San Fernando Valley State College

REORIENTING GRADIENT CENTRIFUGATION

A New Method for the Quantitative
Isolation of Cell Components

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Biology

by

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PREFACE

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ABSTRACT

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A reorienting gradient zonal rotor was designed and constructed to test the capabilities of this type of rotor for isolating cellular components. Problems associated with reorienting gradient centrifugation were investigated with this rotor. Density gradients were measured, accelerated in the rotor, decelerated, and measured again to determine the effect of reorientation on the gradient shape. Sample zones were sedimented and collected to test the effect of reorientation.

Rate- and isopycnic-zonal centrifugations of liver tissue carried out using the reorienting gradient rotor and the collected fractions were analyzed for content and purity. Native glycogen was isolated by combined rate-zonal centrifugation (in a reorienting gradient rotor) and isopycnic-zonal centrifugation. The isolated particles were examined with the electron microscope to record particle sizes and purity of fractions.
A comparison of low-speed reciprocating-seal zonal rotors with reorienting gradient zonal rotors is presented. Similar experimental separations using both types of rotors are compared, and a discussion of the possible applications for reorienting gradient rotors based on these results is also presented.
INTRODUCTION

The functions and properties of cellular components can be ascertained only when these components are isolated from cells in pure fractions or when they are observed and studied in situ. Since many cell structures are particulate, having large molecular weights, or are bounded by membranes, they may be conveniently isolated from disrupted cells by centrifugation. Thus, centrifugation is an extremely important tool in cell biology.

The centrifugal separation and isolation of different cellular components is usually carried out in density gradients in which the sample to be fractionated is initially confined to a narrow zone near the top of the gradient. Particle separations may be based on differences in the size and/or density of the sample constituents (1). Isolations based solely on particle density are referred to as isopycnic separations and are usually employed to isolate cellular particles having a unique density. Isolations based on both particle density and particle size are called rate-zonal separations and can be successfully employed when the desired particles have sedimentation rates which are different from other particles in the cell. Frequently, rate-zonal and isopycnic separations are carried out in sequence to ensure that pure fractions are obtained (2).

Until recently, density gradient centrifugation was carried out in tubes. This method has many disadvantages,
the most serious of which are wall effects (3). These anomalies are caused by particles striking the walls of the tubes as they move perpendicular to the axis of rotation (i.e., parallel to centrifugal force). After the particles reach the tube wall their rate of sedimentation is changed as they slide down the wall instead of moving through the gradient. Another disadvantage is the limitation on sample size. Because of the small size of the centrifuge tube and orientation of the gradient in the tube during centrifugation, only a small sample volume can be placed in each tube. When large samples are to be fractionated, numerous density gradients must be prepared in separate tubes. These tubes must then be handled individually during subsequent analyses.

Zonal rotors have greatly improved this situation and have been used successfully in many laboratories since their initial development by Norman G. Anderson at the Oak Ridge National Laboratory.¹ The single compartment of the zonal rotor greatly exceeds in capacity the combined volumes of the individual positions of available swing-bucket rotors. The rotor is loaded with gradient and sample during rotation, providing conditions of maximum gradient stability; separated fractions are also collected from the rotor during rotation. Finally, since the internal volume

¹. Norman G. Anderson is the Section Head of the Biophysical Separations Laboratory of the Oak Ridge National Laboratory, Oak Ridge, Tennessee.
of the zonal rotor is divided by radial septa into a number of sector-shaped compartments, no wall effects occur during particle sedimentation (3). Zonal rotors employ a rotating seal which provides continuous access to the rotor chamber during centrifugation. Reorienting-graduent zonal rotors, also under development at the Oak Ridge National Laboratory, do not require this seal system but must be loaded and unloaded at rest. Advantages of high capacity, handling ease and lack of wall effects are retained in the reorienting-gradient rotors. In these rotors, the density gradient is caused to undergo reorientation in a manner similar to the reorientation of buckets of a swinging bucket rotor during acceleration and deceleration. While the research potential of high-speed reorienting-gradient rotors has undergone extensive testing and development over the past several years, little effort has been directed to the possible application of this principle in low-speed rotors of high capacity.

