ANALYTICAL BIOCHEMISTRY 30, 230–248 (1969)

Analytical Techniques for Cell Fractions

XIII. Rotor A-XVI, A Plastic Gradient-Reorienting Rotor for Isolating Nuclei[1]

L. H. ELROD, L. C. PATRICK, AND NORMAN G. ANDERSON

Molecular Anatomy Program, Oak Ridge National Laboratory,[2] and Oak Ridge Gaseous Diffusion Plant,[2] Oak Ridge, Tennessee 37830

Received January 28, 1969

Zonal centrifugation in large, hollow rotors has been developed as a general method for separating particles ranging in size from whole cells to protein molecules (1–3). Although most zonal rotors developed thus far are loaded and unloaded dynamically (i.e., during rotation) (1, 3), it is advantageous for many purposes to be able to load and unload them at rest (4). This is the case for large, low-speed rotors for whole-cell separation and for isolation of nuclei, when expensive and often very viscous gradient materials are used, and also for very high-speed rotors, with which fluid line seals cannot be used at top speed.

The general operating principles of gradient-reorienting rotors were worked out by using the small plastic A-VII rotor (4). The method was also applied to high-speed C-series rotors (5) and to the B-III, an experimental aluminum rotor (6, 7). The latter was only moderately successful and did not yield reproducible results. In this paper, the problem of applying the gradient-reorienting principle to a liter-size rotor has been re-examined and applied to the problem of isolating nuclei from rat liver.

ROTOR PERFORMANCE

The results obtained with the A-VII suggested that disturbances may occur in the gradient due to uneven acceleration and deceleration and to rotor instability. Thus, a simple plastic model rotor was constructed in which reorientation could be easily observed. A lower water phase and a red-dyed upper oil phase were used so that disturbances at only a single

[1] Research conducted under the joint NIH-AEC Molecular Anatomy (MAN) Program supported by the National Cancer Institute, the National Institute of General Medical Sciences, the National Institute of Allergy and Infectious Diseases, and the U. S. Atomic Energy Commission.

[2] Operated by Union Carbide Corporation Nuclear Division for the U. S. Atomic Energy Commission.

interface could be followed. The rotor was mounted on an International clinical centrifuge and observed visually. Large disturbances in the form of waves and interface surface were seen even during the most careful acceleration under these conditions, as shown in Figure 1b. It was con-

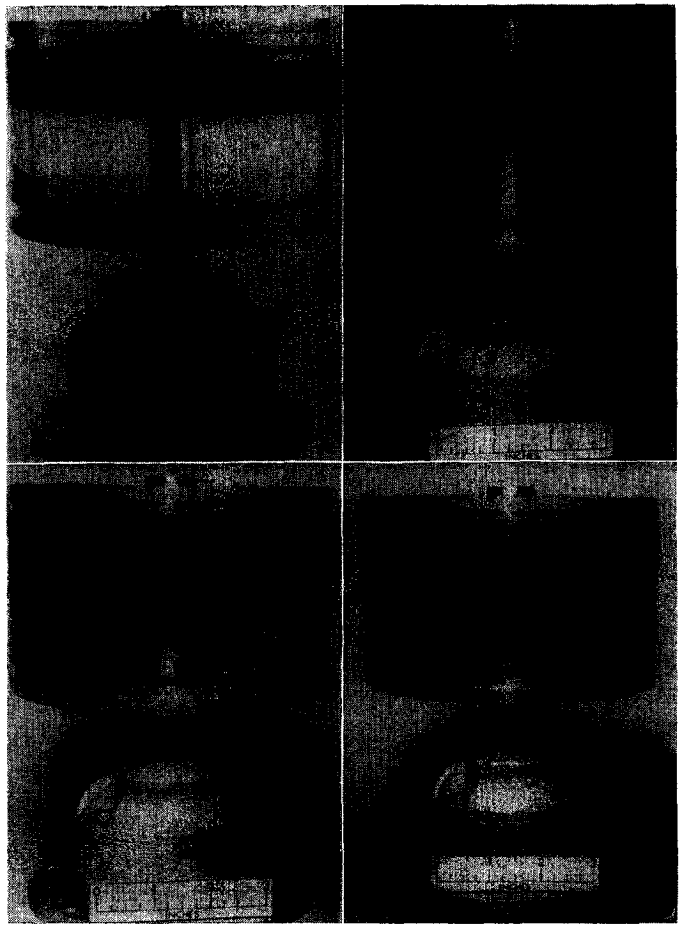

FIG. 1. Model rotor containing two-phase gradient (note disturbances at interface of liquids in B): (A) rotor at rest; (B, C) rotor during acceleration; (D) rotor at speed.

cluded that considerable attention must be directed toward the problem of even and gradual acceleration and deceleration and to the problem of the optimal number of septa.

