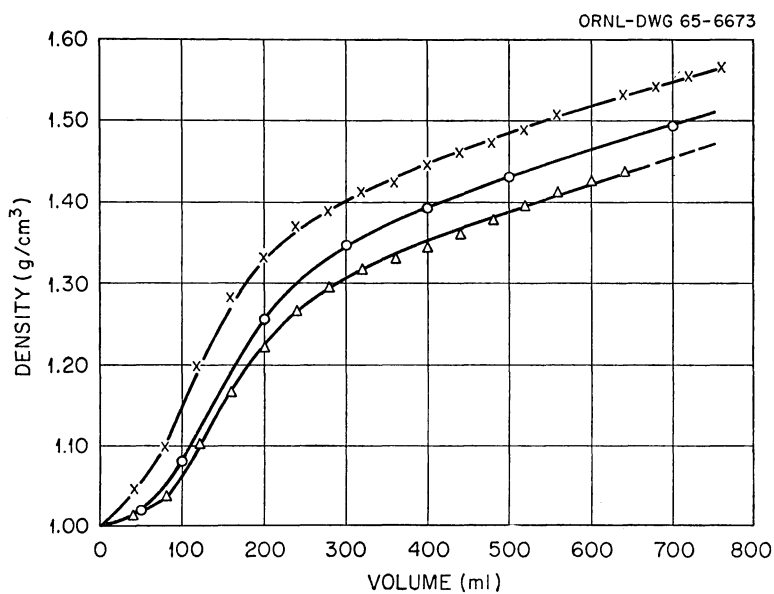
TABLE 3.—Characteristics of B-IX rotor core

Effective core length	24.92 cm
Total chamber volume	749 ml
Fluid stream volume in chamber	79 ml
Gradient volume in chamber	670 ml
Maximum core diameter	8.26 cm
Minimum core diameter	7.84 cm
Core taper	1/2°
Unloading speed (minimum)	1602 rpm

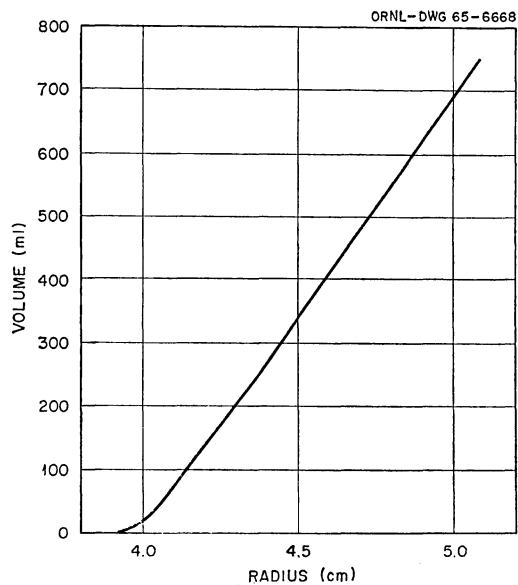
Inlet pressure as a function of rotor speed for a number of gradient volumes and densities is shown in text-figure 6. It is evident that the maximum of 20 pounds per square inch is not exceeded at 40,000 rpm. Gradients set up and recovered from the rotor are shown in text-figure 7. The recovered gradient may be related to radius in the rotor by use of the graph shown in text-figure 8.



TEXT-FIGURE 6.—Inlet pressure in B-IX rotor at an average flow rate of 3 liters per hour. ●: 500 ml of cesium chloride, density, 1.73 g/cm³; Δ: 500 ml, 1.63; X: 700 ml, 1.63.

