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Separation of Cell Components in the Zonal Ultracentrifuge

Abstract. Zonal separation of particles in density gradients contained in hollow or tubeless rotors has been explored with the aim of developing a preparative counterpart of the analytical ultracentrifuge. A rotor with a capacity of 1625 milliliters has been tested up to 22,500 revolutions per minute, with sucrose density gradients used. Excellent separation of subcellular particles has been achieved as well as partial separation of the albumin and globulin peaks of serum.

We wish to develop a true preparative counterpart of the analytical untracentrifuge which will achieve zonal-velocity or equilibrium separation in liquid density gradients of particles having different sedimentation rates or densities. In previous work (*1–3*) the amount of sample and the volume of the gradient have been severely limited by the size restrictions inherent in swinging-bucket rotors. Considerable time is required for density gradient production, and care must be exercised in loading and unloading tubes and in acceleration and deceleration. Further, the stepwise nature of most gradient recoveries limits the resolution attainable. Of these difficulties, the most serious is the limitation on sample and gradient size which has made density gradient centrifugation more of an analytical than a preparative procedure.

For the separation of different cell types and of cell particulates, a system is required in which a reproducible gradient of large volume can be quickly produced and in which centrifugation of a sample can be started a few minutes after sample preparation. To separate particles of molecular dimensions, it is necessary to attain speeds in the ultracentrifuge range. We report here the first results with a rotor and ancillary equipment designed to spin gradients of 1200- to 1500-ml volume at speeds up to 40,000 rev/min (92,000g at R_{max}), but used in these studies to only 22,500 rev/min.

