An Introduction to Particle Separations in Zonal Centrifuges [1]

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SUMMARY

Zonal centrifugation, based either on sedimentation rate or on isopycnic banding in liquid density gradients, is useful for high-resolution separation of subcellular particles. However, the amount of material which may be separated is extremely limited when conventional centrifuges are employed. To increase the capacity and, in certain instances, the resolution of the method, the possibility of employing large-volume hollow rotors has been explored. These are either loaded and unloaded while spinning or are designed to allow the density gradient in the rotor to reorient from a rest position to spin orientation and back with minimal shear and disturbance. The principles of operation and construction of low-speed A series and intermediate-speed B series zonal rotors are reviewed.—Nat Cancer Inst Monogr 21: 9-39 1966.

FOR THE EXPLORATION of cell substructure, high-resolution preparative and analytical centrifugal separations systems are required which cover the size range from whole cells to protein molecules. This is roughly the range of particle sizes that may be resolved in the electron microscope. The Svedberg analytical ultracentrifuge (*1*) is admirably suited for determining the sedimentation rates of particles of viral dimensions and less, down to and including small protein molecules, and for measuring buoyant densities of proteins and all larger particles for which suitable suspending media are available. Analytical centrifuges for determining the sedimentation rates of particles much larger than viruses and preparative zonal centrifuges for physically separating quantities of particles over the entire size range of interest were not available prior to the inception of the work described here. One of the principal purposes of the Joint National Institutes of Health-Atomic Energy Commission Zonal Centrifuge Development Program is to develop the centrifuges and ancillary instrumentation necessary to fill these gaps. The instru-

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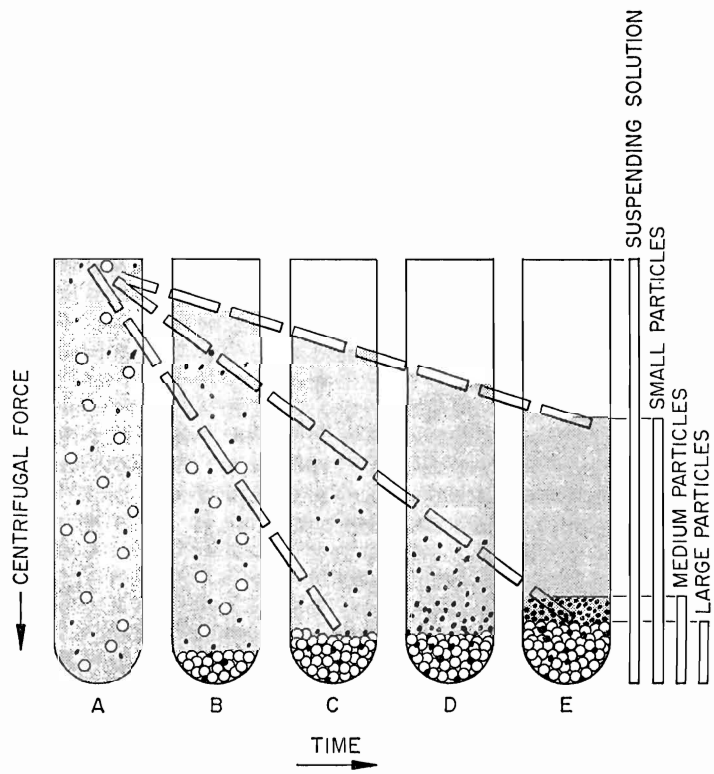
ments developed under this program are also useful, under certain conditions, for separations in the low-molecular-weight range where centrifugal separations have not been previously employed.

DIFFERENTIAL CENTRIFUGATION

The centrifuge has had a long and successful history as a means for isolating particles of biological interest. Most tissue separations have been made by so-called differential centrifugation, in which centrifuge tubes are filled with a homogeneous particle preparation at the outset. Particle sedimentation in a centrifugal field in such a tube is shown diagrammatically in text-figure 1.



TEXT-FIGURE 1.—Sedimentation of a particulate suspension in a centrifugal field. Initially all particles are uniformly distributed through the centrifuge tube (*A*). As centrifugation proceeds (*B*) all particles present sediment at their respective sedimentation rates, resulting first in the total sedimentation of the largest particle species (*C*). The degree of cross contamination in the pellet between two species of particles at the moment when one species has sedimented completely is approximately proportional to the ratio of sedimentation rates. Further centrifugation (*D*) results in complete sedimentation of the intermediate-sized particles (*E*). The distribution of particles in tube E is shown in the bar graph at the *right*.

Since a few particles of every size, including the smallest ones, are present at the bottom before centrifugation starts, it is evident that a pure pellet of the largest particles present cannot be obtained in one centrifugation. In addition, as shown in text-figure 1B through 1E, some of the small particles sediment during the complete sedimentation of larger particles. The intermediate particulate species is likewise contaminated by the smallest sedimenting units. The result is that only a fraction of the most slowly sedimenting species is obtained in a pure form in one centrifugation. Some redistribution according to particle density may occur in the pellet, however. If a very small particle is denser than a large one, this may increase the contamination as the small particles move slowly between the previously sedimented larger, but lighter, particles.

In differential centrifugation the supernatant (entire fluid volume over the pellet) would be removed after a certain period of centrifugation, for example, at tube C of text-figure 1. Repeated resuspension and resedimentation of the first pellet will yield a reasonably pure preparation of the large particles. All supernatants are then combined and centrifuged long enough to sediment the intermediate-sized particles completely. The second pellet is then cyclically purified as was the first. The reason that this method, with its apparent low resolution, has worked so well is that it has been most often applied to the separation of particles whose sedimentation coefficients differ by orders of magnitude. It has been least successful in those instances where very small differences in sedimentation rate exist, as, for example, in the separation of certain large lipid-containing viruses from small cytoplasmic fragments having very nearly the same properties.

In the analytical ultracentrifuge, particles sediment as shown in text-figure 1, but in a special sector-shaped cell with transparent windows. With suitable optical or combined optical and electronic techniques, the moving particle boundaries can be visualized either as a series of steps in a concentration versus radius plot, or as a series of peaks when the first derivative of the concentration versus rotor radius curve is plotted. The problem considered here is the development of centrifuges that would physically separate quantities of particles into fractions identical to those visualized as peaks during analytical centrifugation, *i.e.*, to devise a true preparative counterpart of the analytical ultracentrifuge.

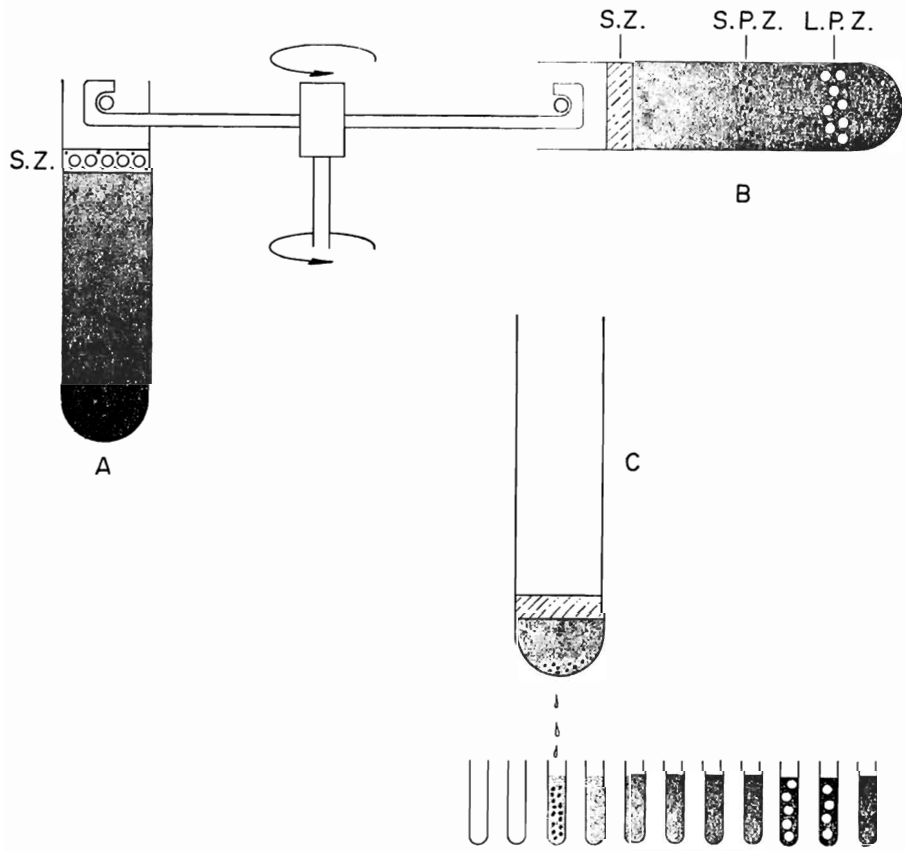
PRINCIPLES OF ZONAL CENTRIFUGATION

To achieve separations of higher resolution, two types of centrifugation have been developed. In the first, particles are separated into discrete zones on the basis of differences in sedimentation rate (rate-zonal centrifugation), and in the second, separation is based solely on differences in buoyant or banding density (isopycnic-zonal centrifugation).

Rate-Zonal Centrifugation

If the mixture to be separated could be confined initially to a narrow zone at the top of a centrifuge tube, the sedimenting particles might be expected to separate into discrete zones according to their sedimentation rates. The difficulty observed experimentally is that convective disturbances will occur unless we contrive to always have every level of the liquid, whether it contains particles or not, lighter than the liquid immediately underneath it, *i.e.*, to sediment the particles through a liquid density gradient as shown in text-figure 2.