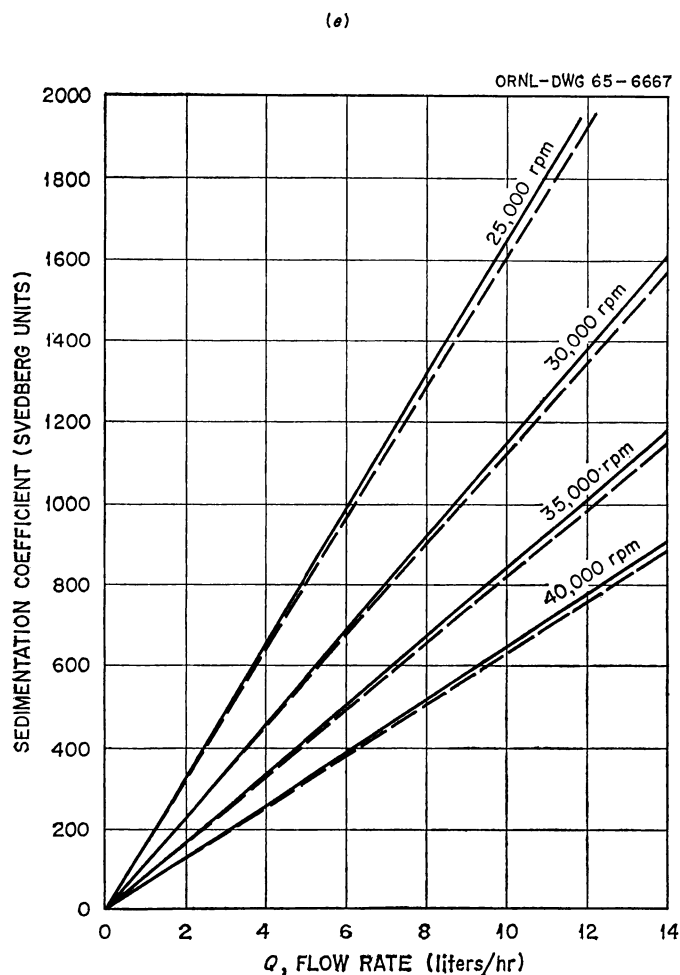


TEXT-FIGURE 7.—B-IX continuous flow centrifuge system. O: 700 ml cesium chloride, density, 1.63 g/cm³; X: 500 ml, 1.73; Δ: 500 ml, 1.63.



TEXT-FIGURE 8.—Relation between recovered volume and the radius of the rotor.

The equations previously derived (5) for continuous-flow rotors have been adapted to the B-IX core by the assumption that (a) particles which sediment to a level having a greater radius than the maximum taper remain in the rotor, (b) the fluid stream is confined to the tapered region of the core, and (c) that the tapered region can be approximated by a uniform annular space with a minimum radius equal to the actual minimum taper radius and a maximum radius corresponding to the mean square radius associated with the taper. The calculated performance of the B-IX rotor is shown in text-figure 9, in which curves are plotted for the particle sedimentation coefficient in Svedberg units versus flow rate at several different speeds. Experimental verification of these theoretical curves is presented elsewhere (?).



TEXT-FIGURE 9.—Calculated performance of the B-IX rotor system. Rotor length, 24.92 cm; mean square core radius, 3.917 cm; minimum core radius, 4.024 cm. Dashed line indicates 95% cleanout; solid line, 99% cleanout.

DISCUSSION

The development of centrifuge rotors in which particles may be isolated by continuous-flow centrifugation combined with isopycnic banding makes the so-called s - ρ separations (1) a single-step procedure. By appropriate choice of centrifuge speed, flow rate, and the gradient imprisoned within the rotor, relatively large masses of material may be isolated. In the development of the B-VIII and B-IX centrifuge cores, the specific problem of interest has been the isolation of virus particles from culture media. The principles, however, are applicable to the isolation of nuclei, mitochondria, ribosomes, and other particulate material. By using a series of such rotors operating at increasing speeds, a mixture of particles may be separated into fractions having decreasing sedimentation rates.

For virus isolation in the preparation of vaccines, the B-VIII and B-IX rotors offer a number of advantages. For example, inactivation and agglutination due to pelleting are avoided, and the material is recovered in a suspension which, after dialysis, is suitable for a rate-zonal run. In addition, a number of runs may be made consecutively. At the end of a given run, the rotor is full of the gradient solution, and reintroduction of culture fluid through the center line will re-establish the starting conditions.