A-XVI Design. To examine the problem of gradient behavior, a plastic rotor was constructed of polycarbonate, as shown in Figures 2 and 3. The rotor was spun in a very heavy flywheel holder mounted in an Inter-

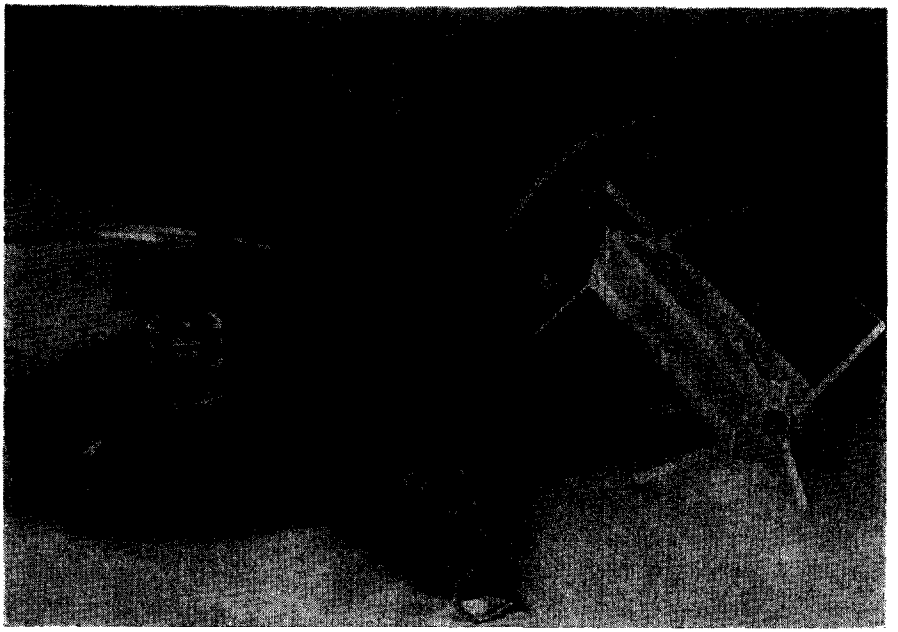

FIG. 2. Side view of disassembled A-XVI rotor.

national Equipment Company PR-2 centrifuge, as shown in Figure 4. The core, which initially was designed for four septa, allowed the gradient to be removed either by suction from the bottom or by displacement through the top. The physical characteristics of the rotor are shown in Table 1. The rotor tapers slightly to match the tapered brass cup on the flywheel.

Gradients. Special care must be taken in gradient-reorienting centrifugation to prevent convection between the sample layer and the overlay. This is best done by using a short density gradient for the overlay. To avoid making a separate density gradient for the overlay, one long gradient may be prepared, and part of it discarded to give a density shelf on which the sample layer may rest.

By using a 285 ml gradient pump as previously described (8), a 0–60% sucrose (w/w) gradient was prepared. The overlay was considered to be the first 50 ml of the gradient delivered by the pump (from 0 to about 6.0% sucrose w/w). After this volume had been delivered by the pump to a small-bore cylindrical reservoir, an additional 35 ml (6.0 to 10% sucrose w/w) was pumped to waste. The pump was then connected to the rotor line, and the rotor was completely filled with the gradient that was now 10–60% sucrose w/w. The sample volume (usually 20 ml), having a density equivalent to 8.5% sucrose w/w, was then introduced on top

TABLE 1
Physical Characteristics of Plastic Rotor A-XVI

Rotor	
External dimensions:	
Height	14.29 cm
Maximum diameter	12.70 cm
Minimum diameter	12.07 cm
Internal dimensions:	
Height at center	13.09 cm
Wall height (edge)	11.47 cm
Diameter at top	9.52 cm
Diameter at bottom	9.53 cm
Weight, empty	1,474 gm
Volume with no core	816 ml
Volume with four-vaned core	672 ml
Flywheel Holder	
Maximum diameter	40.64 cm
Weight	25.43 kg

TABLE 2
Acceleration and Deceleration Schedules Used in PR-2 Centrifuge

Schedule	Rheostat setting	Rheostat advanced when indicated speed is reached
A-1	5	50 rpm
	6	100 rpm
	7	200 rpm
	9	300 rpm
	11	500 rpm
	15	900 rpm
	20	1400 rpm
	25	2000 rpm
	30	3000 rpm
A-2	10	500 rpm
	20	2000 rpm
	30	3000 rpm
A-3	2.5	Driven by a servomotor at constant rate until rotor reaches 3000 rpm, then rheostat turned back to 30 for maintenance power setting.
D-1	0	Coast to stop.
D-2	3	Coast to stop.

of the gradient. The sample was followed by the overlay that had been stored in the reservoir. The introduction of sample and overlay displaces an equal volume of the heavy end of the gradient to waste (Fig. 5).