This technique, originated by Brakke (*2–4*), has been termed rate-zonal centrifugation, or more conveniently *s*-zonal centrifugation, since it depends on the particle sedimentation rate, *s*. In this technique, a centrifuge tube is prepared which contains a continuous liquid density gradient, formed either by diffusion between initially discontinuous layers



TEXT-FIGURE 2.—Rate-zonal centrifugation in a swinging-bucket rotor. The gradient and sample layer are introduced at rest (*A*). The tubes are accelerated to a horizontal position (*B*) and centrifuged until the desired separation is effected. The rotor is decelerated to rest, at which time the gradient and the separated zones (sample zone, S.Z., small particle zone, S.P.Z., and large particle zone, L.P.Z.) are recovered (*C*), usually through a small hole punctured in the bottom of the tube.

or by a gradient-mixing device. At rest, a thin zone of the suspension to be fractionated is layered on top of the gradient. If particles lighter than any portion of the gradient are to be separated, then the sample is placed at the bottom of the gradient. Since the latter method depends on the rate of flotation, it may be termed s_f-zonal centrifugation.

The centrifuge tube is accelerated slowly so that the transition from rest to a horizontal position occurs without disturbing the gradient or the sample layer (*5*). After the required separation has been made, the tubes are decelerated to rest and the gradient is recovered as a series of discrete fractions. This method has been extensively used for the separation of small amounts of nucleic acids, RNA-DNA hybrids, ribosomes, ribosomal subunits, polysomes, and other cell components. The amount of material that may be separated with available high-speed swinging-bucket centrifuges is very small; for the SW 39 rotor the gradient is 4 ml per tube (3 tubes per rotor), and the sample layer is only a fraction of a milliliter. Because of the small starting sample, this method is primarily an analytical one. Its success has resulted from the high sensitivity of both the monitoring devices employed for scanning the collected fractions and of biological assays. For example, nucleic acids can be detected in very small amounts because of their very high ultraviolet absorbance. Generally, insufficient material is collected for chemical analysis by conventional methods, or for use as the starting material in further fractionation studies.

Rate-zonal centrifugation should be clearly distinguished from analytical ultracentrifugation as it is conventionally done with homogeneous, dilute aqueous solutions. In the latter method, the physical properties of the particle and the suspending medium through which it passes do not change appreciably during sedimentation. Calculation of the sedimentation rate may be made without knowing or assuming values for particle density. In rate-zonal centrifugation the sedimenting particle moves through a medium of increasing density, viscosity, and (in most gradients) osmotic pressure. If the particle behaves as a small osmometer, the dimensions and the effective density change during sedimentation. In addition, gradient solutes may be bound to the particle.

If the osmotic properties and permeability are sufficiently well understood, the sedimentation coefficient can be calculated from the position of the particle after centrifugation (*6–8*). For most viruses and many proteins it may be assumed, as a first approximation, that only minor changes in osmotic volume or density occur in sucrose gradients. For mitochondria and other semipermeable structures, changes in the physical properties during sedimentation must be considered.

Previous work on centrifugation through gradients by use of swinging-bucket centrifuge rotors has been extensively reviewed (*4–6*, *9*). While excellent separations have been made in density gradients set up in tubes, the method has had certain disadvantages. For example, considerable time is required to set up the gradients; care must be taken to avoid disturbances during acceleration and deceleration and the gradient must

be recovered with the tubes at rest. Especially in high-speed rotors, the capacity of the centrifuge tube is severely limited. Wall effects may produce clumping, premature sedimentation of particles, and convective disturbances. Because of the limitation on the amount of material which may be separated, the techniques for *s*-zonal separations have not been widely used for the isolation of large particles such as whole cells, nuclei, and mitochondria.

Rate and isopycnic separations of high-molecular-weight DNA present a special problem because, as first pointed out in 1953 (*10*), this material is broken by shearing. In addition, the sample layer should be sufficiently dilute to allow each molecule a free sphere of rotation. Without high-capacity zonal centrifuges, it is difficult to isolate for chemical analysis sufficient mammalian DNA with a very narrow sedimentation coefficient range by the rate-zonal method. Large-capacity systems are also required for the isolation of analyzable quantities of minor DNA satellite bands.

Theoretical studies on zone capacity (*11*, *12*) in rate-zonal centrifugation have been made. In practice the amount of material which may be supported in a zone has been reported to be smaller than predicted (*4*). Difficulty in maintaining adequate temperature control may account for this in part.

The shape of a density gradient may be considered to be convex when the rate of increase in density decreases with radius in the centrifuge, and concave when the rate increases with radius. A linear gradient changes in density linearly with radius. (In a sector-shaped centrifuge compartment, the plot of density with radius is not the same as a plot of density vs. volume.) The following factors favor the use of convex gradients:

1. The capacity of a gradient increases as the difference between the particle and solvent density decreases. If the capacity of a gradient is to be the same along its length—a desirable situation when a single virus species is being purified—then dc/dr should decrease as $\rho' - \rho$ decreases (where dc/dr refers to the rate of change in concentration of the gradient solute c with radius r, and ρ' and ρ are the densities of the particle and the solution, respectively).

2. In a multicomponent system a series of zones will leave the starting zone with little separation between them, but these will become more widely distributed during centrifugation. The greatest gradient capacity is therefore required through, and just below, the sample zone, and the gradient should be steep and convex in this region. However, if the gradient rises too steeply just under the sample layer, particles may be concentrated into an excessively narrow zone before starting through the less steep portion of the gradient.

3. In sector-shaped compartments radial dilution and diffusion combine to decrease the particle concentration (but not the total particle mass), in a sedimenting zone composed of homogeneous particles. As the concentration diminishes, the gradient slope required to maintain stability also decreases. This effect again favors a convex gradient.

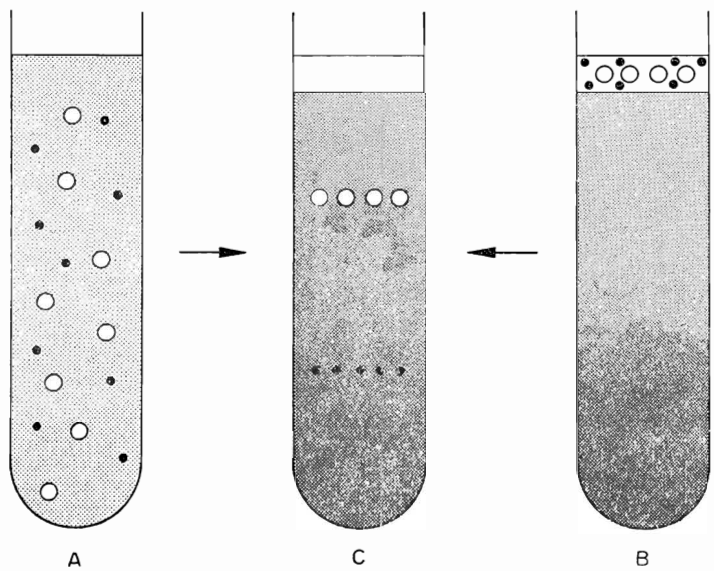
Concave gradients may be employed when it is desirable to band a rapidly sedimenting or dense particle at its isopycnic point while allowing a slowly sedimenting component to sediment through a shallow gradient. Complex gradients have been used for cell component isolation in the present volume. Some of the larger components are banded isopycnically while others are separated on the basis of sedimentation rate.

Isopycnic-Zonal Centrifugation

If sedimentation of particles is continued until they reach a portion of the gradient where the gradient density (ρ_s) and the buoyant density of the particles (ρ_B) are equal, no further sedimentation will occur and the particles may be considered to be at equilibrium (text-fig. 3).

This method, termed isopycnic-zonal or ρ_B-zonal centrifugation (where ρ_B indicates buoyant density), may be used to separate particles differing in buoyant density without regard to particle size (*5*, *13*), except as particle size contributes to diffusivity. The initial position of the particles to be banded is usually unimportant, *i.e.*, they may be uniformly distributed through the tube or they may be introduced as a discrete zone.

Isopycnic-zonal centrifugation was originally used by E. N. Harvey (*14*) and E. B. Harvey (*15*) to stratify *in situ* the subcellular particulates of sea urchin eggs and to separate the eggs into light and heavy halves. Linderström-Lang (*16*) used density gradient columns of water-saturated organic liquids at rest to measure the densities of droplets of aqueous



TEXT-FIGURE 3.—Isopycnic-zonal centrifugation. The particles to be banded at first may be uniformly distributed in a homogeneous suspending medium (*A*) and then separated on the basis of buoyant density as a gradient is formed in the centrifugal field (*C*), or the particles may be layered over a preformed gradient (*B*) and centrifuged to equilibrium (*C*).

solutions. The separation of powders of dried cells and tissues into nuclear and cytoplasmic fractions in nonaqueous density gradients was introduced by Behrens (*17, 18*) and used by Mirsky and co-workers (*19–21*) to determine the partition of enzymes and soluble components between the nucleus and cytoplasm of a variety of tissues. More recently, Meselson, Stahl, and Vinograd (*13*) extended ρ_B-zonal centrifugation to the molecular level with their classical studies on zonal separations of DNA in cesium chloride gradients. The studies that have extended this method to the separation of small amounts of DNA, RNA, DNA-RNA hybrids, viruses, ribosomes, ribosomal subunits, polysomes, and other cell components are now too numerous to be reviewed here. As with rate separations in swinging buckets, the amount of material that may be separated has been severely limited.