In many instances the stream flowing through the centrifuge contains fairly large numbers of particles that are lighter than the virus sought. If the rotor is partially unloaded at intervals, this light band may be removed while the virus band is retained.

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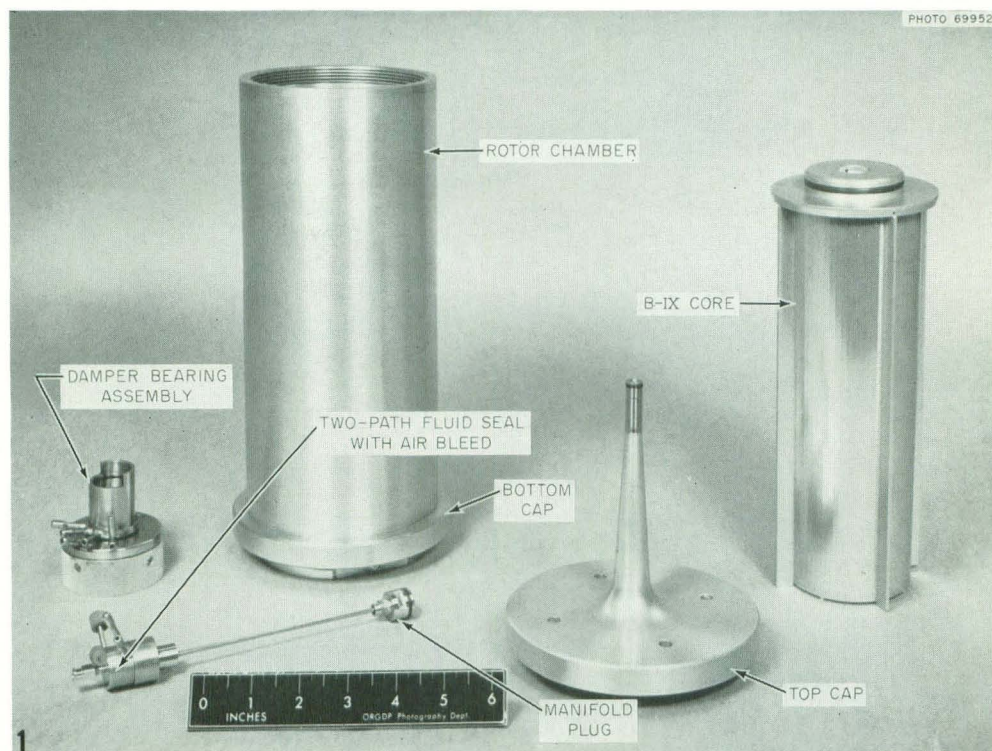
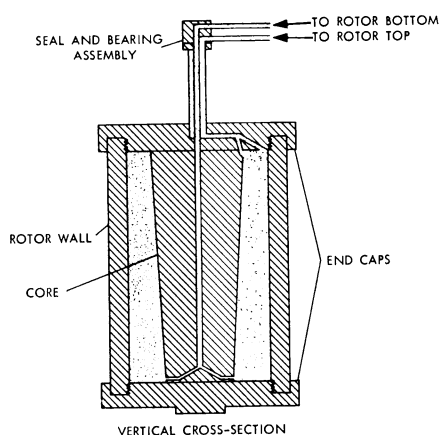
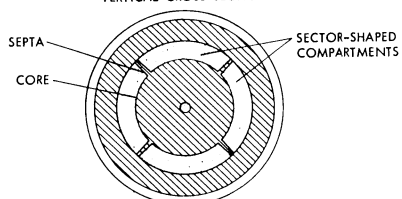


FIGURE 1.—Components of B-IX rotor for continuous-flow centrifugation combined with banding.