The rotor was then either pumped out from the bottom at once or accelerated to various speeds by using the different schedules listed (Table 2). The rotor and all gradient materials were kept at 5°C.

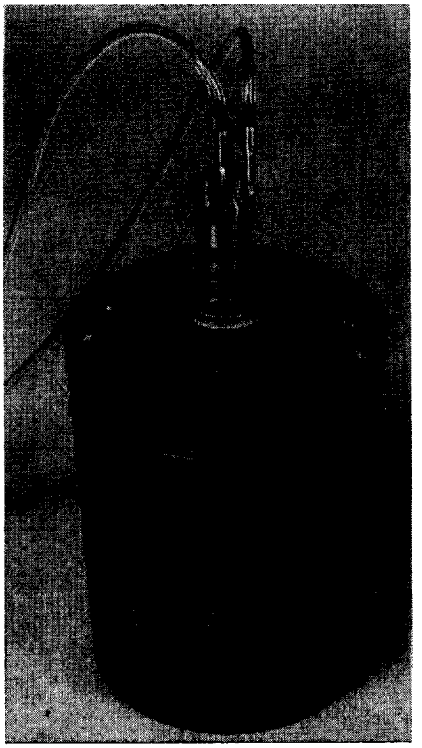

FIG. 3. Assembled A-XVI rotor with line seal in place.

Effect of Acceleration and Deceleration. Acceleration and deceleration schedules were used in the PR-2 centrifuge as shown in Table 2. A strip with 72, 36, and 18 squares was attached to the flywheel cup, as shown in Figure 4. With a stroboscope set on 3600 cycles per minute, these squares appeared stationary at 50, 100, and 200 rpm increments, respectively. The acceleration and deceleration curves could be plotted from data obtained by recording the time at which different speeds were reached during acceleration and deceleration. These schedules and curves serve for a variety of speeds since acceleration can be stopped at the instant that the required speed is reached, and deceleration from any

speed immediately conforms to the portion of the deceleration curve remaining.

Acceleration and deceleration curves obtained with the schedules listed are shown in Figure 6. The averages of four experiments are shown.

The problem of mixing during acceleration and deceleration was at least partially overcome by placing the plastic A-XVI rotor in the mas-