The density at which a particle will band isopycnically (ρ_B) is a function of its density in the medium employed and is altered when the particle is permeable to the solutes used, or when the solutes are bound to the particle. For these reasons, the buoyant density of particles of biological interest is often a function of the suspending medium. Further, the buoyant density measured will usually not be the same as the density value (reciprocal of the partial specific volume) used in rate sedimentation studies.

Interestingly, changes in particle density by specific binding create new separation possibilities. For example, if a reagent specific for a given binding site is heavier or lighter than the particle containing the binding site, it should change the banding density. The effect of stains used to distinguish DNA and RNA, sulfhydryl reagents, and ferritin-conjugated antisera should be investigated in this regard.

Combined s–ρ_B zonal methods

The sedimentation rate of a spherical particle in a centrifuge is given by the familiar equation:

$$s = \frac{2a^2(\rho' - \rho)}{9\eta} \qquad [1]$$

where s = sedimentation rate in cm/sec per unit centrifigal field
a = particle radius in cm
ρ' = density of a solvated particle in g/cm^3
ρ = solution density
η = viscosity of the suspending medium in poises

The rate is therefore dependent on the square of the particle radius (assuming it is a sphere) and the difference between the solvated density of the particle and the density of the suspending fluid. The sedimentation rate is also sensitive to particle shape. However, particles having quite different densities and radii may have the same sedimentation rate provided the product of a^2 and $(\rho' - \rho)$ is the same for all of them.

For this reason, we would like to separate particles on the basis of their

effective size and their density separately. Since the sedimentation rate is a function of both, it is evident that the two parameters can only be effectively separated by a two-step procedure using s-zonal centrifugation for one and ρ_B-zonal centrifugation for the other (*22*). The choice of the order of the separation depends on several considerations and is considered in detail in a subsequent paper in this volume (*22*). The method is useful for studying enzyme activities in microsomal subfractions (*22a* and *22b*) and for the isolation of native glycogen (*23*).

To maximize the particle radius dependence of the rate separations in practice, the difference in density between the particle of interest and the solvent should be maximized, and the separations should be carried out in gradients having the lowest density practicable. This is evidenced from the fact that the effect of radius a on sedimentation approaches zero as the term $(\rho_B - \rho_s)$, where ρ_B is the buoyant density of the particle and ρ_s, the density of the gradient, approaches zero (*i.e.*, particles of any radius may band at the same density). These considerations suggest that the maximal separation for scanning purposes may be obtained first by separation of subcellular particles on the basis of sedimentation rate in a zonal centrifuge and then by separation of the collected fractions on the basis of density alone. Of interest here is the question: Will such separations yield relatively pure subcellular particles? The range of sedimentation rates of subcellular particles (including proteins) is approximately 10^6 Svedberg units, while the banding densities vary from approximately 1 to 2. If the sedimentation coefficients of sedimentable particles are plotted against particle buoyant density in a given medium (such as CsCl) to give a so-called "s–ρ" plot, it is evident that many known particulates would be widely separated (*22*). For example, microsomes and glycogen span nearly the same range of sedimentation coefficients, but differ greatly in buoyant density, and band at approximately 1.18 and 1.62, respectively (*23*). Conversely, mitochondria and microsomes have nearly the same buoyant density but differ very much in sedimentation rate.

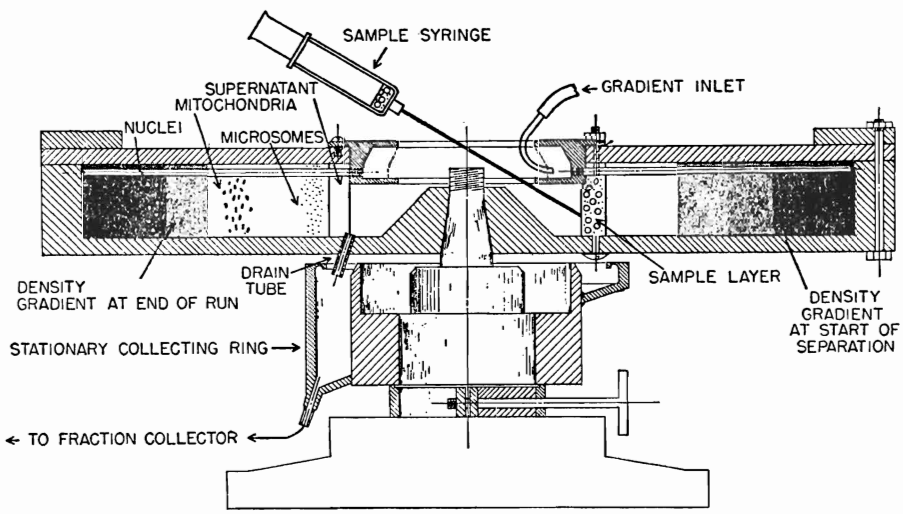
If the necessary centrifuge systems for making high-resolution s–ρ separations can be built, the possibility exists that minor cell constituents may be found which have sedimentation rates and banding densities unlike those of known components. In addition, it may be possible to isolate trace amounts of virus from cell homogenates. If this is feasible, the problem of analyzing the virus-like particles that have been occasionally described in electron micrographs of human tumors can be considered. The experimental possibilities that would be opened up by the development of techniques for high-resolution s–ρ separations are many and are discussed in detail in subsequent papers in this monograph (*22*, *23*). Briefly, the problem is to develop separation techniques that would parallel the electron microscope in the range of particle sizes to be separated, and that would be sensitive to two of the several elements of morphology on which visual characterization depends, namely, size (to a lesser degree shape) and density. The development of zonal centrifuges has been the first step in the exploration of s–ρ_B separation systems.

DEVELOPMENT OF ZONAL CENTRIFUGES

Ideally, one would wish to achieve simultaneously in one rotor system the following: 1) ideal sedimentation and maximum resolution in sector-shaped compartments, 2) rapid gradient formation in the rotor with minimal stirring or convection, 3) sharp starting or sample zones, 4) high rotational speed, 5) large capacity, and 6) rapid recovery of the gradient after centrifugation without loss of resolution. A number of rotors in the A and B series have been designed to fulfill these requirements.

Initially, a swinging-bucket system (A-I) was used to demonstrate that high resolution could be obtained (fig. 1) with glass tubes of modified sector shape (*24*, *25*). Because density gradients are greatly stabilized in a centrifugal field, the A-II rotor system was developed in which the density gradient was introduced into the centrifuge tubes through a central distributor cup (fig. 2) while the centrifuge was running (*26*). In the A-II system, however, the gradients had to be recovered from the tubes at rest.

The possibility that centrifuge tubes could be eliminated entirely was explored by using a hollow centrifuge head filled and emptied *during* rotation. The first rotor of this type (*27*, *28*) was the A-III and is shown in text-figure 4 and figure 3. The gradient was introduced to the edge of the rotor, light end of the gradient first, through radially oriented tubes attached to an annular groove near the rotor center. After the gradient was in position, the sample was introduced manually to its inner



TEXT-FIGURE 4.—Schematic drawing of operation of zonal rotor A-III. The density gradient flows into the spinning rotor through the center annular ring, and thence through small tubes to the rotor edge. The sample layer is then manually layered on the central (*light*) edge of the gradient. After separation of the particles, the gradient is recovered by displacement toward the center drain tube using a dense sucrose solution introduced to the rotor edge.

surface. After particle separation had occurred (text-fig. 4) the gradient was displaced toward the center by allowing a very dense fluid to flow to the rotor edge. The gradient was collected as a stream flowing out the exit tubes in the bottom of the rotor near the axis of rotation. This relatively crude rotor demonstrated the feasibility of the zonal centrifuge. A more refined rotor (A-IV, fig. 4), demonstrated that gradients could be introduced into and recovered from rotors operating at speeds up to 18,000 rpm. Additional "A"-series rotors have been designed and tested (*29*, *30*) and have culminated in A-XII, which is now commercially available.[3]

Based on early studies with the A-III and IV rotors, construction of an intermediate-speed zonal rotor was proposed, and two experimental models were built on subcontract by the Spinco Division of Beckman Instruments, Inc. Rotor B-I, constructed under this contract, was extremely unstable for reasons not understood at that time. It now appears to have had the wrong ratio of moments of inertia (*31*). Rotor B-II (fig. 5), tested extensively under the present Joint National Institutes of Health-Atomic Energy Commission Zonal Centrifuge Development Program, has produced excellent separations (*32–35*) but as originally built did not reach the maximum design speed for reasons that are discussed by Barringer (*31*).

The rotational stability problems were solved in the design of the B-IV rotor [4] (*36*, *37*), and the basic principles of zonal centrifuge operation may be best illustrated by considering this rotor in some detail.

The rotor is filled during rotation at a low speed, as shown diagramatically in figure 6B. The light end of the gradient is pumped to the rotor wall first, followed by denser fluid, which displaces the lighter fluid end of the gradient toward the rotor core. When the gradient is in the rotor, additional dense fluid, termed the "underlay" is pumped in until the light end of the gradient begins to flow out the center line. At this point the sample layer is introduced through the center line (fig. 6C), reversing the direction of fluid flow through the rotor and causing part of the underlay to flow back out through the edge line. To push the sample layer further into the rotor chamber clear of the core, additional light fluid (the overlay) is pumped in as is shown in figure 6D. The connection to the rotor edge is then closed, and the center line attached to a reservoir of water to allow a small volume of fluid to flow into the rotor during acceleration to compensate for rotor expansion. The rotor is then accelerated to a speed sufficient to effect the desired particle separation (fig. 6E).