The objective of the studies reported here was to explore techniques for the separation of cellular components using low-speed reorienting-gradient rotors. The study was divided into 3 phases: (1) the design, construction and testing of a suitable rotor and auxiliary systems for load- and unloading the density gradient; (2) a series of experiments to test the operation of the system; and (3) an analysis of subcellular components of tissues using the
Many factors must be considered in the design of a re-orienting-gradient rotor. The desire for large capacity influences overall size. Because of the movement of materials during the reorientation process an internal configuration of equal height and diameter is desirable. However, mixing is minimized when the gradient is loaded into or unloaded from a tall, narrow rotor. Rotor stability considerations favor a short, wide rotor to avoid precession during centrifugation.

Gradient stability in the rotor was examined in the second phase. Gradient reorientation, that is the transition from rest of a stable gradient to a stable gradient in a high centrifugal force, is shown in fig. 1. The horizontal lines indicate levels of equal density in the continuous density gradient. As the rotor is accelerated, all levels of equal density become paraboloids of revolution about the axis of rotation. With continued acceleration a centrifugal force is reached which is sufficient to cause the isodense levels to achieve verticality (4).

An analysis of the shear forces occurring during orientation and reorientation shows that these forces are small if the rotor is accelerated slowly (4). Changes in the areas of isodense layers do occur but the rate of increase or decrease of adjacent zones is small.

A "cushion," composed of a homogenous volume of high
Figure 1.

SCHEMATIC DIAGRAM OF REORIENTING ROTOR SYSTEM

A. Rotor is loaded at rest with the density gradient. Lines in the rotor indicate isodense layers. Cushion (black at the bottom of the rotor) is loaded last.

B. Gradient is overlayed with sample (one sector at a time).

C. A rotor is accelerated, each isodense layer forms a paraboloid of revolution about the axis of rotation.

D. At operating speed the isodense zones are oriented vertically. Separation of particles in the sample occurs after orientation of the gradient is completed.

E. As the rotor is decelerated, the isodense layers, now containing the separated particles, again form paraboloids of revolution.

F. Rotor is unloaded at rest.
A. LOADING GRADIENT

B. LOADING SAMPLE

C. ACCELERATING

D. ROTOR AT SPEED

E. DECELERATING

F. UNLOADING
density, placed at the bottom of the rotor (where the shear forces due to this change in layer area are greatest) restricts the gradient to an area in the rotor where the shearing is considerably reduced.

The tangential velocity of the gradient at any point is a function of the rotational speed of the rotor and the distance of that point in the gradient from the axis of rotation. As these factors change there is a change of tangential velocity and swirling (or laminar mixing) can occur. Radial septa are necessary to minimize swirling during reorientation of the gradient.

The third phase involved separations of subcellular particles from liver tissue. Liver tissue was selected because there is a wide variety of organelles and other constituents in liver cells. Furthermore, much work has been carried out with the zonal rotors at the Oak Ridge National Laboratories with liver tissue. Results with rotating-seal zonal rotors served as an excellent index for evaluating results obtained with the reorienting gradient system. Sedimentation coefficients and banding densities of the subcellular components of liver are sufficiently diverse to allow separation to be carried out by either rate-zonal or isopycnic centrifugation.

Glycogen exists in liver as distinct particles of varying size (2). A procedure for the isolation of glycogen particles by a two-step centrifugation method using
tissues extracted with water has recently been described (2). The first centrifugation in this method is a rate-
zonal separation, using sucrose or cesium chloride for the density gradient. This first step separates the tissue into zones of particles having similar sedimentation coefficients. Glycogen collected in selected fractions is then separated from other cellular components of similar sedimentation coefficient by an isopycnic centrifugation. The density gradient is formed using saturated CsCl. This centrifugation separates particles into zones of equal density. Separations using this method indicate that glycogen molecules exist in a continuous gradation of particle sizes. Thus, glycogen may be employed as a convenient tool for testing the reorienting technique for the separation of polydisperse particles.
MATERIALS AND METHODS

Design and Construction of the Rotor.

