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STUDIES ON ISOLATED CELL COMPONENTS

VIII. HIGH RESOLUTION GRADIENT DIFFERENTIAL CENTRIFUGATION[1]

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THE purpose of the studies described in this paper has been to lay the groundwork for the development of a technique for analytical medium-speed centrifugation in which quantitative separation of nuclei, whole cells, mitochondria, microsomes, soluble proteins, and lipid inclusions may be achieved in one or two steps. Since it is not possible, at present, to make quantitative measurements of all particulate fractions during centrifugation in a manner analogous to that used in the ultracentrifuge, it is necessary to rely on methods which allow fractions to be recovered, free of cross-contamination, for analysis at the end of the centrifugation. Fortunately, the procedures which are necessary to achieve convection-free sedimentation in suspensions of cell components are readily adapted to do this. The method to be described has been developed over a period of several years and is based in part on previous work done on starch gradients (N. G. Anderson, 1949, unpublished) and on techniques described by Behrens [1] and by Brakke [3, 4].

It appears not to have been generally realized that the separation of tissue brei constituents in a centrifugal field cannot be predicted from a simple uncritical application of Stokes' law. If one assumes, for example, equivalent radii of 4 μ for nuclei and 1 μ for mitochondria, their sedimentation velocities should differ by a factor of approximately 16, depending on the densities of the particles. Actually, no such clear-cut difference in sedimentation rate is observed, and a few nuclei are often found still in suspension after many of the mitochondria have sedimented. In methods wherein a brei layer is used over a denser clear layer of solution such as the one described by Hogeboom *et al.* [8], one observes mitochondrial contamination of sedimented nuclei even when many nuclei are still to be found in the upper layer. This and other anomalous effects may be shown to be caused by several artefacts which are common to nearly all isolation procedures.

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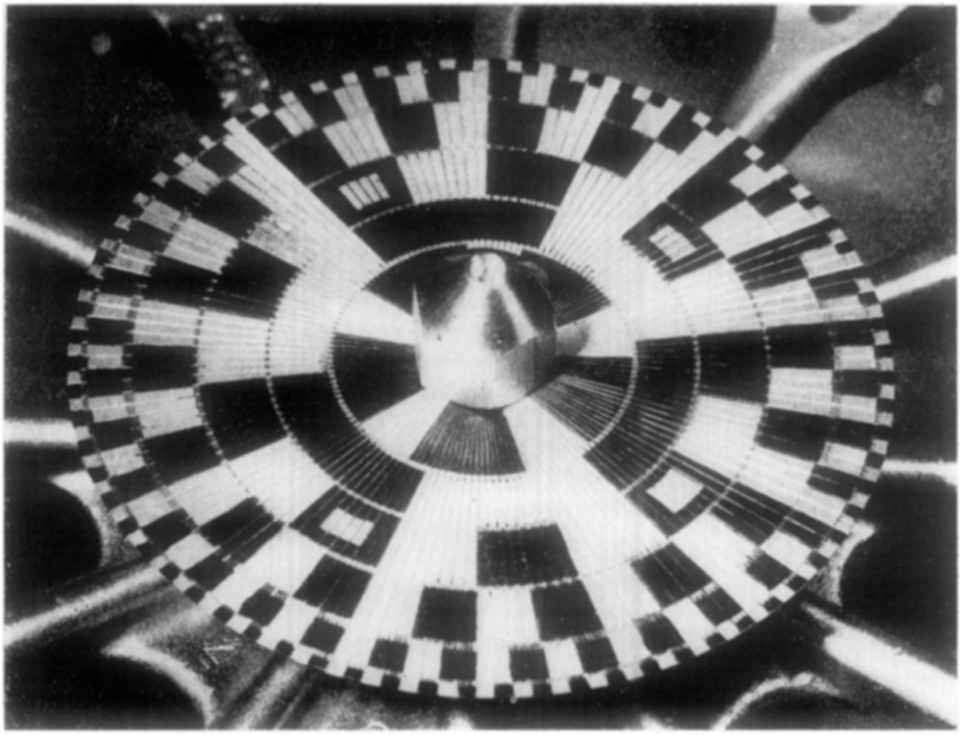


Fig. 1. Stroboscope disc for accurate speed determinations.