After deceleration to low speed, the gradient is displaced by a dense solution toward the core and out through the center line (fig. 6F). The gradient flows through an ultraviolet absorbance monitor and into a fraction collector. Part of the gradient may be pumped through auto-

[3] The A-XII rotor is available from International Equipment Company, Needham Heights, Mass.

[4] The B-IV zonal ultracentrifuge is available from the Spinco Division of Beckman Instruments, Palo Alto, Calif.

mated systems to assay for enzyme or total protein activity (fig. 6). A radioactivity analyzer can also be used in this system. The operation of the "A" series, dynamically loaded (*i.e.*, loaded and unloaded during rotation) rotors (A-V, VI, IX, and XII) is similar in principle to that of the B-IV rotor described.

It is evident that the sample layer may also be introduced first to the rotor edge and then be picked off the rotor wall by the inflowing gradient. This method has two disadvantages: It allows some particles to sediment to the rotor wall at once, and it increases mixing by both diffusion and laminar flow as the gradient streams in under the sample layer, which is initially spread over the entire inner wall of the rotor.

It is also evident that the gradient may be backed into a compartment containing a dense fluid, which is withdrawn through the outer edge line. The gradient may also be recovered in the reverse direction, that is, through the connection to the outer edge. Of these methods, the one used in the continuously rotating zonal rotors described in the present report is believed to give the highest resolution.

The design of the core, considered in greater detail in a subsequent paper (*37*), is critical for high resolution separations. In a dynamically loaded rotor it serves to divide the rotor volume into sector-shaped compartments, to connect the center and edge of each compartment with the seal lines, and to guide the sample in and the gradient out of the rotor with minimal mixing and loss of resolution.

ROTORS WITH REMOVABLE SEALS

As previously suggested (*32*) the seal system of a dynamically loaded rotor may be removed during high-speed operation, making a bearing on the end of the rotor to which the seal is attached unnecessary if the rotor is self-balancing. The problems associated with the construction and operation of such rotors have been largely solved with the development of rotors B-X and B-XI (*38*). It is probable that zonal centrifuges based on this design will find very wide application in biochemical and virological studies. They are not, however, adaptable to continuous flow operation in their present form.

REORIENTING GRADIENT ROTORS

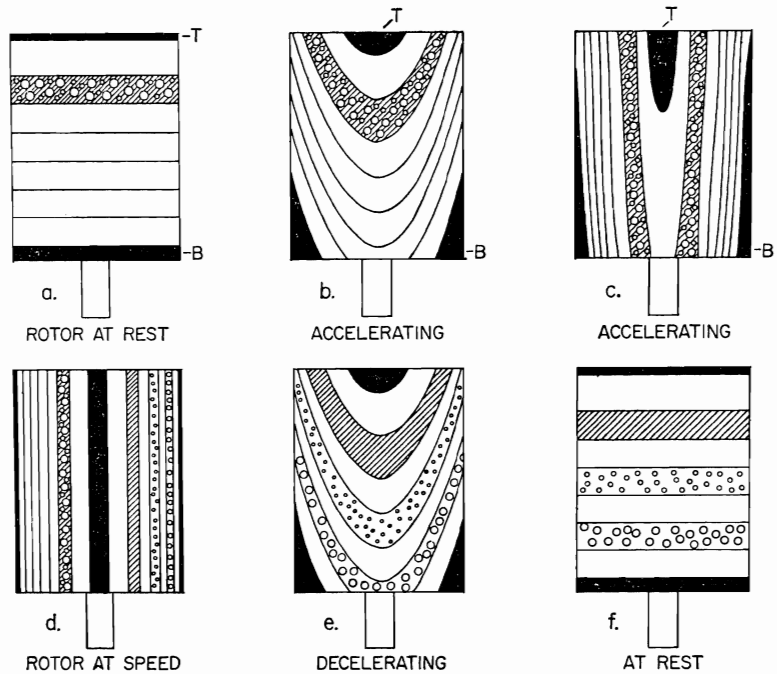
Dynamically loaded zonal rotors have been shown in practice to give very high resolution separations (*23*, *33*). It is unlikely, however, that suitable rotating seals can be built which would operate in a satisfactory manner in the range of 100,000 rpm and faster. We have therefore examined two alternate possibilities (*a*) gradient reorientation in the rotor during acceleration and again during deceleration so that gradients may be set up and recovered at rest (*39*), and (*b*) removable-seal rotors in which the seals are attached to the rotor only during filling and emptying

of the rotor at low speed. The possibility of building reorienting-gradient rotors arises from the following considerations.

The shearing forces occurring in a liquid confined in a closed cylinder during the transition from rest to a stable orientation in a high centrifugal force field may be considered qualitatively by examining text-figure 5. The horizontal lines indicate levels, or surfaces, of equal density in a continuous density gradient.

During acceleration, each isodense surface becomes part of a paraboloid of revolution. At a given rotational speed, all isodensity curves are identical, being merely transposed vertically. Acceleration results in a series of configurations shown diagramatically in text-figures 5b and 5c. At high speed, where the ratio between the centrifugal force and the acceleration due to gravity in a downward direction is very high, the isodensity surfaces will be nearly vertical (text-fig. 5d).

Deformations occurring at the various levels may be best understood by describing the changes occurring in layers originally at the top, middle,



TEXT-FIGURE 5.—Schematic diagram of reograd rotor system. (*a*) Rotor is filled at rest with density gradient and sample layer. To indicate extremes of zone deformation, a thin upper layer, *T*, and bottom layer, *B*, are also indicated. (*b*) During acceleration, each zone forms a paraboloid of revolution about the axis. *Note T* and *B*. (*c*) Near operating speed, the zones approach a vertical orientation. (*d*) At a sufficiently high speed, the zones become nearly vertical. Separation of particle zones is shown at *right*. (*e*) During deceleration, zones again form paraboloids of revolution. (*f*) At rest, various zones may be recovered by draining rotor contents out the bottom, or displacing the gradient out through the top. Reprinted from Anderson *et al.* (*39*).

and bottom of the rotor. The fluid originally against the upper rotor cap becomes squeezed into a small paraboloid of revolution during acceleration (text-fig. 5b) and then occupies the center of the rotor at high speed (text-fig. 5d). A zone in the middle of the rotor (5a) at rest increases in area during acceleration, and then decreases in area slightly as an approximately vertical position is approached. The zone at the very bottom of the rotor at rest (text-fig. 5a) decreases markedly in surface area during acceleration (text-fig. 5b), but covers the entire surface of the rotor wall at high speed (text-fig. 5d). The greatest area changes, therefore, occur in those zones near the top and bottom when at rest but near the center and the edge at high speed.

The reoriented gradient, before the particles have sedimented appreciably, is shown at the left (text-fig. 5d), and after sedimentation at the right. The distribution during deceleration is shown in text-figure 5e, with the distribution at rest shown in text-figure 5f. The separated zones are recovered by draining the gradient out of the bottom of the rotor, or by displacing it out the top.

A mathematical analysis of the areas of isodensity surfaces (*40*) shows that shearing in the center of the gradient in a sector-compartmented rotor is not excessive. While increases and decreases in area occur, the difference in rate of increase or decrease in the areas of adjacent zones is rather small. By placing a dense "cushion" or underlay in the bottom, and an overlay of light fluid above the sample layer at the top, the sample layer and density gradient may be restricted to that part of the rotor where least shearing occurs. As the fluid layers change position during acceleration and deceleration, their tangential velocity will change, since the velocity at any point in the rotor is a function of both the rotational speed and the radius of the point. Fluid in the upper layer, originally near the edge of the rotor, decreases in tangential velocity relative to previously underlying fluid during acceleration, for example. Vertical septa, therefore, are considered necessary to prevent swirling during reorientation of the gradient.

High-speed reorienting-gradient zonal rotors are still in a very experimental stage although rotors with a capacity of 100 ml have been successfully tested to 141,000 rpm (310,000 $\times$ g at R_{max}), and a 10 ml rotor with a design speed of 400,000 rpm (1.2 million $\times$ g) is under development. The details of this work will be included in future publications. These rotors are useful for both rate-zonal and isopycnic-zonal centrifugation.

Extensive experimental studies will be required to determine whether the removable-seal design or the reorienting gradient configuration will give the best resolution with high-speed rotors designed expressly for protein or nucleic acid separations.

CONTINUOUS-FLOW CENTRIFUGATION

In the centrifuge systems described thus far it has been assumed that the starting sample contains a high concentration of particles. In many

instances this is not the case, and some preliminary method for concentrating particles must be employed. This is especially true of culture fluids of animal viruses where multiliter volumes may contain only a few milligrams of viral particles.

While continuous-flow centrifugation has been widely used industrially and for a few laboratory separations such as the recovery of bacteria from multiliter cultures, the full capabilities of the method do not appear to have been realized. In an ideal continuous-flow centrifuge it is evident that initial separations should be as good as those observed in a single sedimentation in differential centrifugation. The problem is to develop rotor systems that will function efficiently over a very wide range of particle sizes, and which, in addition, will not form aerosols of the sample material during its passage through the rotor. Of immediate concern in the present program is the development of contained high-speed continuous flow centrifuges that can be used to recover pathogenic virus particles from large volumes of culture fluid.

In addition, a continuous-flow centrifuge is needed where the particles removed from the fluid stream are not compacted, but are allowed to sediment into a gradient imprisoned in the rotor chambers (*33*). This would allow buoyant density to be used as an additional separation parameter and would also enable the experimenter to make repeated separations without disassembling the rotor.