The design of the rotor was finalized after it was decided that loading and unloading the gradient were the most critical manipulations in the procedure. The elongated internal configuration was implemented to reduce mixing during this period. The rotor design was flexible in that the rotor could be adapted for use in the international model PR-2 refrigerated centrifuge or the Servall model RC-2 refrigerated centrifuge. Critical speeds of the PR-2 between 700 and 900 rpm set the lower limit of operation. The Servall RC-2 had an upper limit of 15,000 rpm. Even at this higher speed, rotor shear stress was well within the strength limitations of the design.

The W-1 rotor was constructed from 6061 aluminum with a T-6 heat treatment. The top was sealed to the rotor with an O-ring. Two base adapters were constructed so that the rotor could be used on the PR-2 and the RC-2 centrifuges. The rotor was bolted to the drive system in both cases using the standard mechanisms supplied with the centrifuges (fig. 2).

The core (constructed from 2024 aluminum) held septa which divided the rotor into 12 sector-shaped compartments. The septa and core were anodized to facilitate assembly and cleaning. The compartments were interconnected at the bottom of the rotor where the septa did not contact the rotor.
Figure 2.

Assembled W-1 rotor with PR-2 adapter base plate.
A transparent top was designed for use below 5,000 rpm (fig. 3). A protective ring was constructed from aluminum to guard personnel in the event of failure of the lucite at speed. This top permitted visual observation of the gradient during centrifugation and was also used to observe separations of particles and to determine the optimal time and rpm necessary to affect a separation. Using this top, rate-zonal and isopycnic separations were distinguished.

A loading and unloading apparatus was designed to fill and empty all of the rotor compartments simultaneously. This apparatus consisted of a distributor cap which held and located 12 stainless steel tubes in the center of each compartment. The upper ends of these tubes were connected to a manifold which in turn was connected to a peristaltic pump for loading and unloading the gradient (fig. 4).

Since bubbles in the line connecting the loading device to the pump are difficult to avoid, a bubble trap was incorporated in the manifold to insure that all 12 loading tubes would fill their compartments simultaneously. The disassembled rotor is shown in fig. 5.

Operation of the Rotor

In a typical run, the fully assembled, prechilled rotor was locked onto the centrifuge drive shaft. The loading device was lowered into position in the rotor and connected to the peristaltic pump. The 900 ml gradient was
Figure 3.

W-1 with the lucite top in position.
Figure 4.

W-1 rotor with loading assembly in position.
Figure 5.

Partially disassembled W-1 rotor. Core and septa are anodized. Rotor is shown with base plate for PR-2.
loaded into the rotor in approximately 20 min using a two-chamber mixing device to prepared linear gradient. The light end of the gradient was loaded first. After the gradient was loaded into the rotor, a 100 ml "cushion" of 67% (w/w) sucrose was pumped to the bottom of the rotor. The sample was then loaded into the rotor (fig. 1B), and the rotor slowly accelerated over a 15 min period to the speed required to affect the desired separation. At completion of centrifugation the rotor was slowly decelerated to rest over a period of 15 min. The interval from 500 rpm to rest was the most critical phase of deceleration and was accomplished in no less than 5 min. The loading device was again lowered into position and the gradient pumped out, starting with the heavy end. The eluting gradient was monitored continuously at either 254 mµ (using a LKB Uvicord I), 260 mµ (using a Beckman DBG spectrophotometer), or 575 mµ (using the Bausch and Lomb Spectronic 20 colorimeter). The eluent was collected in most instances in about 65 fractions of 16 ml each in tubes maintained in ice. The density gradient was determined refractometrically using aliquots of collected fractions.

Separation of Subcellular Components of Liver

The separation of subcellular components of liver tissue was carried out under a variety of conditions in order to ascertain the most satisfactory combination of gradient, rpm, and centrifugation time. Male Sprague-
Dawley rats were stunned, decapitated and exsanguinated. The livers were homogenized in cold 5% (w/w) sucrose to yield a final dilution of 1:5 (w/v). Homogenates containing 3 to 4 gm of liver were layered into the rotor in a final volume of 15 to 20 ml.