METHODS AND MATERIALS

In the present work two centrifuges were used: an International Equipment Company Size 2 centrifuge modified by the installation of refrigerating coils, suitable insulation, two observation ports on the hinged cover, and a stroboscopic lamp mounted inside the centrifuge; and, more recently, a specially modified Model PR-2 refrigerated centrifuge[1] from the same manufacturer. Alterations in the latter include installation of a large observation port of shatterproof glass, an electrical contactor[2] on the main shaft for continuous stroboscopic observation during sharp changes in speed, steel jacks to raise the centrifuge off the rubber castors to reduce vibration, and a stroboscopic lamp[3] mounted inside the centrifuge. By the use of a stroboscope[4] and an auxiliary high-intensity lamp[5] alone or in conjunction with the contactor described, a flexible system is obtained for the observation of centrifuge tubes at

[1] International Portable Refrigerated Centrifuge, Model PR-2, International Equipment Co., Boston, Mass.

[2] Contactor Type No. 1535A. General Radio Co., Cambridge, Mass.

[3] Strobotron, Type 631-P1, manufactured by Sylvania Corp. for General Radio Co.

[4] Strobotac, Type 631-B, General Radio Co.

[5] Strobolux, Type 648-A, General Radio Co.

all speeds, during rapid changes in speed, and for the accurate (> 0.02 per cent) determination of speed, 60-cycle line frequency being used as standard. Cutout shields allow the centrifuge tubes to be observed during centrifugation. An aluminum disc (Fig. 1) with concentric rings divided into 144, 72, 36, 18, 6, and 10 divisions, alternately black and silver, is placed in the center of the centrifuge head for speed determinations with a lamp flashing frequency of 3600/minute. Speeds are read off at 50-rpm increments with a minimum of repetition of patterns, and 25 rpm increments (zero or hundreds plus 25 or 75) appear as clear lines in a grey outer ring. The speed increments observed with any set of black divisions is found by dividing the flash rate (3600) by the number of black divisions. Thus the disc described gives stable patterns for the various rings at multiples of 50, 100, 200, 400, 1200, and 720 rpm respectively reading from the outside in. Minor modifications such as placing silver centers in four divisions of the 400-rpm increment ring differentiates 400 rpm from 800 rpm. With head number 253 used in these experiments, a stable image of the head is also obtained at 300-rpm increments. The sequence of patterns obtained on acceleration and deceleration is readily memorized. To obtain accurate acceleration and deceleration curves, the times at which various speeds were reached were recorded on a printing chronograph.[1]

CENTRIFUGATION ARTEFACTS

Hydrodynamic effects.—Two types of hydrodynamic effects are seen. The first, here termed the *streaming effect*, occurs in very low gravitational fields and is in fact easily studied in stationary tubes. It does not appear to involve movement of the particles out of their original suspending medium. The second, termed the *turnover effect*, occurs during centrifugation and is observed in layered systems when the suspended particles pass an interface between solutions having different densities.

The streaming effect is seen when a 10 per cent liver brei is layered over sucrose solutions having a slightly higher density. As shown in Figs. 9–14, material soon begins to fall in streams from the brei layer through the denser layer *without any centrifugation*. With the systems described either by Wilbur and Anderson [15] or by Hogeboom *et al.* [8], much material reaches the bottom of the tube within 5 minutes. Microscopic examination shows that nuclei, whole cells, and mitochondria are all present in the sediment. In further studies, breis were prepared in Solution I [15] in such a manner as to contain 20, 10, 5, and $2^1/_2$ per cent rat liver by weight, and were layered over Solution III [15]. Decreasing anomalous sedimentation was seen with decreasing liver concentration, or with increasing density of the under layer (Fig. 9, 10). When a 10 per cent brei in Solution I was layered

[1] Tracergraph, No. SC-5A, modified to print consecutive times from starting time. Tracerlab, Inc., Boston, Mass.

over 0.88 *M* sucrose containing the same salts as found in the homogenizing medium, only a few small streams of descending material were seen, and these appeared only after an interval of about 30 minutes. Little qualitative difference was noted between tubes maintained at room temperature and those kept at 4° C during observation. Similar results were obtained with suspensions of rat erythrocytes (Figs. 12–14).

The streaming effect appears to be the result of local statistical fluctuations in the number of particles per unit volume which produce local concentrations of particles sufficiently high to increase the density above that of the lower fluid. The heavy particles (nuclei, whole cells, mitochondria) then begin to sediment as a group, dragging along the fluid between them and associated smaller particles. As the droplet moves through the denser fluid, diffusion equalizes the sucrose concentration.