The B-V rotor system (*41*) has been developed for high-speed continuous flow separation of particles which can be pelleted, while the B-VIII and B-IX rotors (*42*) have been successfully developed for continuous-flow centrifugation combined with isopycnic banding.

These rotor systems allow particulate material to be isolated from rather large volumes of vaccines. The distribution of mass through a density gradient may then be determined during approach to equilibrium (rate-zonal centrifugation) or at equilibrium (isopycnic-zonal centrifugation) in the B-IV rotor. The importance of characterizing of the particulate material in vaccines for human use and of attempts to prepare vaccines containing only those antigens required for protection has been previously stressed (*33*).

OTHER GRADIENT SEPARATIONS

The centrifuge systems described in this volume make possible a number of separations in addition to those already described. These will be discussed in greater detail elsewhere and are merely listed here. These include:

1. *Particle immobilization during chemical dissection*

Bacterial cells, or subcellular particles, may be banded isopycnically in a gradient containing detergents, enzymes, solubilizing agents either uniformly distributed through the gradient, at the particle banding level, or in an overlay from which they sediment or diffuse into the particle band. As the particles disrupt, DNA or other material may be released and

separated in the gradient on the basis of sedimentation rate or banding density.

2. *Particle dissection by sedimentation through reagents immobilized in a density gradient*

The sequential removal of globulins and histones by sedimention of nuclei through a gradient of increasing ionic strength and acidity is an example of sequential gradient particle dissection.

3. *Gradient resolubilization*

In an alcohol-water gradient an alcohol-precipitated protein will sediment until it reaches a level where it can dissolve. If the centrifugal field is not too high, it will remain at the level where it dissolves. This method is applicable to many proteins and to RNA-DNA separations (Anderson, 1956, unpublished). In addition, the method can be used to concentrate small amounts of protein, nucleic acid, or virus from large volumes of fluid with the B-IX rotor. The precipitating agent is added before the fluid is run through the rotor. During centrifugation the precipitate sediments into the immobilized gradient in the rotor and either dissolves or bands isopycnically. This method is also applicable to low-molecular-weight materials.

4. *Precipitation in a gradient followed by isopycnic banding*

Water-insoluble organic substances in a water-miscible organic solvent may be layered over a suitable aqueous density gradient in which either crystalline or amorphous particles of the organic solids will band. With proper choice of solvents, all levels of the gradient and sample layer can be made denser than water, which is layered over the sample. As water diffuses into the sample layer, precipitation occurs and particles move out through the gradient to their isopycnic level. Similar systems using water-soluble materials layered over gradients prepared from dense water miscible organic solvents have also been explored. These concepts extend the usefulness of zonal centrifuges into the field of small molecules.

ANCILLARY STUDIES

Although the central problem in this program has been the development of high-resolution centrifuge systems, other preparative and analytical methods have been explored. An automated analytical system for bases, nucleosides, and nucleotides previously developed (*43*) has been extensively modified for high pressure studies on columns of very small bore (*44*). A prototype of a complete system for the analysis of mixtures of sugars has been completed and tested (*45*). The final design work on a model suitable for general laboratory use is now under way (*46*). In addition, new methods for countercurrent separations are being devised (*47*). To monitor the effluent from lipid separation columns, a flame ionization analyzer

using a fine wire to transport samples into a hydrogen flame has been explored (*48*).

SEPARATION OF SUBCELLULAR PARTICLES IN ZONAL CENTRIFUGES

The studies recorded in this volume were begun by experimental biologists interested in fractionating real cells. It soon became evident that certain basic problems concerned with the physical chemistry of subcellular particles should be considered along with purely empirical approaches. The following discussion poses some of the problems that have emerged. The view taken here, admittedly the result of personal bias and limitations, is that in nearly all instances we must attempt to measure each parameter of a subcellular particle directly by methods that are, if possible, independent of other measurements.

The reasons for this approach can be made clear by considering systematically the relationships between the models that may be considered mathematically and the real objects that we wish to separate. The following paragraphs describe a series of cases and indicate some of the problems associated with the zonal centrifugation of each type of particle. The viewpoint is that of a physiologist rather than of a physical chemist.

Case I. Particles that are ideal, smooth, round, uncharged, unhydrated, and impermeable.

Case II. Polymeric molecules that are hydrated, charged, and may not be round or smooth, such as proteins or nucleic acid molecules, but are often homogeneous with respect to molecular weight.

Case III. Particles composed of aggregates of polymeric molecules; these occur in a range of sizes and may show marked changes in frictional ratio with size but do not exhibit osmotic behavior.

Case IV. Vesicles that exhibit osmotic behavior.

Case V. Vesicles that exhibit osmotic behavior and also size or volume changes in response to physiological variables.

Case VI. Particles that are hydrated, exhibit large volume changes in response to physiological variables, but do not show osmotic behavior.

In *Case I* (*see* text-fig. 6) all the classical equations of centrifugation apply.[5] The diameter, $2a$, corresponds to the hydrodynamic diameter (distance between shearing planes on opposite sides of the molecule), the particle does not absorb water, and the solvated layer is negligible. The physical properties of the suspending medium or density gradient do not in any way affect the physical properties of the particle. The forces acting on a particle are buoyant (F_b), centrifugal (F_c), and frictional (F_f) forces. These may be defined as

$$F_b = -v\rho\omega^2 r \qquad [2]$$

$$F_c = m\omega^2 r \qquad [3]$$

$$F_f = -6\pi\eta a\omega^2 rs \qquad [4]$$

[5] The author is indebted to Dr. Rodes Trautman for invaluable assistance in the development of this section.

where

v = volume of particle in ml
ρ = density of the solution (g/ml)
ω = angular velocity in radians per second
r = distance from axis of rotation to the particle
m = anhydrous mass of particle
η = viscosity of the solution
a = particle radius
s = sedimentation coefficient
f/f_o = friction factor
$\omega^2 rs$ = velocity of the particle (cm/sec)
h = particle solvation
d = density (g/ml) of solvated layer

The net force per particle is zero ($F_b + F_c + F_f = 0$) once particles have been accelerated to their average velocity during a sedimentation velocity experiment; hence

$$s = \frac{4a^2(\rho^d - \rho)}{18\eta(f/f_o)} \qquad [5]$$

where ρ^d is the density (g/ml) of the anhydrous particle.

Case II. Proteins and nucleic acids in solution are hydrated. If the particle solvation is h (ml) of density d (g/ml) the solvated particle density ρ' (g/ml) is

$$\rho' = \frac{m + hd}{v + h} \qquad [6]$$

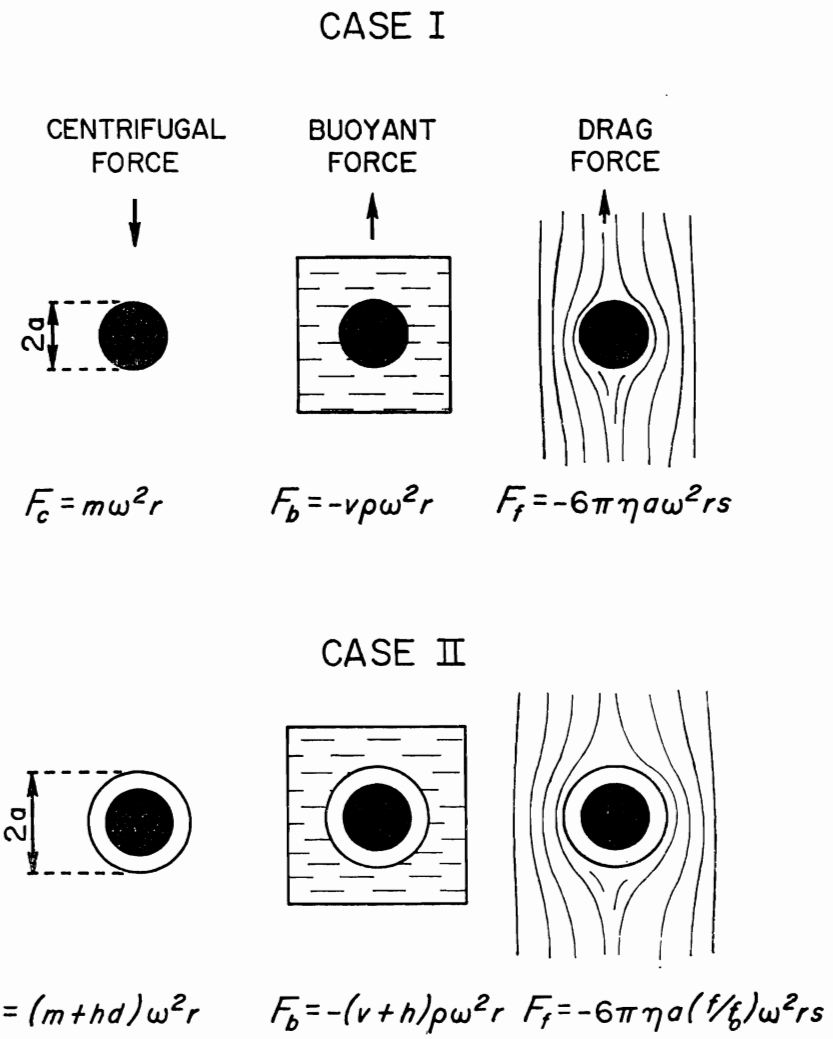
The radius a (cm) of an equivalent sphere having a volume equal to the volume of the solvated particle is

$$4/3\pi a^3 = v + h \qquad [7]$$

As shown in text-figure 6, *Case II*, the forces acting on the solvated particle are the centrifugal force, $F_c = (m + hd)\omega^2 r$, the buoyant force, $F_b = -(v + h)\rho\omega^2 r$, and the friction force, $F_f = -6\ \pi a(f/f_o)\omega^2 rs$.