The fractionation of liver was carried out using a 10 to 60% (w/w) sucrose density gradient. Centrifugation was completed in 140 minutes at 5,000 rpm using the PR-2 to affect a rate-zonal separation and 5 hr at 5,000 rpm to affect an isopycnic separation. Portions of the first 15 fractions were stained with new methylene blue and examined microscopically to identify cell nuclei, unbroken whole cells, and red cells. Fractions were also analyzed at 575 mp to determine the spread of the red cells. The protein content of each fraction was determined by the method of Lowry et al (5). Selected fractions were also stained with Janus green B and examined with the light microscope for mitochondria.

**Separation of Native Glycogen**

The rate-zonal fractionation of liver was carried out using a density gradient of 10 to 30% (w/w) sucrose. Liver homogenates were prepared as described above. Centrifugation was carried out at 5,000 rpm for 4 hr. Selected fractions were then used for the isolation of the glycogen by isopycnic banding in CsCl gradients using a Beckman Spinco type 30 rotor. Tubes containing 16 ml of saturated CsCl
layered beneath 11 ml of sample were centrifuged at 24,000 rpm for 3 hr. The banded glycogen zones were photographed prior to collection of the glycogen.

The rate-zonal separation of liver glycogen was also carried out using density gradients formed with polyvinylpyrrolidone (PVP). The gradient ranged from 5 to 20% PVP. Livers were homogenized in 5% PVP and centrifuged as above. The carbohydrate content of each fraction was determined by the phenol-sulfuric method of Dubois et al. (6). The optical density at 490 mp was recorded using a Bausch and Lomb Spectronic 20 colorimeter. Since PVP gave a slight color, samples containing 5 to 20% PVP only were also measured at 490 mp after the phenol-sulfuric acid procedure, and appropriate corrections were made.

Samples of glycogen, banded isopycnically, were prepared for electron microscopy by the following method. One drop of suspension was allowed to remain on a carbon-coated grid for 1 min. The grids were then exposed to osmium tetraoxide vapor for 30 sec, and dried by touching the edge of the grid with filter paper. The grid was then floated face down on a drop of 2% phosphotungstic acid for 30 sec and blotted dry. Grids were examined with a Zeiss EM 9A electron microscope.
RESULTS

Loading, Unloading and the Reorientation of the Gradient

The stability of the gradient during loading and unloading was tested in the rotor with a 900 ml linear density gradient of sucrose-CoCl₂. The sucrose served to form the density gradient while CoCl₂ provided a color gradient. The gradient was monitored at 575 mp by Bausch and Lomb Spectronic 20 colorimeter fitted with a continuous flow cell. After the gradient was loaded, the rotor remained at rest for 20 min. Changes in absorption were again monitored as the gradient was pumped from the rotor. The results of this loading and unloading test are presented in fig. 6A. The unloading curve, descending curve, is a mirror image of the loading curve. These curves indicated that the gradient was not altered during the loading and unloading process.

The effects of reorientation on the gradient were tested by loading the rotor with an identical sucrose-CoCl₂ gradient while monitoring the absorption at 575 mp. The loading apparatus was then removed, the rotor slowly accelerated to 1,000 rpm and then slowly decelerated to rest. The loading device was replaced and the gradient monitored as it was pumped from the rotor. Fig. 6B shows the results of this stability check which was made on the PR-2. The unloading curve is again the mirror image of the loading curve. Thus, very little mixing in the gradient occurred,
Figure 6.

Gradient stability during loading, unloading and reorientation. Curve A shows gradient shape during loading and unloading without acceleration of the rotor. Twenty min elapsed between the loading and unloading procedures. Graph B also shows gradient shape during loading and unloading; however, in this instance the rotor was accelerated to 1,000 rpm and then decelerated to rest in 20 min. (See Materials and Methods.)
during the transitions from rest to 1,000 rpm and back to rest.