The *turnover effect* occurs in layered systems when particles migrate through the interface between the two layers and appear at the top of the lower layer. The top of the lower layer is then made denser by virtue of the particles it now contains and "turns over", moving as a body to the bottom of the tube. This effect is most easily observed in a two-layered system in which a dilute suspension of erythrocytes is used in the upper layer. As shown in Figs. 15 and 16 when the erythrocytes have been centrifuged long enough to move the last of them a few millimeters, many are already at the bottom. Similar effects are observed with multilayered or discontinuous gradients. The results of Kuff and Schneider [11], which yield a picture of layers with material accumulated above each interface, are also due to the turnover effect. It is therefore not surprising that these authors found little microscopically observable difference between the various levels.

Swirling.—It is evident that the acceleration and deceleration of fairly large volumes of fluid (20–100 ml) without mechanical disturbance is very unlikely. The transition from a vertical tube position rotating around an external axis parallel to the tube, to a horizontal position characteristic of useful rotational speeds involves forces which, in many centrifuges, produce a rotation of fluid at the top and bottom of the tubes opposite in direction. The result is often sufficient to produce considerable swirling. A similar condition exists during deceleration.

A somewhat less obvious cause of swirling occurs when a centrifuge is switched off. As shown in one curve in Fig. 3, the maximum rate of deceleration is reached almost instantaneously. Stroboscopic observations with simple dyed layer systems shows that considerable mixing occurs. In non-layered systems the presence of mechanical disturbances is readily demon-

strated by attempting to decelerate centrifuge tubes containing very dilute suspensions of red blood cells in 100-ml centrifuge tubes. Although stroboscopic observation during sedimentation showed a fairly sharp trailing boundary, it was rarely possible to bring the tubes to rest while maintaining the boundary in a completely undisturbed condition.

Convection.—No attempt has been made to study thermal convection currents other than to attempt to minimize them by working from 0–4° C. It should be pointed out that the air resistance in a swinging bucket head such as the one employed here is large and that a considerable amount of power is dissipated. This, together with the discontinuous operation of the cooling system makes it highly unlikely that the temperature in the tubes is either constant or uniform throughout the tube length. The effect of small variations in temperature is greatly magnified as the gravitational field is increased since no increase in the forces opposing movement of the fluid (viscosity) occurs. In a field of 1000 *g*, for example, a change from 0.0 to 0.1° C will effectively change the density of water by 6 mg/ml.

Wall effects.—The major wall effects occur because particles in a centrifuge do not sediment in a parallel fashion, but rather fan out from the axis of rotation. In layered systems, especially those involving thin brei layers close to the axis of rotation and fairly long tubes, a considerable portion of the particles hit the side of the tube before reaching the bottom. Collision with the wall tends to make particles agglutinate and either adhere or slide as a mass to the bottom. The use of sector-shaped tubes to eliminate this difficulty has long been standard practice in the ultracentrifuge. These tubes have not previously been used in the low-speed centrifuge owing, probably, to nonavailability of glass tubes of a satisfactory design.

Aerodynamic effects.—In all instances the centrifuge tubes have been kept closed at the top with either Parafilm[1] or plastic tops.

HIGH RESOLUTION GRADIENT DIFFERENTIAL CENTRIFUGATION

A separation of brei components approaching the ideal has been achieved by use of (a) a continuous-density gradient to minimize hydrodynamic effects and (b) sector-shaped centrifuge tubes to eliminate wall effects, (c) by carefully controlling the centrifuge operation to prevent mechanical disturbances, and (d) by keeping the particles suspended at all times.

Centrifuge operation.—It is evident from the foregoing that considerable care is required during acceleration and deceleration. The most critical

[1] Parafilm, grade "M". Marathon Corp., Menasha, Wisconsin.

period is during the transition of tube orientation from vertical to horizontal. To start the centrifuge the variable transformer (variac) is set to supply enough current to allow the centrifuge to reach a final speed of 210 rpm. At this setting rotation usually begins very slowly but may require a very gentle start by hand. When a speed of 100 rpm is reached, as shown by the stroboscopic control disc, the variac is advanced one division and left there for 10 seconds. The variac is then advanced one division every 5 seconds until a setting is reached which is two divisions above the equilibrium setting for the speed desired. When the desired speed is reached, the variac is turned back to maintain it. Deceleration is accomplished by turning the variac back one setting each 5 seconds until a setting 1 division below the starting setting is reached. This provides a bias current which is sufficient to give a very slow terminal deceleration. Acceleration and deceleration curves for a number of operating speeds are shown in Figs. 2 and 3. For comparison, the deceleration curve observed when the centrifuge is switched off at 3000 rpm is included. The value, $(RPM)^2 \times 1.118 \times 10^{-5}$, which gives the radial centrifugal force when multiplied by the radius, is plotted against time. By integrating the areas beneath the curves it is possible to obtain the number of seconds of operating speed centrifugation equal to that done during acceleration and deceleration. Plots of these are shown in Fig. 4. When the centrifuge compressor is not operated off the same line as the centrifuge motor[1] and a constant voltage transformer is used[2], the values obtained vary by no more than ± 4 per cent. Since acceleration and deceleration generally constitute a minor part of the centrifuge run, a small change in the curves will produce negligible effects.