In a density gradient the behavior of these particles will differ from that for *Case I* to the extent that the amount of bound gradient constituents varies during sedimentation, and to the degree that the hydrodynamic radius changes during movement through the gradient.

Case III. The best example of *Case III* is liver glycogen. It is composed of small, apparently oval, and homogeneous subunits (γ particles) that are quite dense (*23*). These appear to exhibit quite normal sedimentation properties. Clusters of γ particles are assembled into β particles, which sediment more slowly than measurements of their greatest diameter predict. The β particles are assembled into still larger α clusters, which sediment more slowly than predicted. Isopycnic banding density is very nearly the same for all sizes, and no evidence for



TEXT-FIGURE 6.—Diagrammatic representation of forces acting on an anhydrous particle (*Case I*) and a hydrated particle (*Case II*) in a centrifugal field. *Arrows* indicate the direction of centrifugal force (F_c), buoyant force (F_b), and drag force (F_f). *See* text for definitions of terms used.

osmotic behavior has been observed. With this particle, F_c and F_b are probably the same as for *Case I*; however, the friction force, F_f, varies in a peculiar way with the maximal radius, and a different frictional ratio (f/f_o) must be used with different-sized particles.

Case IV. Vesicles that exhibit pure osmotic behavior and remain perfectly spherical while changing volume in a gradient have not as yet been described. All real particles tend to (*a*) have a nonosmotic volume, (*b*) become nonspherical when excessively shrunken, and (*c*) take time to reach equilibrium during migration through the gradient, the time being dependent on the permeability to water of the surface membrane of the

particle. The application of the available physical data on mitochondria to the calculation of the sedimentation rates in sucrose gradients has been described (*6*). It is assumed that all particles behave in the same manner, and that the calculations correctly describe the particle diameter and density at every level in the density gradient. Until these values can be determined by independent methods they must be treated with caution since the sedimentation equations describe individual particles, whereas permeability and osmotic studies are done on large numbers of particles and give no indication of particle heterogeneity with regard to physical properties.

Case V. Experimentally, mitochondria exhibit osmotic volume changes and volume changes in response to experimental physiological variables. Thus, in isosmotic solutions, removal or addition of adenosine triphosphate, thyroxin, and a variety of other substances causes mitochondria to change markedly in volume (*49*). It cannot be assumed that the physiological mechanisms involved in these apparently nonosmotic volume changes are unchanged during sedimentation through a density gradient. Before the sedimentation of such particles can be adequately studied it will be necessary to measure particle sedimentation rate and diameter *continuously* during sedimentation through a gradient. Centrifuge systems specifically designed for such studies are in the design stage.

Case VI. Nuclei obtained from rat liver are hydrated, exhibit large volume changes in response to certain physiological variables, and do not show osmotic behavior. The volume in solution is largely a reflection of the number and type of ions present (*50*). In distilled water, nuclei swell greatly and ultimately dissolve. Low concentrations of calcium chloride (0.01 M) cause the nuclei to shrink below the intracellular volume. Further increases in the concentration of this salt produce swelling again. If nuclei are taken from very hypotonic (0.01 M) $CaCl_2$ into hypertonic (0.88 M) ion-free sucrose, marked swelling is observed. Liver nuclei are permeable to relatively large molecules (*51*) and behave as an open anionic gelwork.

Since nuclei bind divalent cations much as negatively charged ion exchange resin beads do, a distinction must be made between the total amount of a given ion present in a nuclear suspension, and the concentration present in solution after equilibrium between free and bound cations has been reached. Thus, if a concentrated tissue homogenate is made 0.05 M with respect to calcium, relatively little change in nuclear volume or nuclear internal structure may be seen. If this homogenate is layered over a sucrose gradient containing 0.005 M $CaCl_2$, the nuclei sedimenting through the gradient will soon sweep through a sufficient volume of solution to come into equilibrium with a 0.005 M $CaCl_2$ solution, will shrink, and become granular. In many instances sucrose solutions, especially if prepared with tap water, may contain enough salt to prevent nuclei from swelling excessively. Nuclei appear to be in equilibrium with some cytoplasmic proteins (*52*) and may gradually lose these during zonal purification. The point is that the physical properties of nuclei cannot be

assumed to be constant when sedimented out of a tissue brei and through a gradient.

Evaluation of the sedimentation rate of nuclei or other subcellular particles from two-position measurements (the starting position in the rotor and the position when unloading is begun) must be considered as crude approximations until (*a*) the diameter and density of the particles have been measured independently at a number of levels in the gradient, (*b*) the position of a given particle species has been measured continuously during centrifugation, and (*c*) the variation of sedimentation rate with gradient position has been determined.

That at least some part of the nuclear structure is sensitive to osmotic pressure is suggested from the observation that nuclei isolated in isotonic sucrose-dextran gradients retain their capacity for protein synthesis (*53*) while those isolated in conventional sucrose gradients do not.

The proof that a mathematical model of a subcellular particle actually applies to the particle must be determined experimentally. For this reason, the computer program developed for use with the B-IV zonal ultracentrifuge applies rigorously only to *Case I*, and the equivalent sedimentation coefficients, S^*, determined in Svedberg units, when used in connection with real particles, are for comparative purposes only. It is quite evident that the development of computer programs that can be shown to apply directly to each species of subcellular particle will require further work. The program applies to particles in *Case II* if the water of hydration equilibrates rapidly with the gradient medium and if the particles therefore *change* their solvated density during zonal centrifugation

DISCUSSION

As an introduction to particle separation in zonal centrifuges, certain basic problems and some of the rotor systems developed to solve these problems have been presented. Broadly stated, the aim has been to develop high-resolution methods for separating cells and sedimentable subcellular particles in quantities sufficient for chemical analysis or for further fractionation studies. Initially, low-speed, test centrifuge systems (early A series) were used to explore the concept of dynamically loaded and unloaded zonal rotors. These were useful for "proof-of-principle" purposes but suffered from a number of operational defects. As the feasibility of this approach was demonstrated, more refined rotor systems were developed.

It appeared unlikely that one rotor system could be built which would give optimal separations over the entire particle size range of interest. Therefore four classes of rotors have been explored: Series A for particles in the size range visible in the light microscope, Series B for particles as small as plasma macroglobulins, Series C for rate separations of small proteins and large-scale nucleic acid and protein-banding studies, and Series D for exploratory studies in very high centrifugal fields. Details of the development and experimental application of some of the rotors

in the A and B series are presented in subsequent papers in this monograph. The presentations are limited to results obtained with rotors driven by conventional, and widely used, centrifuge drives. To make the techniques developed more generally available, drawings and detailed information on construction and operation have been released to the public by the Atomic Energy Commission, and two of the centrifuges described here are now commercially available as a result of this policy.

Much of the experimental work done under this program has been recorded in semiannual reports (*54–57*).[6]

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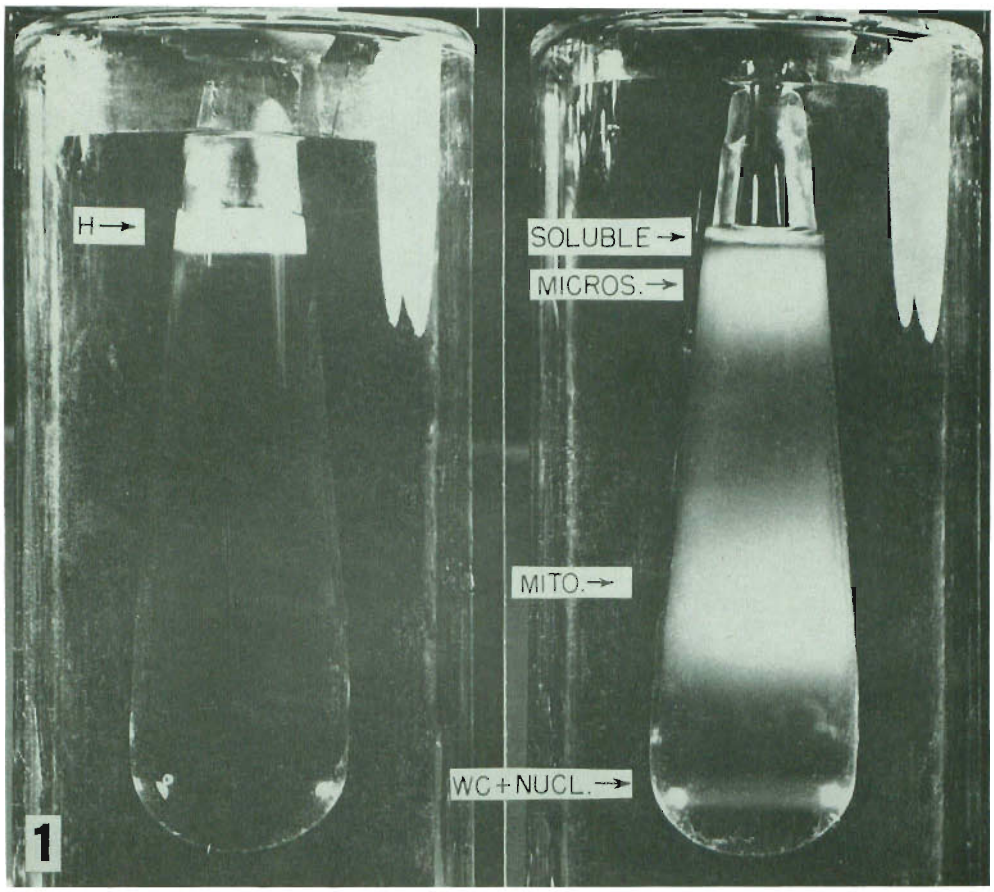

FIGURE 1.—Fractionation of rat liver subcellular particulates in a sucrose density gradient using pyrex tubes of modified sector shape. In tube on *left*, homogenate or brei has been layered over gradient at rest. On *right*, tube is shown after prolonged centrifugation. Whole cells and nuclei are banded near the *bottom* of the gradient, mitochondria in the *middle*, and microsomes near the *top*. A small zone of cleared soluble material is visible at the very *top*. The gradient volume was 65 ml; the sample volume, 2 ml; and the centrifugation time, 18 hours at 3000 rpm. Reprinted from *Science* by permission (*24*).