Zone Spreading and Laminar Mixing

The rotor was tested several times using blood cells to determine if a zone remained intact during the reorientation and what degree of mixing occurred. A 10 ml sample of blood in 5% (w/w) sucrose solution was layered onto a 10 to 60% (w/w) sucrose density gradient. The sample zone was centrifuged at 3,000 rpm for 30 min with the lucite top in position. Under these conditions the red cells were banded into a isopycnic position. The rotor was decelerated and the gradient unloaded and collected.

The blood sample banded into a zone approximately 1 mm wide which corresponded to a volume of 30 ml of gradient. After deceleration and unloading the blood was contained in less than 100 ml of gradient.

A swirling effect ("laminar mixing") occurred within each sector-shaped compartment when deceleration was too rapid. This effect is shown in fig. 7. This swirling was considerably reduced by extending the period of deceleration after reorientation.

Fractionation of Liver

Results of the rate-zonal fractionation of liver are shown in fig. 8. Nuclei were isolated free of contamination by other cell components in fractions 2 through 6 with
Figure 7.

Laminar mixing occurring during too rapid deceleration of the rotor. The dark, circular zones seen in each of the 3 sector compartments shown are blood cells which had been sedimented to their isopycnic position. Laminar mixing was reduced considerably by increasing the deceleration time.
the peak occurring in fraction 3. Red blood cells present in the liver homogenate banded out in fractions 7 through 12 with a sharp peak in fraction 10.

Microscopic examination of the fractions containing red blood cells showed some contamination by nuclei in fractions 7 and 8. The next population in the density gradient could best be described as small masses of cell fragments. These fragments varied in size, some being large enough to produce a small peak as they went through the monitor.

The next zone consisted mainly of mitochondria. Large amounts of material (labeled soluble phase) were found displaced only slightly from the starting zone.

Isopycnic fractionation of liver is shown in fig. 9. The cell fragments peak, located in fraction 11, was displaced further toward the heavy end of the gradient, as was the mitochondrial peak. Also in fractions 28 through 33 a peak was recorded which probably contained microsomes. The soluble phase was displaced much further into the gradient during the isopycnic centrifugation.

The results of the combined rate-zonal and isopycnic isolation of liver glycogen are shown in fig. 10. Glycogen was spread throughout the gradient during rate-zonal reorienting gradient centrifugation (fig. 10, top). At the heavy end of the gradient a peak in glycogen concentration occurred in fraction 4. This peak was probably
Figure 8.

Rate-zonal fractionation of rat liver homogenate at 5,000 rpm for 140 min. Recording at 260 μm located fractions containing nucleic acid (or protein); recording at 625 μm represents results of quantitative protein analysis of collected fractions. Recording at 575 μm localized red cells in the gradient. Selected fractions were analyzed for sucrose content refractometrically.
caused by glycogen associated with membrane fragments. A gradual increase in glycogen is noted as one progresses toward the light end of the gradient. Selected fractions were used to band out glycogen isopycnically in CsCl. The glycogen banded at a density level of 1.6. These tubes were then photographed (fig. 10, bottom). A faint band in the first fraction eluted from the gradient was not sharp enough to be photographed. Fraction 57 also did not show a clear band after isopycnic centrifugation.

Electron microscopic examination of the glycogen band from each tube showed that a continuous spectrum of glycogen particle sizes had been isolated. Particle sizes ranged from 3 μm up to 200 μm. Fig. 11 shows a series of electron photomicrographs selected to show the spectrum of decreasing particle sizes isolated beginning at the heavy end of the gradient. It is apparent from an examination of these photomicrographs that distinct populations of particle sizes were separated into various fractions as a result of the rate-zonal centrifugation. The particles in any given sample were similar in size. The small particles which contaminate all of the samples are probably breakdown products resulting from the period after rate-zonal centrifugation.

Fig. 12 shows glycogen particles at a magnification of 80,000. Three levels of structure can be observed. These three levels of structure have been described previously (2). Fundamental particles (gamma particles) are 2.5 to
Figure 9.

Isopycnic-zonal fractionation of rat liver homogenate at 5,000 rpm for 5 hr. See legend of fig. 8 for explanations.
Figure 10.