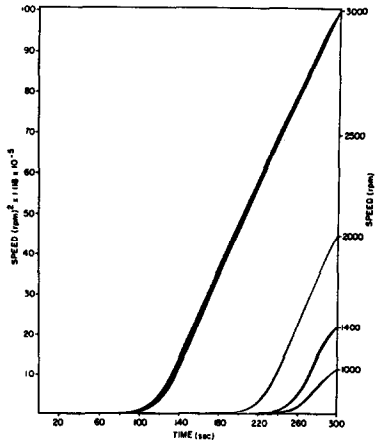
Production of continuous gradients.—The centrifuge artefacts discussed under *hydrodynamic effects* are minimized by dilute breis and continuous density gradients with sugars or other nonelectrolytes, or proteins. In the experiments described here sucrose has been employed.

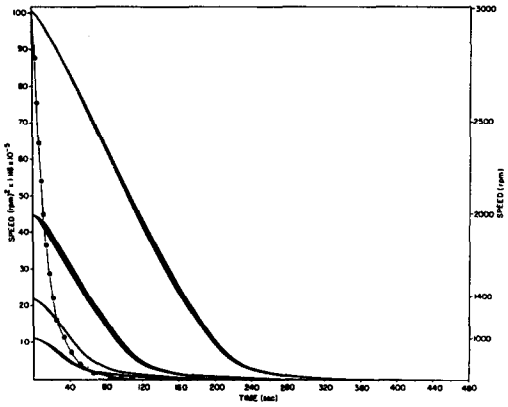
Several methods for producing gradients have been explored. These include modifications of those described by Behrens [1] and the adaptations currently employed in gradient elution procedures [2]. These have not as yet been found as satisfactory as a mechanical system employing differentially driven glass syringes (Fig. 5). This gradient engine produces a slightly sigmoidal concentration versus delivery curve which is of advantage in

[1] Instructions for placing the centrifuge motor and the compressor on separate circuits are available from the manufacturer.

[2] Sola Constant Voltage Transformer, Catalogue No. 30809, Secondary 8.7 amps, 115 volts. Sola Electric Co., Chicago.

separating particles of the size distribution found in liver breis. The solutions delivered by the syringes flow together at a T of 18-gauge stainless steel tubing and then into a small chamber of 1.2-ml volume containing a magnetic mixer. The gradient is delivered to the bottom of a sector centrifuge tube





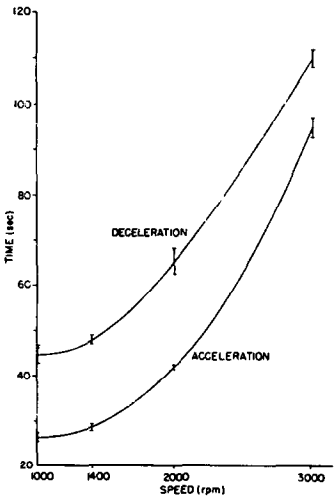


Fig. 2 (upper left). Curves showing controlled accelerations. The value RPM^2 × 1.118 × 10^{-5} which is proportional to the gravitational force is plotted against time. The area under each curve is proportional to the total force applied during acceleration. The curves show the maximum and minimum values observed at each speed.
Fig. 3 (right). Curves showing controlled deceleration plotted as in Fig. 2. The thin-line curve shows free deceleration as it occurs when centrifuge is suddenly switched off.
Fig. 4 (lower left). Centrifugation during controlled acceleration and deceleration. The results are expressed as equivalent seconds at running speed.

by an 18-gauge stainless steel tube, the lighter solution being delivered first. Each gradient requires approximately 1 $^1/_2$ hours to produce. The details of the gradient engine will be described elsewhere.