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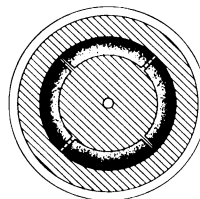
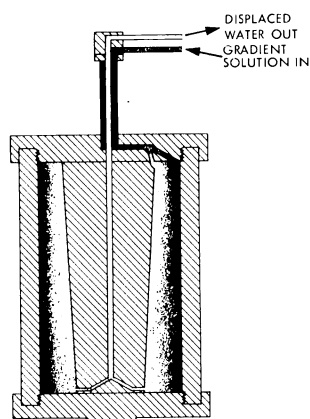


VERTICAL CROSS-SECTION



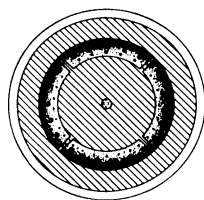
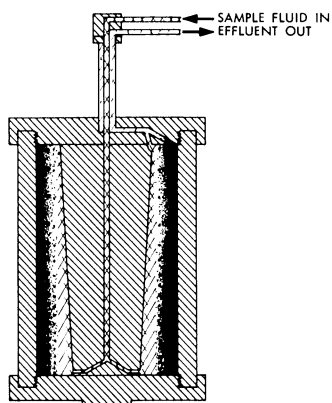
HORIZONTAL CROSS-SECTION

(a)

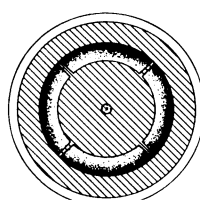
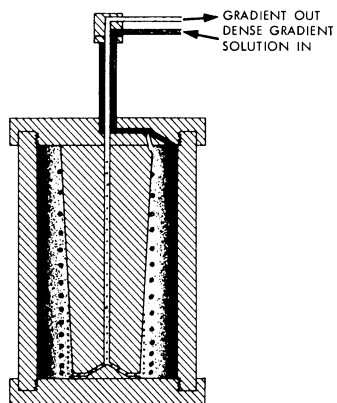


(b)

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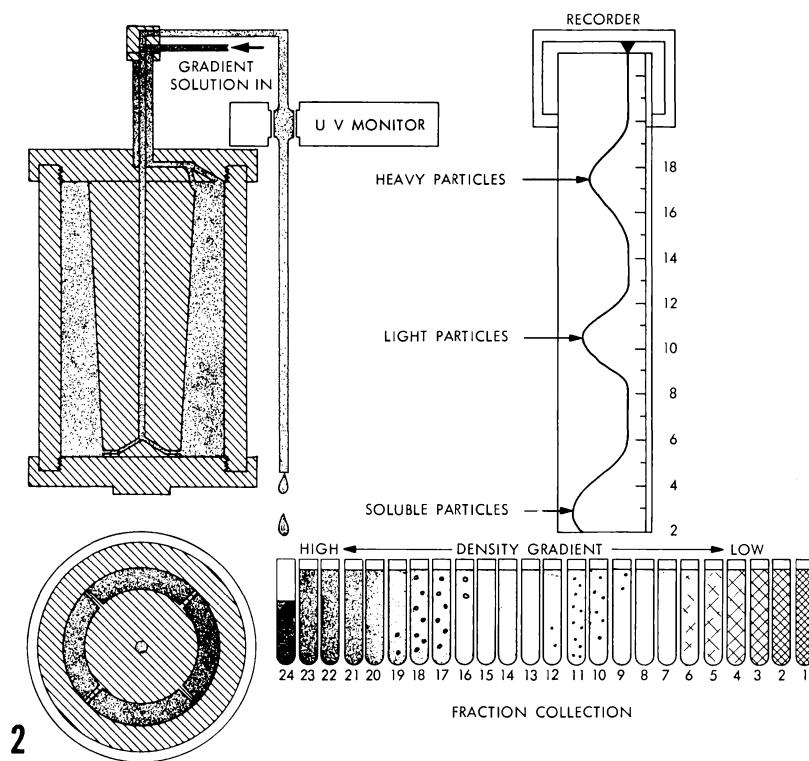


(c)



(d)

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(e)

FIGURE 2.—Diagrammatic representation of isopycnic particle banding in a continuous-flow centrifuge rotor. At each stage both vertical and horizontal cross-sections are shown. In (a) the rotor is completely filled with water through the center line (if the rotor is at rest) or the edge line (if it is rotating). During rotation, usually at 3000 rpm, the gradient solution is introduced to the rotor edge (b). During acceleration and at operating speed (c) the particle-rich stream to be cleared flows out the edge line. Particles left in the rotor band isopycnically in the gradient. When the experiment is completed, the gradient is recovered (d) at low speed (3000–5000 rpm) by displacement with a dense fluid pumped to the rotor edge. The gradient and the zones suspended in it are monitored for ultraviolet absorbance and collected as a series of discrete fractions (e).