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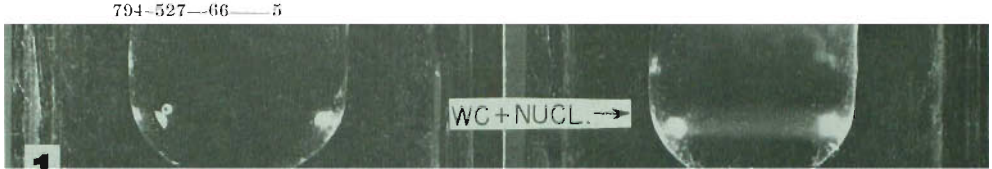


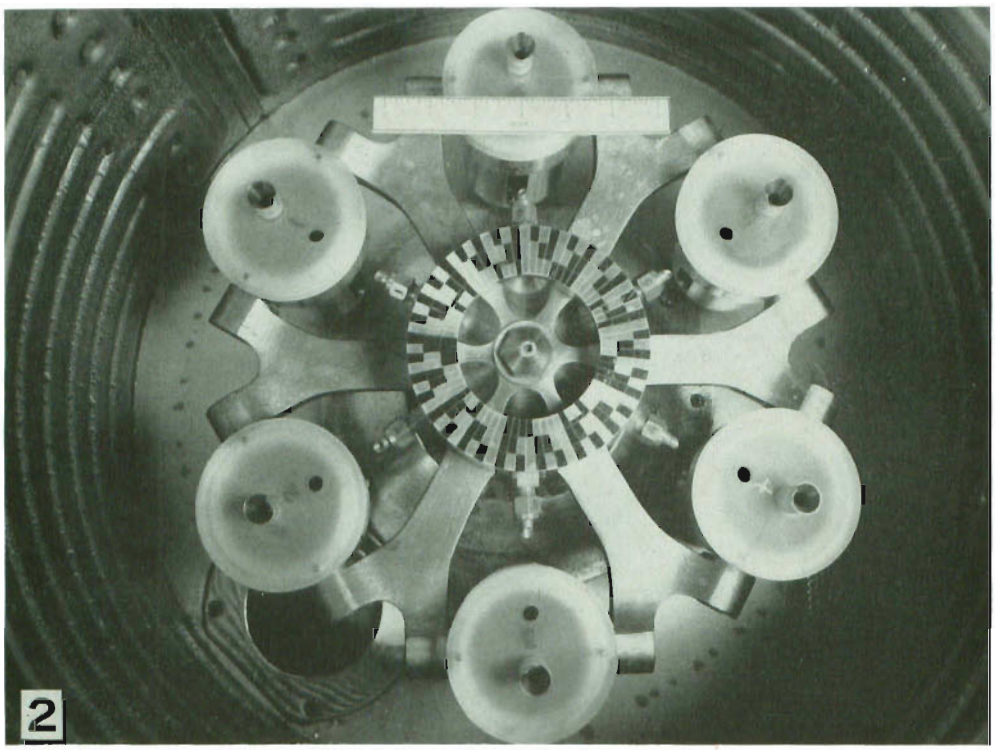
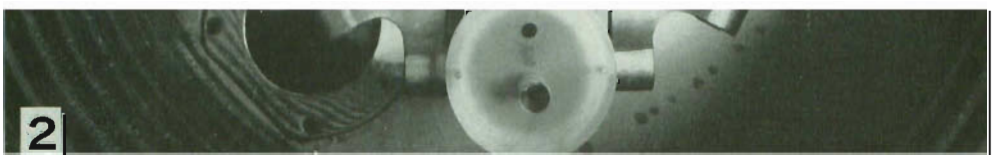


FIGURE 2.—Zonal centrifuge system A-II. During rotation the flared tubes, on the top of each centrifuge cup, tip over and make contact with the center distributing vessel. As fluid is introduced into the distributing vessel during rotation, it is proportioned to the bottom of each centrifuge cup. In this manner, identical density gradients are set up in all tubes at one time. Modified from Albright and Anderson (*26*).



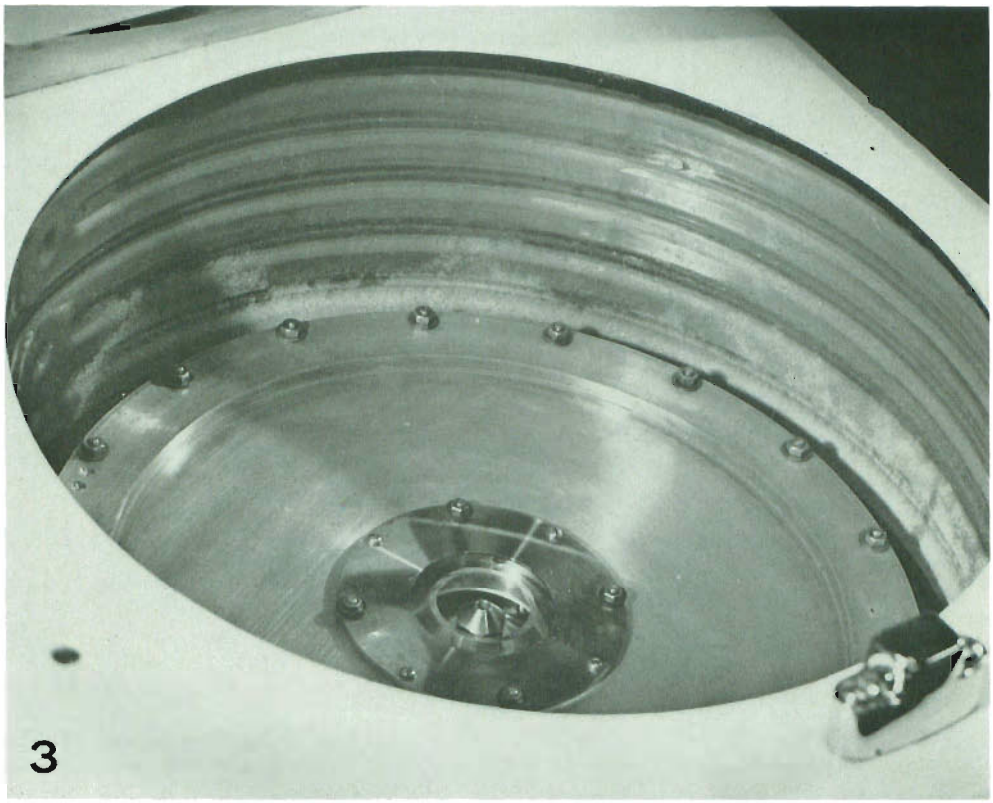


FIGURE 3.—Zonal rotor A-III mounted in International PR-2 refrigerated centrifuge.

FIGURE 4.—Zonal rotor A-IV. Rotor used to demonstrate gradient introduction and recovery from rotor at speeds up to 18,000 rpm. Used in Spinco Model K centrifuge.

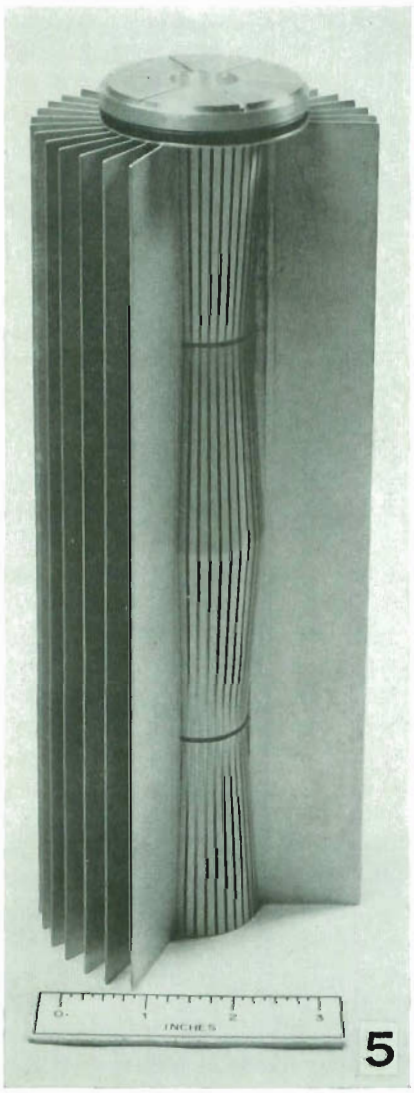
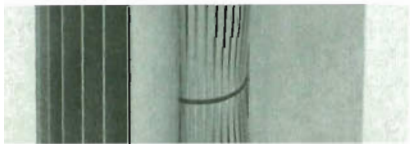


FIGURE 5.—Partially assembled core of B-II zonal centrifuge rotor.