A very dense solution (generally 80 per cent sucrose) is passed through the mixing system after the gradient is formed to fill the hemisphere formed by the bottom of the sector tube. This layer serves as a cushion to prevent

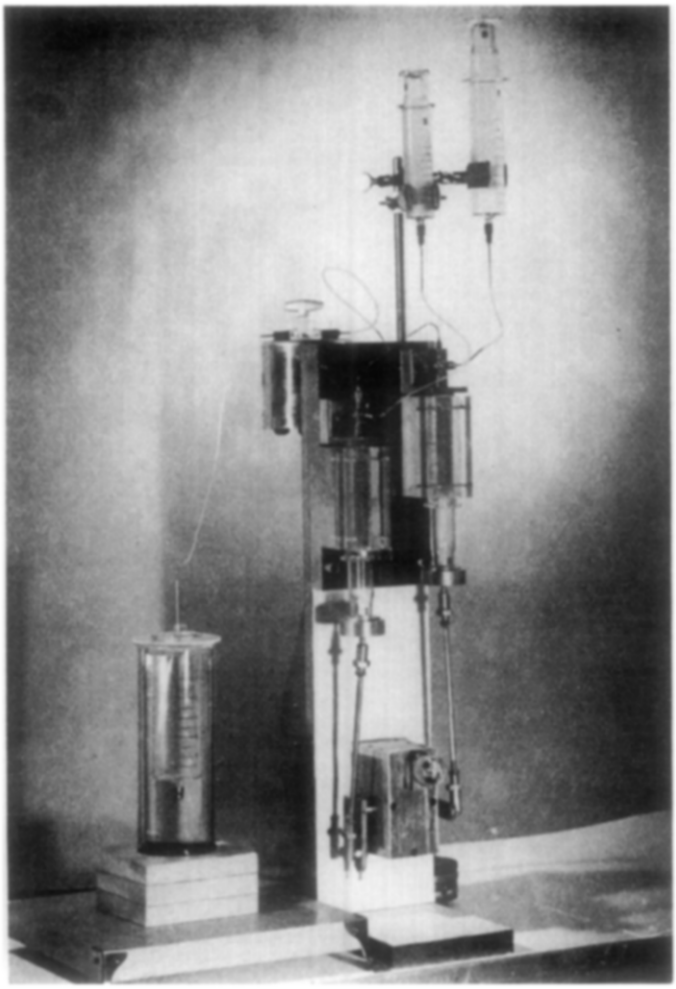
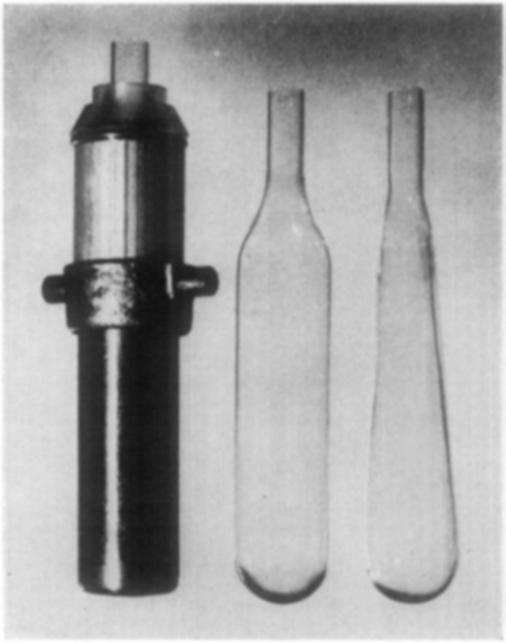


Fig. 5. Model III gradient engine. Two differentially driven pistons continuously mix two solutions of different density to produce a continuous controlled gradient.

large particles from reaching the bottom of the tube. The tubes are kept in ice water during filling and then kept cold until used.

Use of sector tubes.—Round-bottomed, sector-shaped tubes were blown in a suitably machined graphite mold. Two designs have been used. Type A (Fig. 6) is constricted at the top into a round tube 1.4 cm in diameter. This serves to center the tube in the plastic cap shown and to allow the various layers to be removed easily after being raised by a dense fluid introduced to the bottom of the tube by a long stainless steel needle. Although this method of recovering the various fractions is satisfactory for many purposes



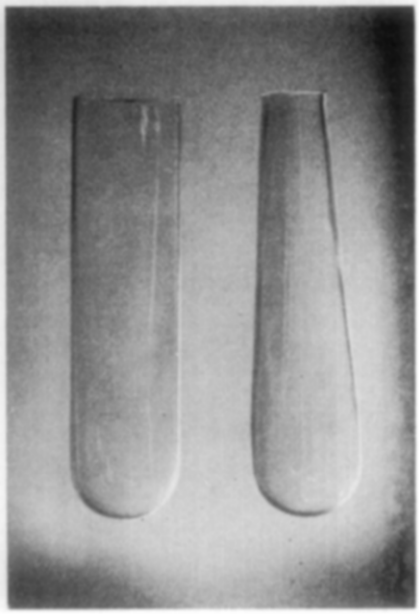


Fig. 6 (left). Type *A* pyrex sector tubes. Two side views illustrate modified sector design. Volume up to constriction is 68 ml. The plastic shield cap shown centers the tube. The aluminum ring decreases the radius sufficiently to allow the tubes to be swung in the Model PR-2 centrifuge. Fig. 7 (right). Type *B* pyrex sector tubes. The flat upper rim allows these tubes to be used with the gradient sectioning device shown in Fig. 8. Useful capacity is 68 ml.