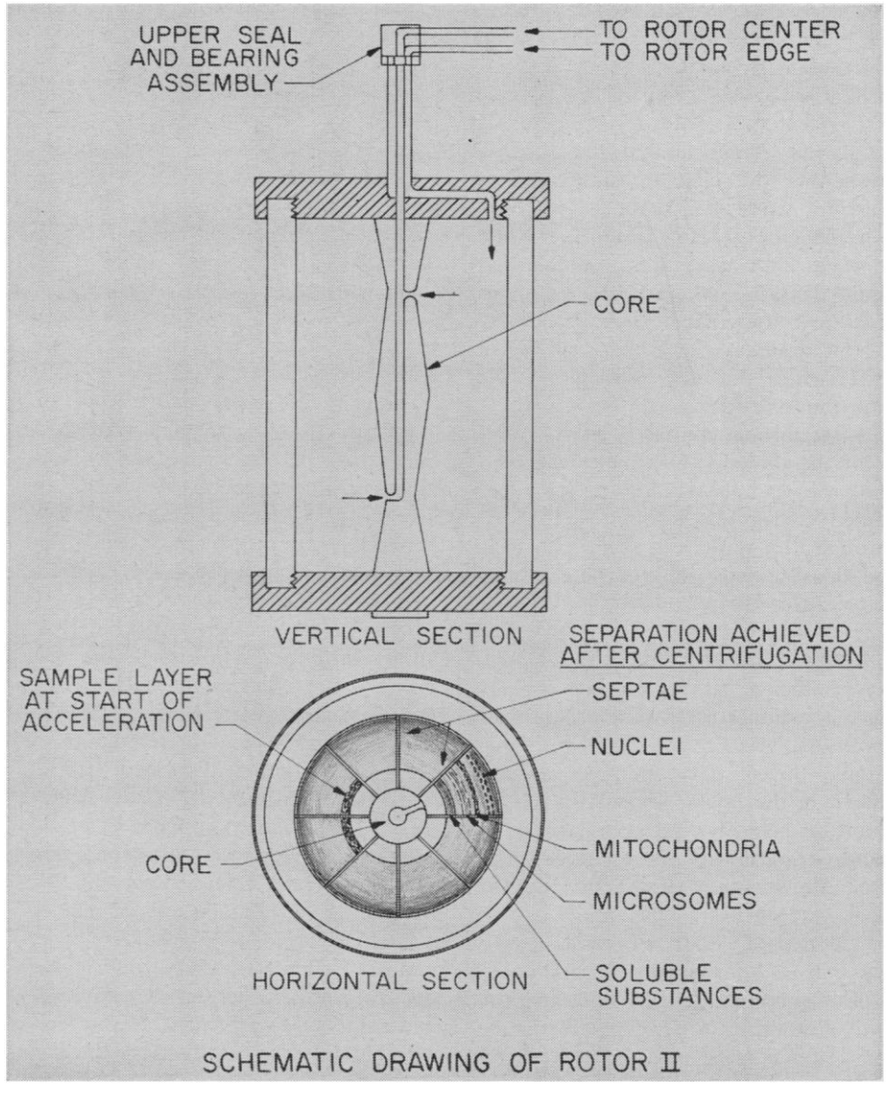

Fig. 1. Schematic drawing of density gradient ultracentrifuge rotor. Gradient is pumped into edge of rotor through upper seal and coaxial tubing. When the light end of the gradient reaches the center of the rotor and begins to flow out through upper seal, the direction of flow is reversed, and the sample gradient is backed into the rotor center. In the horizontal section, division of the rotor interior into sector-shaped compartments is shown. The sample layer is shown in two compartments (left) as it appears immediately after introduction into the rotor. The separation achieved after prolonged centrifugation is also shown (right). The gradient is recovered by pumping 66 percent (wt./wt.) sucrose to the edge of the rotor, displacing the gradient to the center and out through the upper seal assembly.

The basic principles employed in the present zonal ultracentrifuge (ZU-1), which are in many respects similar to those used in the gas ultracentrifuge for isotopic separations, have been developed with the use of four low-speed centrifuge systems (*2*, *4*).

With the background of experience thus gained, we proposed a zonal ultracentrifuge rotor (*5*) and associated systems for high-speed operation with the rotor spinning in a refrigerated, evacuated chamber. The design is shown schematically in Fig. 1. Two rotors have thus far been built. The results recorded here were obtained for rotor II which has a capacity of 1625 ml.

The rotor is hollow and contains an axial core. The core is slotted to hold a number of septa which divide the internal space vertically into compartments having the sector shape required for ideal sedimentation. At the two points along its length where the core is constricted, there are exit ports at the points of greatest constriction. The rotor is supported and driven from be-

low in a refrigerated, evacuated chamber. The upper shaft is composed of two coaxial tubes, which connect through a Teflon-tungsten carbide seal to the external fluid lines. This arrangement allows fluid to be pumped into either the edge or the center of the rotor while it is rotating. In operation the empty rotor is accelerated to between 2000 and 5000 rev/min. A liquid density gradient is then pumped into the rotor edge, with the light end of the gradient first. When the light end reaches the core it is constricted into the exit ports, and it flows up and out through the core line. At this point the direction of flow is reversed, and the sample to be separated is forced under pressure into the rotor through the core, displacing some of the dense peripheral fluid out of the rotor. After the sample is in the rotor, a fluid lighter than the sample is run into the rotor to move the sample layer sufficiently far out in the rotor to form a cylinder of material free of the core. As viewed from the top, the sample layer occupies the position shown in the lower left of Fig. 1. The rotor is then accelerated to the speed required to effect the separations desired, shown schematically in the lower right part of Fig. 1. The rotor is then decelerated to a speed at or below 5000 rev/min for unloading. If a dense fluid is now pumped to the rotor edge the entire gradient will be displaced toward the core. As each density zone approaches the core it is constricted vertically until it flows out the core exit ports and out through the upper seal assembly. It is evident that the gradient and the various separated zones may thus be recovered in order in a continuous stream.

In the experiments described here a 1200-ml gradient between 17 and 55 percent sucrose, which was linear with radius, was used. The remaining rotor volume peripheral to the gradient was filled with a "cushion" of 66 percent sucrose. The sample was made up in 8.5 percent sucrose and introduced to the center of the rotor as a 25-ml, linear gradient (*6*) between pure 17 percent sucrose and 8.5 percent sucrose plus sample except in the case of the rat liver experiment, where a 50-ml sample gradient was used. The small sample was necessary to avoid overloading the continuous recording systems (*7*) used to analyze the gradient at the end of the run.

To see whether the gradients could be introduced into the spinning rotor and be recovered essentially unchanged, a sucrose gradient was analyzed refractometrically before and after passage through the rotor. Experimentally a 1200-ml gradient could be introduced into the rotor at 2500 rev/min and recovered at the same speed with little change except for inward diffusion of the "cushion."

With this gradient system experiments were performed to determine whether a sample layer could be introduced and recovered without excessive broadening. A total of 12.5 ml of a 5-percent solution of bovine serum albumin was used in the 25-ml sample gradient. Theoretically, this sample zone should be 0.8 mm thick as positioned in the centrifuge. A recording of the recovered gradient's optical density showed that the zone had broadened slightly. The width at half height was 1.3 mm as calculated back to its position in the centrifuge. This and similar experiments with other protein solutions showed that the sample zone did not broaden excessively during movement into and out of the centrifuge.