(Top) Distribution of glycogen from rat liver following rate-zonal centrifugation in reorienting-gradient rotor. Each value is given as the per cent of the total fractionated glycogen. (Bottom) Isopycnically banded glycogen (dark zones below density beads) from selected fractions obtained by rate-zonal centrifugation. (See Materials and Methods.)
3.5 μm in diameter. Subclusters are made up of gamma particles and are 20 to 25 μm in diameter. These subclusters make up alpha particles which can be up to 200 μm in size.

The photomicrographs and the glycogen distribution curve indicate that particle size decreases as the concentration of particles increases.
Figure 11.

Electron photomicrographs of negatively-stained glycogen isolated by centrifugation in a sucrose gradient. The numbers adjacent to each photograph indicate the fraction from which the glycogen was isolated by isopycnic banding. (See fig. 10.)
Figure 12.

This is photomicrograph of fraction 6 at a magnification of 80,000. The large aggregates of about 2.6 cm are alpha particles. Circle No. 1 shows a beta subcluster. Circle No. 2 shows a fundamental particle (gamma particle).
DISCUSSION

The final design of the rotor realized the following advantages over centrifugation in tubes: (1) ideal sedimentation in sector-shaped compartments, (2) uniform and simultaneous loading and unloading of all compartments, (3) large sample capacity, and (4) an operating procedure requiring a minimum of handling after separation has been effected.

The sector-shaped compartments eliminate the effects caused by the sedimenting particles striking the walls of the tubes and changing their sedimentation rates. Therefore, the sedimentation rate of specific particles is uniform and predictable, and mixing due to particles sliding along the walls is eliminated.

All compartments are essentially loaded with the same and, therefore, identical gradient. The unloading procedure is the reverse of the loading and the effect is that of collecting the same zone at 12 places simultaneously. The rotor must be completely level during unloading so that an isodense layer does not reach the bottom of the rotor on one side before the other side.

The large sample capacity is probably the most appealing feature of the rotor. Up to 4 gm of liver tissue were treated in a single run of the rotor. This feature of large capacity can be used to isolate components present only in minute quantities in the cell-components which...
could only be treated analytically by conventional methods of centrifugation.

The gradient recovery procedure of the W-1 system overcomes the difficulties associated with recovery of gradients from centrifuge tubes from a swinging-bucket rotor. The density gradient is not moved from its position in the centrifuge while recovery of the gradient takes place. The only disturbance to the gradient is the insertion of the recovery apparatus.

A comparison of the operation of reorienting gradient zonal rotors with zonal rotors with fluid-line seals (reciprocating seals) can be made from our results and those of Anderson (8). The reorienting gradient rotor is loaded and unloaded at rest so the complexity of design is far less than a rotor which is loaded and unloaded while rotating. The operating procedure and the supervision during centrifugation are minimized with a reorienting gradient rotor.

The reorienting gradient rotor avoids complication but the reorientation process is a source of mixing which the reciprocating-seal rotors lack. This mixing can limit the resolution obtainable using a reorienting gradient rotor under certain conditions. The banding and recovery of blood demonstrated the mixing due to reorientation and the unloading technique. The blood zone during centrifugation was approximately 1 mm wide, representing a volume of 30 ml of gradient. After deceleration, the blood was collected
in a volume of under 100 ml. This indicates considerable zone widening occurs (from 10 ml of sample to 100 ml of gradient). The reasons for this zone spreading are two-fold. When fractions of blood were subjected to analysis by a Coulter counter, it was observed that fractions from the heavy side of the blood zone were composed of larger cells than fractions from the light side. Thus, the thickness of the zone during centrifugation was the result of differences in sedimentation properties of the red cells. The thickness of the zone was a result of separation of cells and not dilution.