it lacks precision commensurate with the resolution of particles obtained. Unfortunately, the design of the upper portion of the tube is such that the tube will not stand high centrifugal forces. When used in No. 340 shields and No. 325 trunions in head No. 253 with the 3.9-cm spacer sleeves shown in Fig. 6 to decrease the radius of the tubes, half of the tubes shattered at 2400 rpm. Their use is therefore restricted to lower speeds. They have been found especially useful for the isolation of nuclei.

Type B tubes (Fig. 7) allow full advantage to be taken of the inherent strength of the sector design. The upper rim of the tube is ground flat and then fire-polished carefully to remove any tiny cracks. A sliding section device is used to recover samples from Type B tubes (Fig. 8). A stainless steel tube passes through the lower lucite block, bends at right angles in an eliptical hole matching that of the sector tube, and passes to the bottom of the tube. As a dense liquid (80 per cent sucrose, sucrose with $CaBr_2$, or a dense fluorocarbon) is passed to the bottom of the sector tube, the gradient

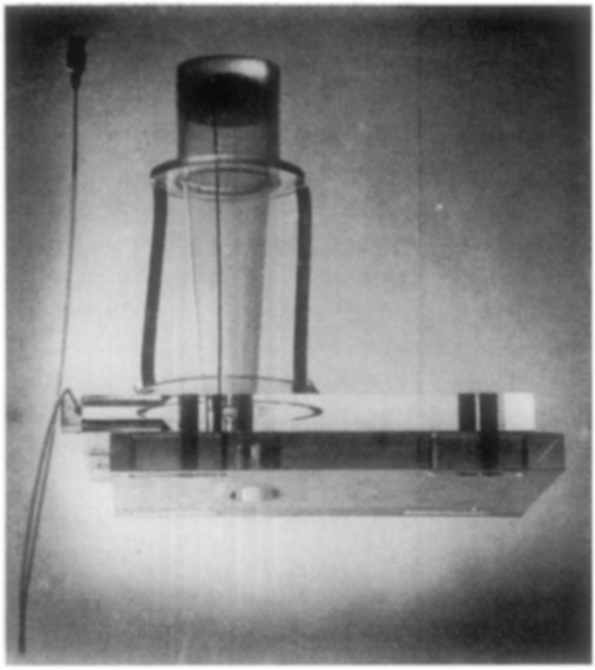
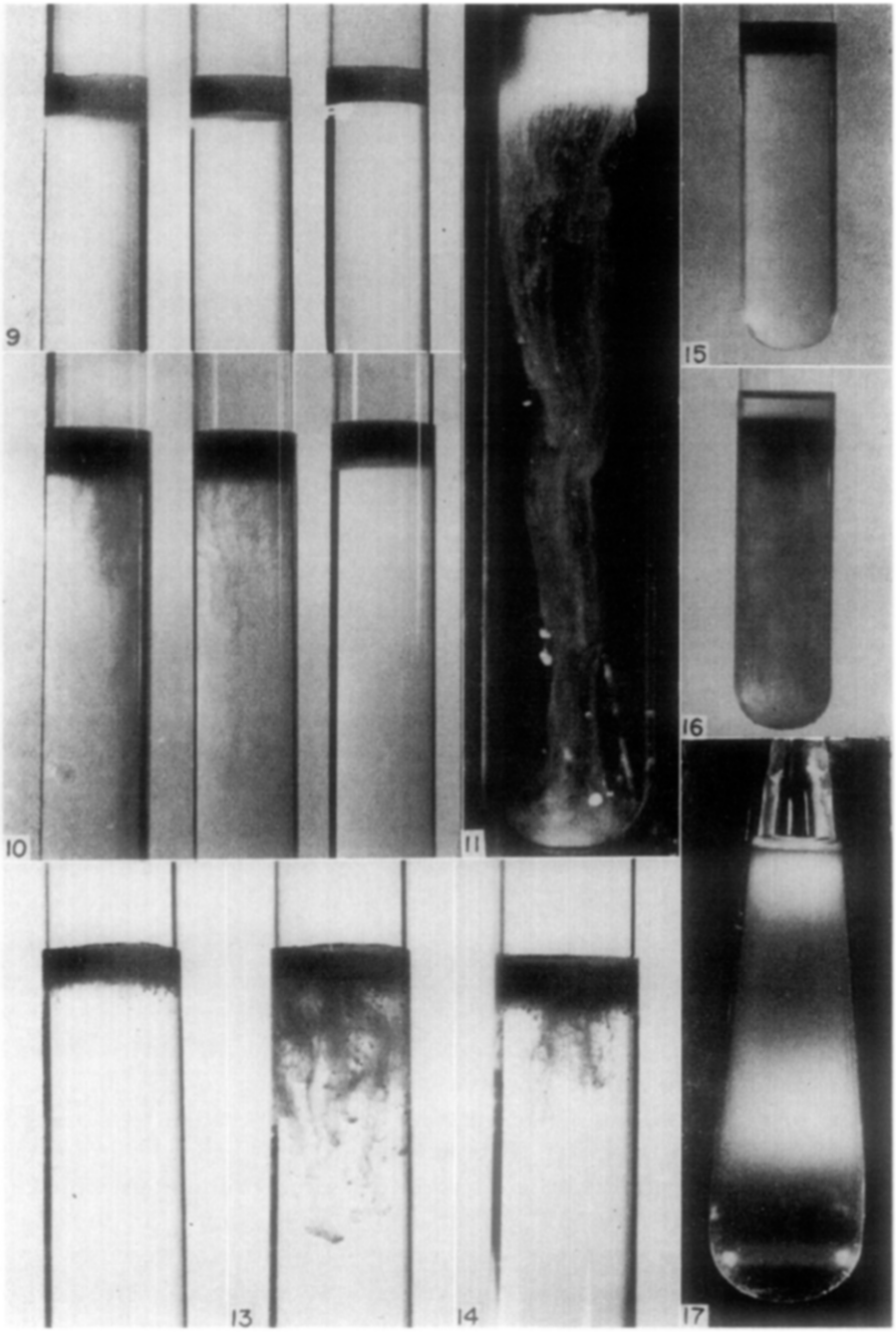