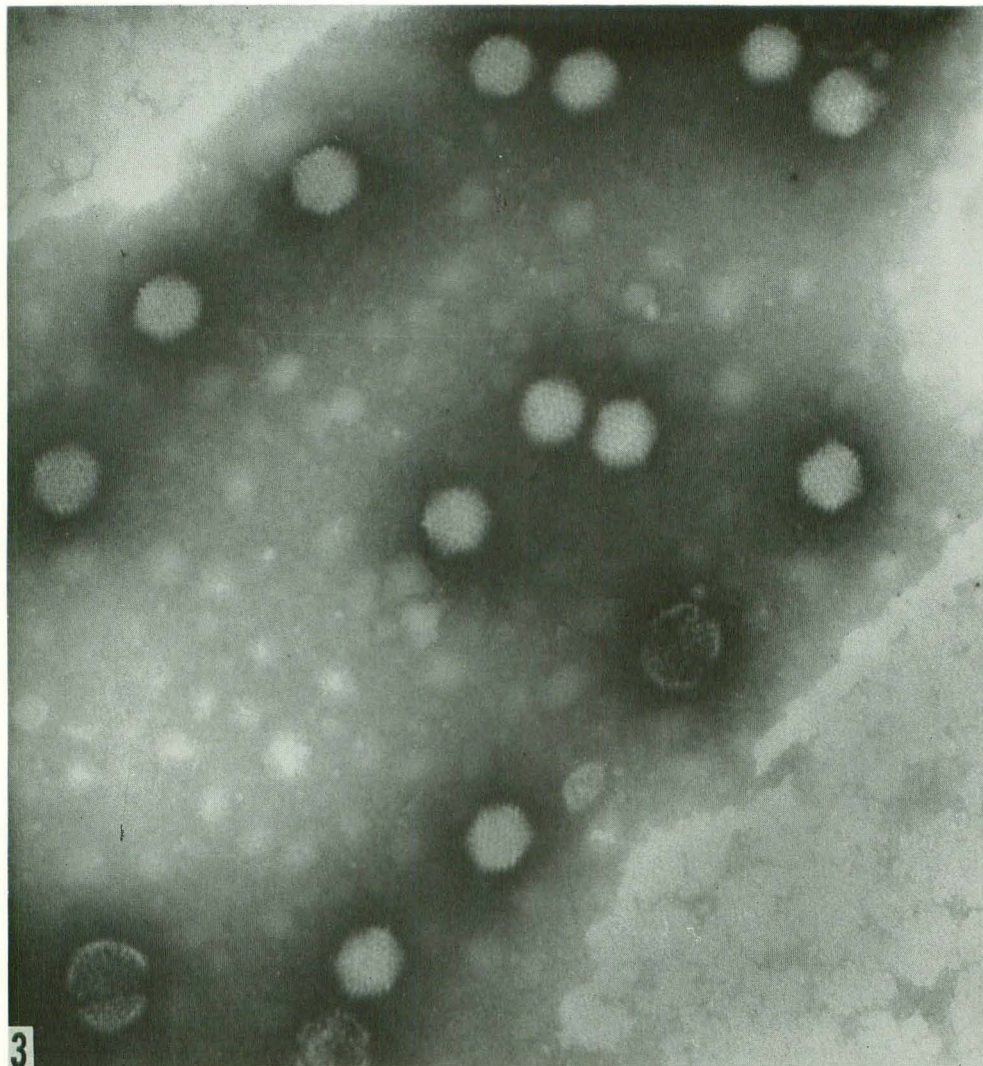


FIGURE 3.—Electron micrograph of adenovirus 2 collected from B-VIII rotor.