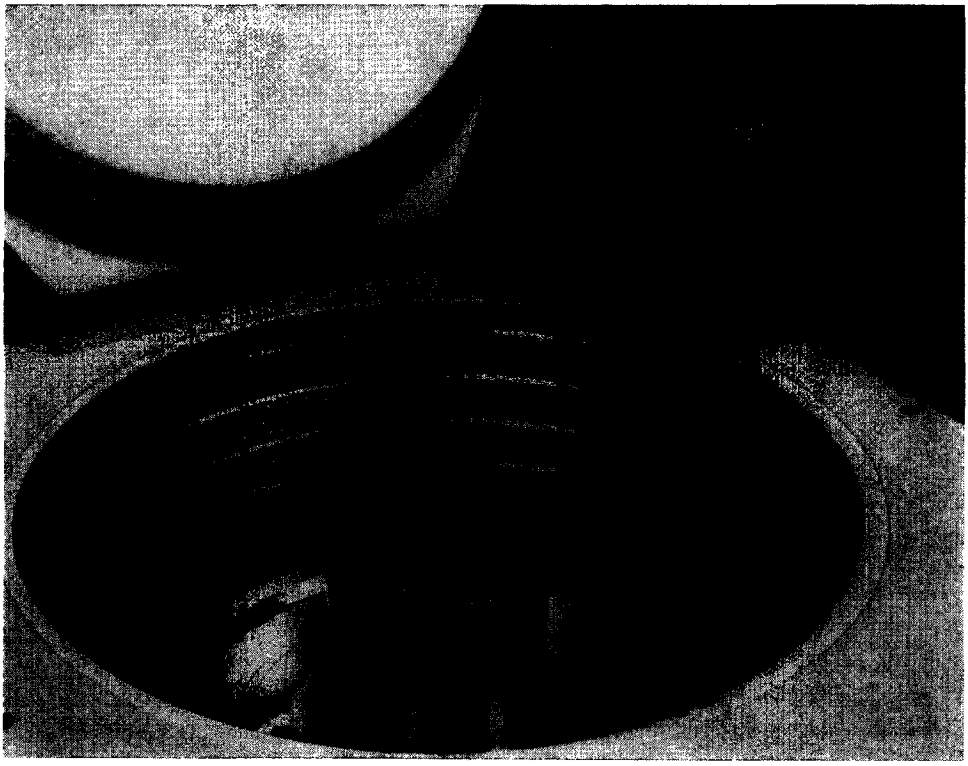

FIG. 4. Rotor and flywheel in place in the centrifuge.

sive flywheel (Fig. 4). The mass provides inertia during acceleration and momentum during deceleration that tends to smooth out surges in the drive system and to allow the rotor to drift slowly to stationary. Figure 7 shows sucrose gradients that were accelerated according to the three different schedules and all decelerated according to the same schedule. A comparison of the three gradients indicates that the rate of acceleration is not an important contributor to the mixing that occurs during reorientation of gradients.

The most critical area for careful control is between rest and 800 rpm.

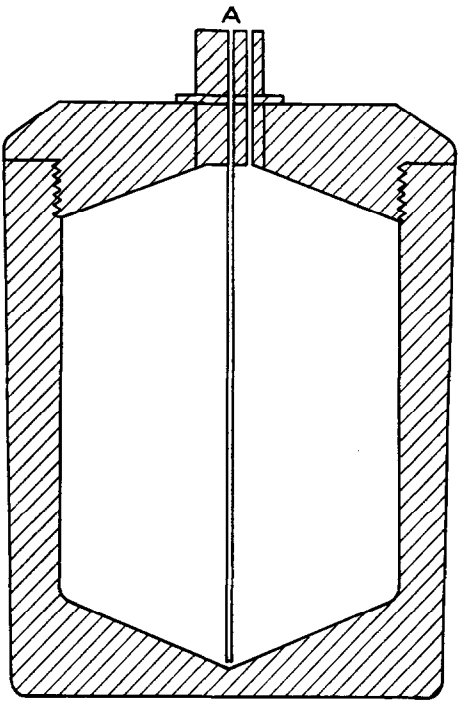

FIG. 5. Schematic representation of loading and unloading sequence in A-XVI rotor (separation of bands from a particulate sample and reorientation of gradient are illustrated): (A) empty rotor; (B) gradient in place; (C) sample in place; (D) overlay in place; (E) reorientation during acceleration; (F) rotor at speed and (G) at rest after deceleration; and (H) unloading.

When the rotor has attained a speed of 800 rpm, the gradient has reoriented sufficiently to be stable during rapid acceleration.

The top speed of the present system is 5000 rpm. Higher-speed versions appear to be necessary for the isopycnic banding of intermediate-density cellular components. Such systems with less massive flywheels and with improved drive and speed control systems are under development.

Effect of Acceleration Schedule on Gradient and on Sample Bands. For experimental studies, a 20 ml sample consisting of bromophenol-dyed bovine serum albumin (BSA), 6.5 mg/ml, and alcohol-extracted ragweed pollen, 5.44 mg/ml, was used. The sample was made to 8.5% w/w sucrose. The ragweed pollen was used to provide a band deep in the gradient (45 w/w % sucrose; see Figure 8). Figure 8 shows the absorbance profile and per cent sucrose at which the bands were recovered after being spun at 3000 rpm for 15 min. When width at half-height of the peaks is compared with sample volume, the BSA shows a spread to about 1.9 times