Fig. 8. Gradient sectioning device for use with Type *B* tubes. The gradient is raised by introducing a very dense solution to the bottom of the tube through the stainless steel needle shown.

is slowly raised. Samples of any desired volume up to 5 ml are obtained by sliding the upper lucite block across the lower one. The blocks are aligned by guide pins and are polished to allow visual inspection of the gradients. The entire arrangement fits on an ice bath in such a way that the sector tube is kept cold.

DISCUSSION

The technique described allows a brei to be fractionated into several discrete fractions (nuclei and whole cells, mitochondria, microsomes, supernatant or soluble phase, and lipids) in one step with the quantitative recovery of each fraction (Fig. 17). Data on the sedimentation rates of the various fractions together with photomicrographs will be presented in subsequent papers, as will also a revised method for nuclear isolation. No cross-contamination is observed except for a few particles in the soluble fraction which appear to be mitochondria with an associated lipid droplet. The method is applicable to a wide variety of problems and has already been applied to the isolation of blood platelets and the separation of *Neurospora* conidia according to size. It is now possible to consider studies concerned with quan-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titative changes in the relative proportions of the various fractions. The errors introduced by the presence of whole cells can be corrected at least partially by actual cell counts.

The anomalies occurring in an angle-head centrifuge and their prevention by sucrose gradients have been described by Pickels [12], and the low resolution of the horizontal swinging-tube centrifuge in the absence of a stabilizing gradient has been demonstrated by Kahler and Lloyd [10] who found that glycerol gradients would stabilize the system sufficiently to allow accurate sedimentation rates of polystyrene latex spheres to be obtained. Brakke [3, 4] adapted sucrose density gradients to the isolation of viruses, using a system in which the material to be fractionated was layered on top. He was well aware of anomalous behavior which occurs without a gradient. The gradients were produced by diffusion in a layered system. The author attempted the fractionation of liver breis on soluble starch gradients in 1949 (unpublished).