The second increase in sample volume occurred as a result of the reorientation and unloading process. Swirling of the gradient occurred at the end of reorientation and during the remaining deceleration. It is essential to eliminate as far as possible this swirling due to Coriolis forces. The steep gradient withstands these forces during the first part of deceleration until the reorientation process begins. However, as reorientation takes place the gradient becomes less effective in resisting swirling. Finally, when the isodense layers become horizontal, tangential flow causes "laminar mixing" of the sample zone. By decelerating the rotor very slowly during and after re-orientation, the swirling can be greatly reduced. Also, since the laminar mixing actually occurs inside each isodense layer after reorientation is completed, the mixing is not between sample zones unless they are adjacent in the
The swirling which occurred as the rotor was decelerated was observed to widen the blood sample zone. When this swirling was controlled (by lengthening the deceleration time during and after reorientation, the sample zone was much sharper. The exact width of the band could not be measured because it was now parallel to the walls of the rotor, but after collection the blood was confined to less than 80 ml of gradient.

The unloading process further diluted the sample zone because the stainless steel tubes are relieved on the bottom at a 45° angle and, therefore, collected gradient from a zone approximately 0.5 mm deep.

The dilution of the sample zone with gradient immediately adjacent to the zone is not a serious problem if the various zones resulting from centrifugation are sufficiently separated. By preparing a gradient which will result in a complete separation of zones, these zones will be maintained even though dilution does occur during reorientation and unloading.

The fractionation of liver demonstrated the resolution capabilities of the reorienting gradient rotor. Similar fractionations in zonal rotors have produced five fractions: (1) nuclei uncontaminated with intact cells, (2) membrane fragments, (3) a mitochondrial fraction, (4) microsomes, and (5) soluble materials. The relative purity of fractions can be determined from the sharpness of peaks.
observed in the eluting gradient. Figs. 8 and 9 indicate the resolution which can be obtained in the reorienting gradient rotor. The isopycnic fractionation compares extremely well with a similar fractionation accomplished in the zonal ultracentrifuge rotor B-II (7).

It is impossible to make a quantitative comparison of separations since the purity of fractions can only be estimated. However, the similarity in peak heights indicates that the particles in the respective zones were present in relatively similar concentrations.

A comparison of figs. 8 and 9 shows that in both separations the large subcellular components sedimented to the same position indicating that they reached their isopycnic positions in the gradient. The mitochondria were not at their isopycnic position after 140 min (previous work (7) has shown the isopycnic position of liver mitochondria to be 43.5% (w/w) sucrose), yet after continued centrifugation the mitochondrial zone was wider. One possible explanation for this observation is that other subcellular components sedimented at the same rate but have a different density. This would explain the widening of the zone during the isopycnic separation.

The isolation of glycogen by reorienting gradient centrifugation was chosen because a direct comparison could be made with similar isolations in a reciprocating-seal rotor (2). The relative purity of fractions could be ascertained by a comparison of photomicrographs of the glycogen
particles.

Liver glycogen exists as a continuous spectrum of particle sizes. This spectrum has been characterized using a combined rate- and isopycnic-zonal centrifugation procedure (2). This method of isolation was used with the reorienting gradient rotor (W-1) instead of the B-IV so that a direct comparison could be made.

Fig. 10 shows that the maximum volume of gradient was used to affect the separation. The distribution curve for glycogen agrees very closely with similar curves obtained using the B-IV. The membrane-associated glycogen caused a sharp peak in fractions 2, 3, and 4. The gradient near the starting zone contained a large amount of the total glycogen indicating that there was a considerable amount of degradation of larger particles or that a large amount of glycogen in the liver exists as small particles.

Fig. 11 can be compared with a similar composite from the work reported above. The corresponding photomicrographs are almost identical in respect to particle size and concentration. The relative purity of fractions can be compared if one assumes that the small contaminating particles are the result of degradation of larger particles. Using this assumption one can observe a high degree of purity of particle size in the results from the reorienting gradient isolation of glycogen.

These experiments illustrate the kinds of separations that can be accomplished in a relatively simple reorienting
gradient zonal rotor. The results of a separation are simultaneously preparative as well as analytical; the separated particles can be used for subsequent chemical and morphological analyses, or the distribution of these particles in the gradient can be used to detect physiological changes with the cell.
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