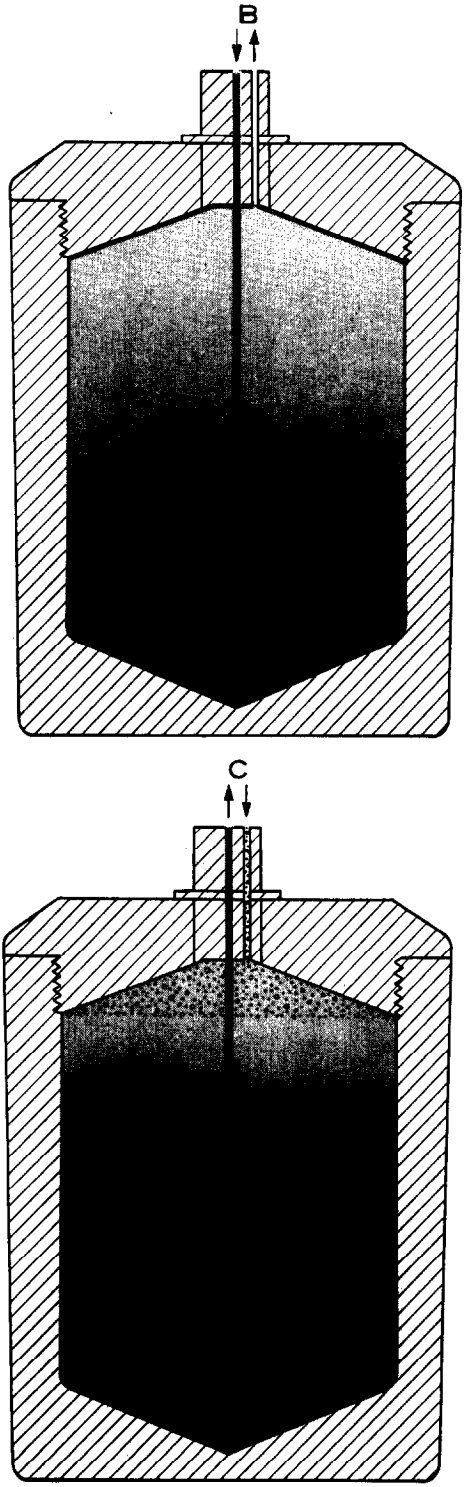

FIG. 5. (*Continued*)

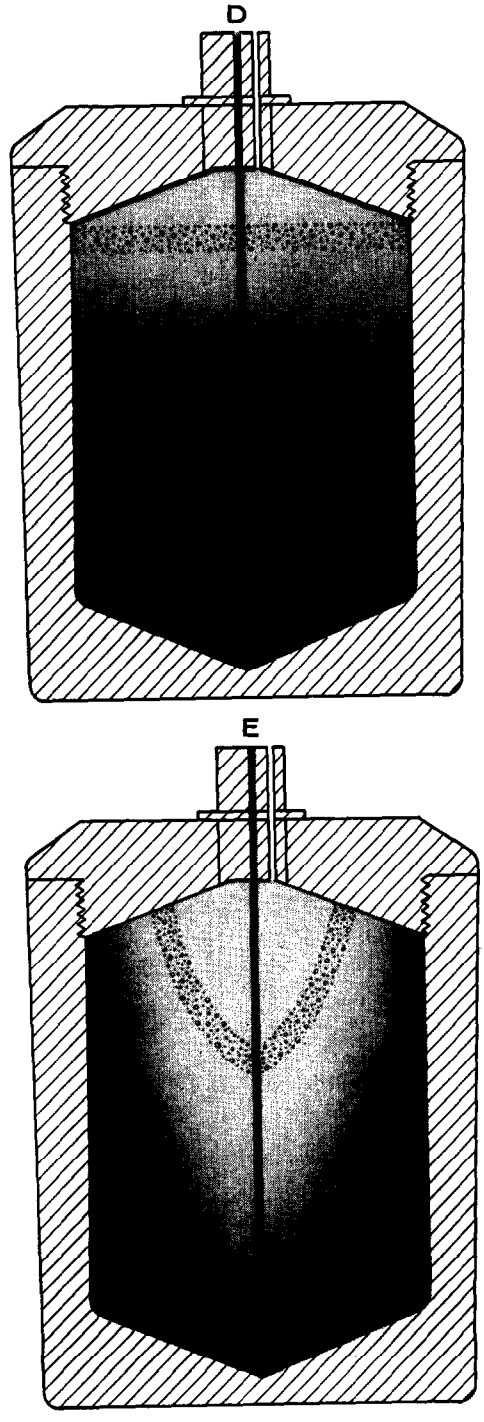

FIG. 5. (*Continued*)