It should be noted that isopycnic gradient centrifugation for the stratification and separation of marine egg halves and quarters was first described by the Harveys [6, 7]. Gradients of nonaqueous solvents were extensively used by Behrens for the isolation nuclei [1]. Later, Thomson and Mikuta [14] utilized a discontinuous gradient in the separation of rat liver cytoplasmic fractions. Kuff and Schneider [11] have advocated the adaptation of the Brakke technique to the separation of cytoplasmic fractions. Unfortunately in adapting the method to mitochondria and Golgi bodies [13] discontinuous gradients were used and, "the interfaces were carefully maintained". The turnover effect accounts for most of the sedimentation under such conditions and for the accumulation of material above each interface. It is not clear whether the separation of Golgi bodies is due to differential or to isopycnic centrifugation.

Fig. 9. Ten per cent rat liver brei in Solution I layered over (left to right) Solutions II, III, and 0.88 *M* sucrose, containing the same salts used in the isolation medium.
Fig. 10. Same tubes 8 minutes later. No centrifuging.
Fig. 11. Twenty per cent rat liver brei 5 minutes after being layered over Solution III.
Fig. 12. Citrated rat blood 28 seconds after being layered over Solution II.
Fig. 13. Same as Fig. 12 72.5 seconds after layering.
Fig. 14 Citrated rat blood 4 minutes after being layered over 0.88 *M* sucrose.
Fig. 15. Citrated rat blood in 0.25 *M* sucrose (1:4) layered over 30 per cent sucrose.
Fig. 16. Same as Fig. 15 after 5 minutes centrifugation at 100 *g*.
Fig. 17. Separation achieved in continuous sucrose gradient. Nuclei and whole cells form band near bottom of tube, while mitochondria and microsomes are clearly separated above this. Note clear soluble layer above microsomes.

The results presented here suggest that the sedimentation observed in a horizontal tube centrifuge as ordinarily used is not a simple function of the sedimentation rates of individual particles. It does not appear profitable therefore to attempt to predict from Stokes' law what particles would sediment ideally [5] or to infer from apparent sedimentation rates the size of particles whose density is not accurately known [14].

The terminology applied to medium- and low-speed centrifugation is ambiguous and deserves comment. Particles may be isolated by sedimenting them to the bottom of a tube, by sedimenting them part way down through a stabilizing gradient, or by centrifuging them to levels of similar density in a density gradient. The methods in which particles are consecutively sedimented at increasing speeds or times has been termed differential centrifugation. In methods involving gradients, however, the term *density gradient centrifugation* has been used for two very different systems without distinguishing between the use of gradients for stabilization and the separation of particles by allowing them to reach density equilibrium [9]. The following classifications are therefore proposed:

(1) *Differential centrifugation.*—Fractions are collected consecutively at the bottom of the tube. The separation achieved depends on differences in sedimentation rate.

(2) *Gradient differential centrifugation.*—Particles are separated according to their sedimentation rate by centrifuging them through a density gradient which prevents convection currents and other anomalous effects. Fractions may be collected at the bottom, top, or along the gradient. For high resolution, the top layer would be made to approach the theoretically desirable condition of infinite thinness with infinite dilution.

(3) *Isopycnic centrifugation.*—Separation is achieved by suspending the particles in a solution having the same density as the fraction desired. Fractionation therefore depends on particle density. Centrifugation is continued until denser or lighter particles have been sedimented to the bottom or floated to the top.

(4) *Isopycnic gradient centrifugation.*—Particles are separated by sedimenting them through a density gradient until each species finds its isopycnic level and is recovered at that level. Centrifugation is continued until this equilibrium condition is established.

The term *equilibrium centrifugation* is not used for the last method since it has been previously applied to the method in which an equilibrium between sedimentation and diffusion is reached in the ultracentrifuge.

SUMMARY

A number of artefacts which prevent ideal sedimentation have been found during the centrifugation of rat liver breis. These include hydrodynamic effects which cause local areas containing a number of large particles to move as a unit dragging along associated smaller particles, turnover effects at density boundaries, convection currents due to heat and mechanical agitation, and wall effects. Both breis and suspensions of red cells have been used in these studies.

A separation approaching the ideal is obtained by the use of more dilute breis, careful layering over a continuous density gradient sufficiently steep to prevent the anomalies mentioned, and a rigid adherence to a gradual acceleration and deceleration schedule. Gradients of known characteristics are produced by a mechanical gradient engine.

A stroboscopic illumination system has been devised for studying the behavior of layered systems in a refrigerated centrifuge having an observation port. A speed-versus-time recording system allows integration of the centrifuging done. Speeds are accurately measured to 0.02 per cent.

New sector-shaped centrifuge tubes which minimize wall effects and a sliding device for recovering all levels of the gradient from the tube after centrifugation are described. Breis may now be quantitatively fractionated in one or two steps.

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