In steep density gradients, such as those used here, some particles may approach their isopycnic position. This appears to be the case with red blood cells which band sharply at the 44.7 percent sucrose (wt./wt.) level.

The separation obtained with a brei (25 percent, wt./vol.) of perfused rat liver in 8.5 percent sucrose is shown in Fig. 2. The nuclei which are free of cytoplasmic contamination are in the zone marked "nuclei," whereas nuclei associated with cytoplasmic "tabs" or in whole cells appear just to the right of the level marked "cell fragments."

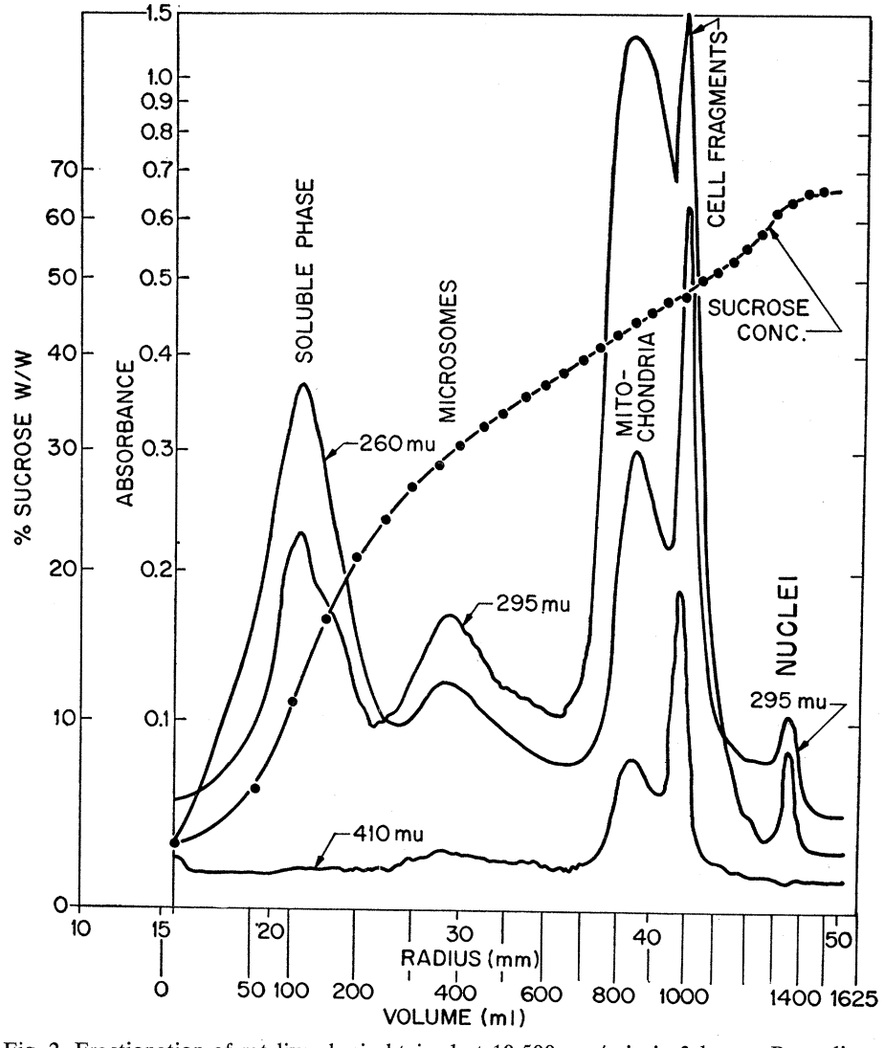

Fig. 2. Fractionation of rat liver brei obtained at 19,500 rev/min in 3 hours. Recordings at 2600 and 4100 A were made after dilution (in a continuously flowing system) with an alkaline buffer (*p*H 10). The recording at 2950 A was made directly (without dilution) on the gradient flowing out of the centrifuge and through a quartz flow cell.

After more prolonged centrifugation (4 hours at 20,000 rev/min), the nuclei are more sharply banded away from the "cell fragments" or membrane fraction. The results of enzymatic studies on these fractions will be reported elsewhere. While much theoretical and experimental work remains to be done before optimal fractionation conditions are achieved, these results suggest that quantitative separations of gram quantities of tissue can now be achieved.