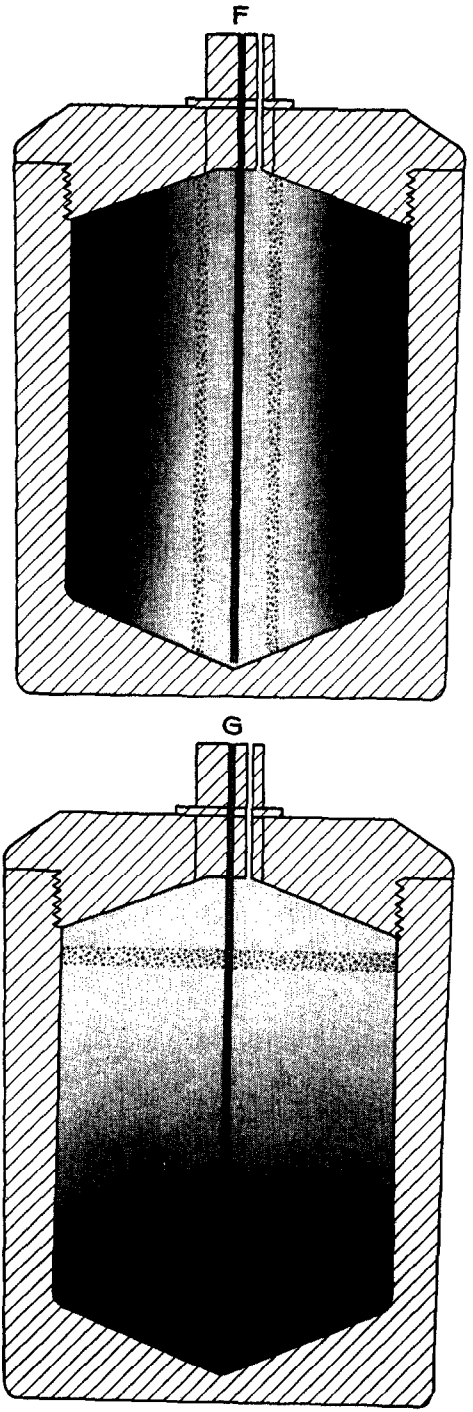

FIG. 5. (*Continued*)

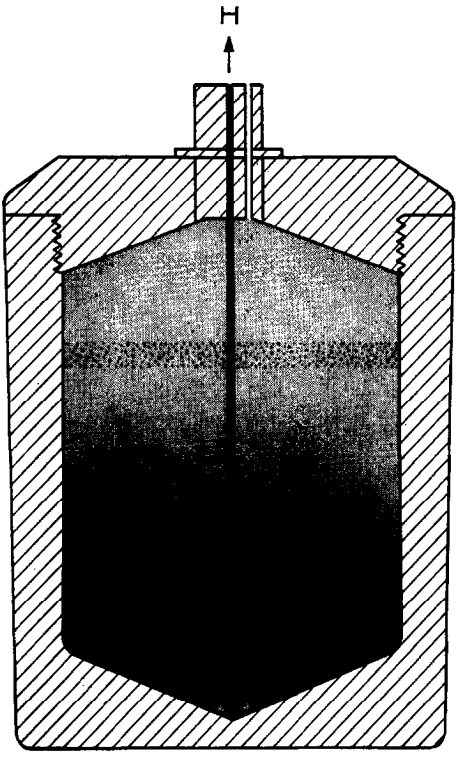

FIG. 5. (*Continued*)

the original volume and the pollen spread to 2.75 times the original volume. Our experience has shown that the pollen band would have been sharper had the run been longer, but 15 min at 3000 rpm had been selected as a standard condition.

Experiments to show the effects of acceleration on the sample band were performed. The rotor was loaded with a gradient (as described under *Gradients* above) and with a sample of BSA. The rotor was unloaded after setting statically for a 15 min period. These studies were compared with additional studies in which the loaded rotor was accelerated to 3000 rpm for 15 min.

The results, based on width of the peak at half-height compared with sample volume, show that the BSA sample that had been spun at 3000 rpm spread to 1.9 times the original volume, whereas the sample that remained static spread to 1.6 times the original volume. These results show that, although a density gradient is used for the overlay, there is some mixing between the sample and the overlay.