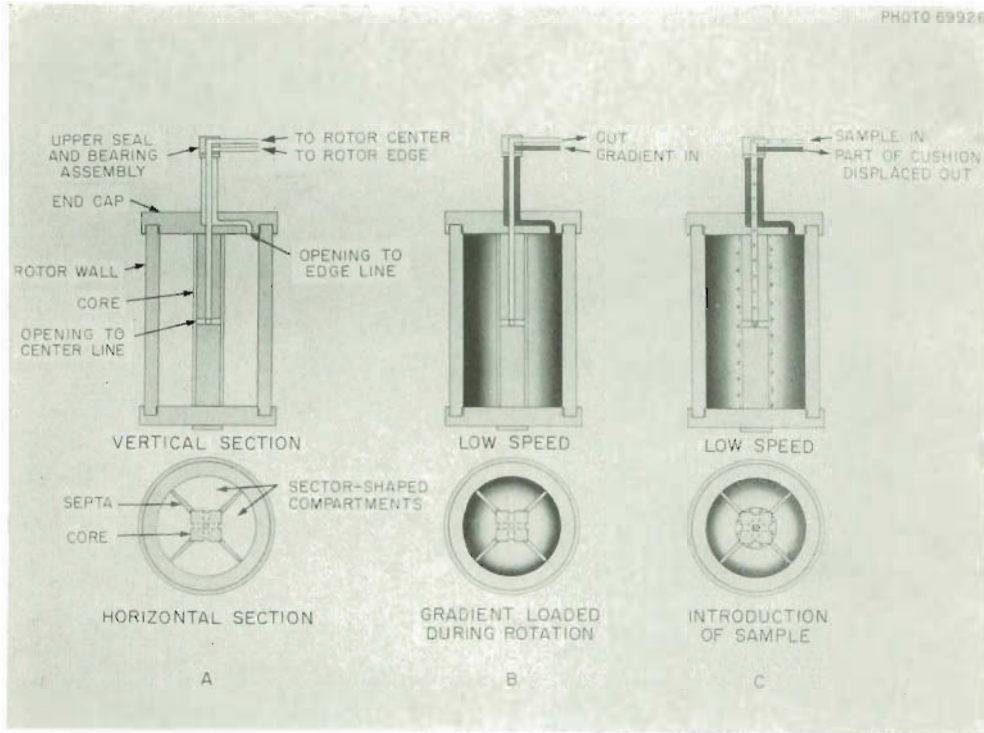


PHOTO 69926
UPPER SEAL AND BEARING ASSEMBLY
TO ROTOR CENTER
TO ROTOR EDGE
END CAP
OPENING TO EDGE LINE
ROTOR WALL
CORE
OPENING TO CENTER LINE
VERTICAL SECTION
OUT
GRADIENT IN
LOW SPEED
SAMPLE IN
PART OF CUSHION DISPLACED OUT
LOW SPEED
SEPTA
CORE
SECTOR-SHAPED COMPARTMENTS
HORIZONTAL SECTION
GRADIENT LOADED DURING ROTATION
INTRODUCTION OF SAMPLE
A
B
C

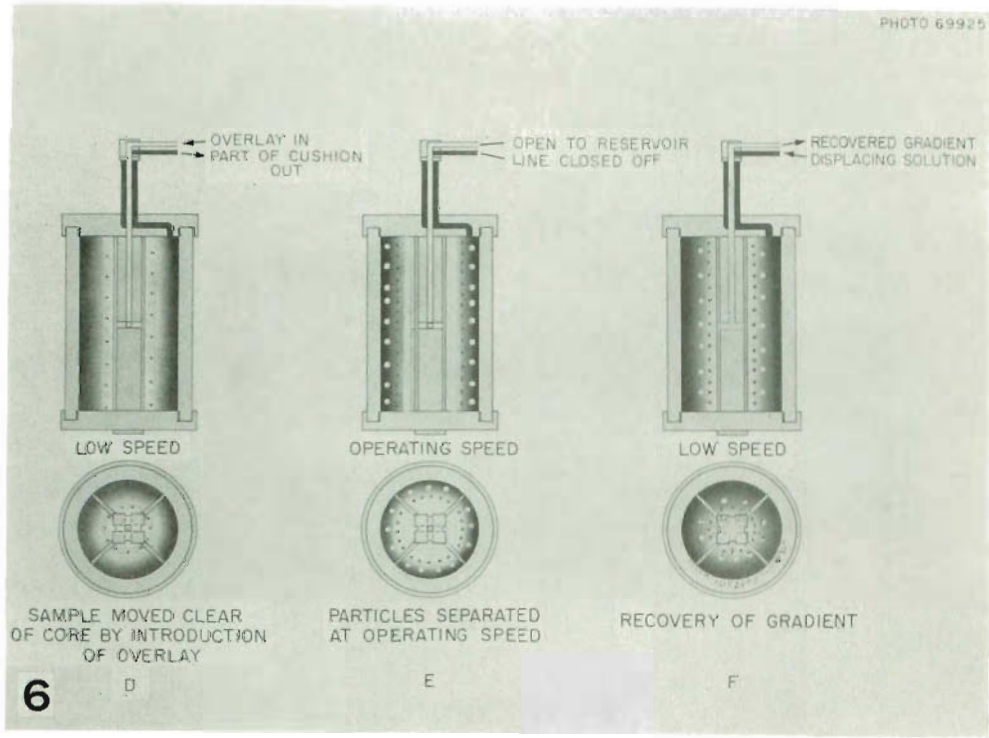
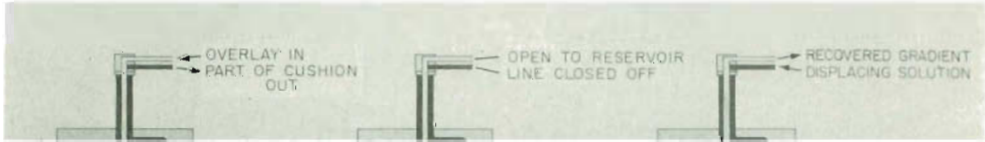


PHOTO 69925
OVERLAY IN
PART OF CUSHION OUT
OPEN TO RESERVOIR LINE CLOSED OFF
RECOVERED GRADIENT DISPLACING SOLUTION
LOW SPEED
OPERATING SPEED
LOW SPEED
SAMPLE MOVED CLEAR OF CORE BY INTRODUCTION OF OVERLAY
PARTICLES SEPARATED AT OPERATING SPEED
RECOVERY OF GRADIENT
D
E
F
6


OVERLAY IN
PART OF CUSHION OUT
OPEN TO RESERVOIR LINE CLOSED OFF
RECOVERED GRADIENT DISPLACING SOLUTION

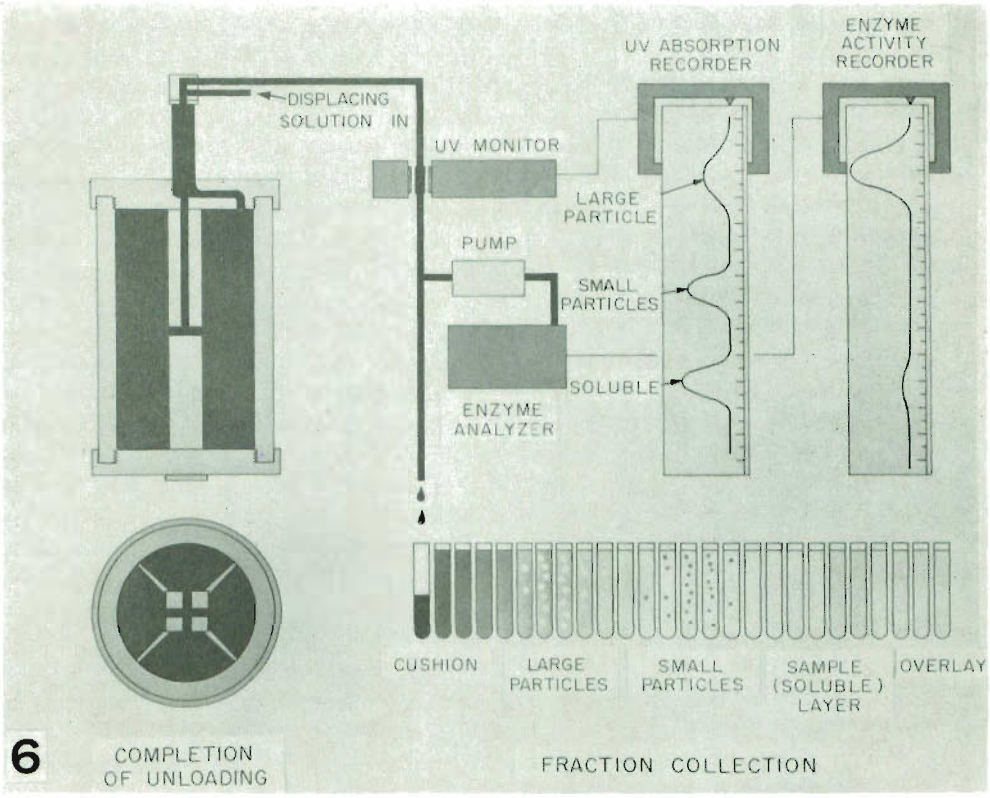


FIGURE 6.- Diagrammatic presentation of operation of the B-IV zonal centrifuge. (A) Schematic diagram of zonal centrifuge rotor. Rotation is about the vertical (*long*) axis. Rotor is shown at each stage in both side (*upper*) and cross (*lower*) section. *See* text for description of operation.