In addition to liver cell fractions, monkey heart cells (*8*) have been fractionated in studies preliminary to attempts to isolate viruses directly from homogenates. Partial separation of the albumin and globulin peaks in rat serum was obtained in 9 hours, suggesting that molecular separations can be easily effected by this very gentle method when higher speeds become available.

It should be emphasized that rotor II used in these experiments was designed to study (i) rotor and gradient stability, (ii) performance of fluid-line seals which allow liquids to be pumped into and out of the centrifuge during rotation, (iii) the fractionation of cell populations into different cell types, (iv) the separation of subcellular organelles and viruses, and (v) the fractionation of protein and nucleic-acid mixtures on the basis of sedimentation rate. The rotor is necessarily of a compromise design that is not ideally suited to doing any single one of the above. These results therefore do not demonstrate the resolution ultimately attainable.

It appears that zonal or density gradient centrifugation in hollow rotors may prove to be a general separation method for particles ranging from whole cells, cell particulates, and viruses to small proteins. The theoretical aspects of large, tubeless-rotor density gradient centrifugation will be discussed in detail elsewhere.

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Positive and Negative Reinforcement from Intracranial Stimulation of a Teleost

Abstract. Tests in which an animal receives or avoids direct electrical stimulation of its brain according to its position in the tank as well as tests of free operant behavior demonstrate the existence of both positively and negatively rewarding areas in the brain of the goldfish.

Since Delgado *et al.* first obtained negative reinforcement by stimulation of certain areas in the brain of the cat (*1*) and Olds and Milner showed that stimulation of certain areas of the brain of the rat is positively reinforcing (*2*), similar areas have been found in the brains of several mammalian species. Areas in which stimulation is positively reinforcing have been found in cat (*3*), dog (*4*), and primate (*5*), and areas in which stimulation induces aversion have been found in rat (*6*) and primate (*7*). If these areas are related to the normally functioning reward systems of animals, they would be expected to exist in any species with a well-developed nervous system. An attempt was made to demonstrate that such areas exist in the brain of the goldfish, *Carassius auratus*, a more primitive animal than those previously studied.

Monopolar electrodes, about 10 inches of 10-mil Formvar wire exposed only at the cut end, were implanted in the brains of goldfish anesthetized with tricaine methane sulfonate (1:10,000). The electrode, in a stereotaxic instrument, was lowered into the brain through a hole made by a dental drill and fixed to the head with dental cement and small anchoring wires. The stereotaxic instrument served only to regulate the depth at which the electrode was implanted; the placement in all coordinates was variable and not reproducible. The electrode was attached by a brass swivel to a suspending wire centered over the fish tank. Stimuli of 0.5-second pulses of 60-cycle current were delivered between the implanted electrode and an indifferent electrode in the fish tank.

After testing, 2 ma of direct current were passed through the electrode for 15 seconds, the fish was killed by decapitation, and the head was fixed in formalin containing ferrocyanide to stain the iron deposit. Frozen brain sections (40 to 140 μ) were photographed by the method of Guzman *et al.* (*8*) to record the electrode placement.

Each fish was subjected to two test procedures. The first procedure was a series of side preference tests at currents between 5 and 150 μa. Each test consisted of ten consecutive 5-minute intervals at a set current intensity. During each interval, the animal received a stimulus every 1 to 2 seconds when it was on one side of the tank, and no stimulus when it was on the other side. The side associated with the stimulus was reversed between each of the intervals, and the presence or absence of a light gave the fish a cue as to which side was associated with the stimulus

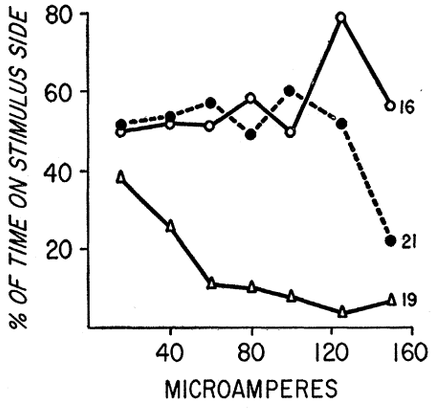

Fig. 1. The performances of fish 16, 19, and 21 in side preference tests (see text) at various current intensities. A percentage above 75 indicates positive reinforcement, below 25, negative reinforcement.

Fig. 2. Sagittal section of the goldfish brain 1 mm from the midline, showing the location of the tips of the electrodes in fish 16, 19, and 21.