To test the value of a density gradient overlay, additional studies were

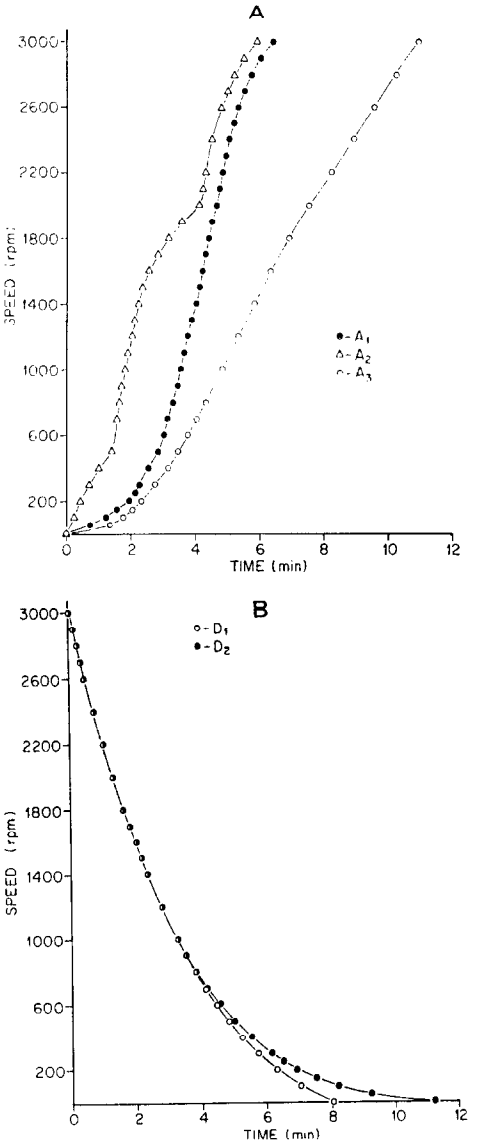

FIG. 6. Curves showing rates of acceleration (A) and deceleration (B) according to schedules in Table 2.

performed by using samples and gradients that were duplicates of those above except that the gradient overlay was replaced by an equal volume of distilled water. The peak width at half-height showed a spread to 3.0 times the original volume. Thus the gradient overlay markedly decreased mixing between the sample and overlay.

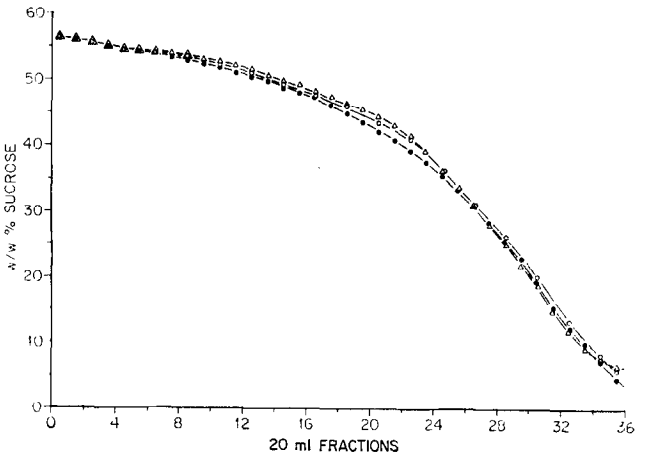

FIG. 7. Comparison of three gradients accelerated to 3000 rpm according to different schedules (A-1 to A-3) and decelerated according to schedule D-2: (○) A-1; (△) A-2; and (●) A-3.

Internal Configuration. Shear stress (9) and Coriolis forces (10) are two factors that tend to degrade gradients during reorientation. The following experiments were performed to determine the optimum number of septa needed to maintain the integrity of reoriented sucrose gradients in this system.

Gradients were duplicated as nearly as possible by using the same pump, pump lines, and solutions. A gradient was loaded into the rotor

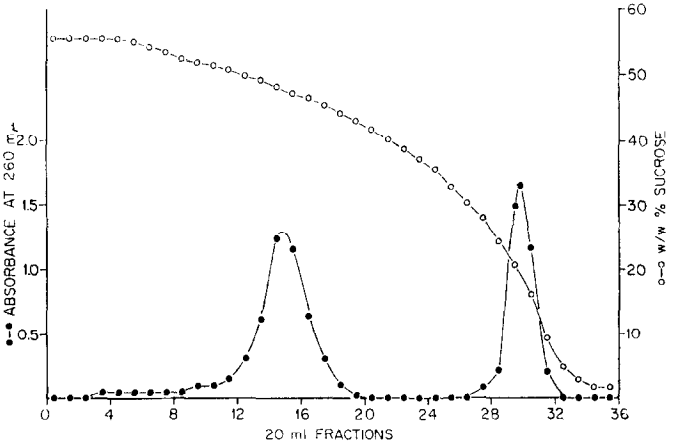

FIG. 8. Absorbance profile of separation of bovine serum albumin and ragweed pollen.

with four septa in place. The rotor was accelerated to 3000 rpm according to Schedule A-3, and then decelerated according to Schedule D-2. When the rotor came to rest, it was again accelerated to 3000 rpm. This was repeated until the gradient had gone through four complete reorientations. The gradient was pumped out and the w/w % sucrose of each 20 ml fraction was determined refractometrically.

This procedure was repeated by using two septa and no septa. Finally, a gradient was pumped into and out of the rotor without spinning it for comparison purposes. The results are shown in Figure 9.

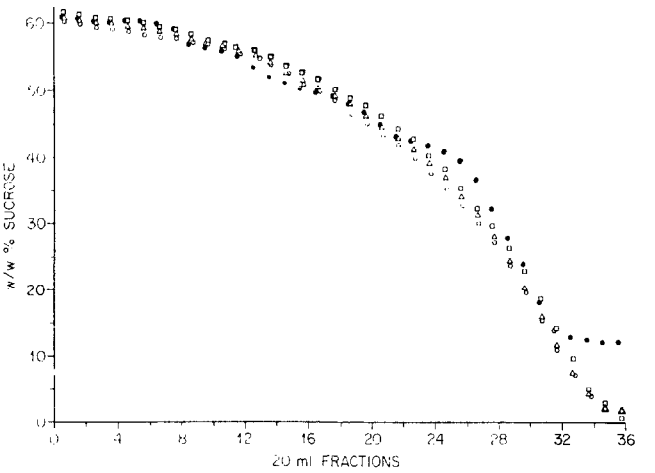

FIG. 9. Gradients reoriented four times each with no septa (●), two septa (○), and four septa (△) compared with a gradient that was not reoriented (□).

These experiments show that sucrose gradients can be reoriented repeatedly without gross detrimental effects if the conditions are properly controlled. It is also indicated that, although two septa seem necessary to preserve the shape of gradients, there is no significant improvement by using more than two septa. These observations may be valid only for the system used here. Factors such as size of the rotor, characteristics of gradient materials, and ratio of height to width of the rotor may contribute toward gradient preservation as well as the number of septa.

Isolation of Nuclei. Nuclei are usually isolated by pelleting them through a layer (or layers) of sucrose that is dense enough (2.2 *M*, 1.28 gm/cc) to prevent the pelleting of other cellular components (11–15). There are at least two disadvantages to this method: (*1*) the nuclei may be aggregated at the bottom of the container; and (*2*) when large viscosity or density steps are included in the path of sedimenting heterogeneous particles, particulate matter may build up at the interface and

interfere with normal sedimentation. Nuclei, for example, may be trapped in a band of light debris and not move through the gradient.

Nuclei from various tissues have been isolated by using sucrose gradients, both continuous and discontinuous. The usual procedure is to layer the sample (16–25) on top of the gradient and then exert enough force to move the nuclei to a density in the gradient that is equal to the density of the nuclei (i.e., isopycnic).

Of the large formed elements of cells, nuclei exhibit the highest isopycnic density. In sucrose solutions they have been shown to band at densities between 1.344 and 1.361 gm/cc (20). The sucrose solutions used to attain these densities have a very high viscosity at low temperatures and are quite difficult to manage. Part of the viscosity problem can be overcome by using heavy water as the solvent for the solutions (20). In the studies previously reported, a B-XV rotor was modified by enlarging the fluid lines in the spindle and core to allow 64% w/v, 1.368 gm/cc sucrose in deuterium oxide to be pumped into and out of the rotor. Because the B-XV must be loaded and unloaded dynamically, either 1.66 liters of the heavy deuterium oxide sucrose solution or an equal volume of another equally dense fluid is required to unload the rotor. When the A-XVI rotor is used for the isopycnic banding of nuclei, a much smaller amount of the deuterium oxide solution is required; and, if the gradient is pumped out from the bottom, no pushout material is required.

Nuclei have been successfully isolated isopycnically from rat liver homogenates in the A-XVI by using water and sucrose gradients 0–60% w/w and a 64% w/v sucrose-deuterium oxide cushion. The nuclei appeared to be in good condition (i.e., intact, nongranular, and unclumped), but some cellular debris had been trapped along with the nuclei at the density step between the 60% w/w sucrose and the cushion (Fig. 10A).

As was elegantly shown by Johnston *et al.* (26), it is possible to isolate nuclei from homogenates on a sedimentation-rate basis, that is, by unloading before the nuclei have reached their isopycnic point. In the A-XVI system, this means bringing the rotor to rest.

A 10% w/v homogenate of fresh, unperfused rat liver was prepared in cold 8.5% sucrose, 1.5×10^{-3} *M* $CaCl_2$, and made to pH 7.5 with Tris acetate. (The Tris acetate stock was made by adjusting the pH of a 0.5 *M* solution of Tris base to pH 8.5 with glacial acetic acid.) The homogenate was given 50 strokes by hand in a Potter-Elvehjem homogenizer with a plastic pestle, then strained through four layers of cheesecloth.

The rotor was loaded with a gradient and a 20 ml sample as described earlier under *Gradients*. All gradient materials contained 5×10^{-4} *M* $CaCl_2$ and were made to pH 7.5 with Tris acetate. The rotor was accelerated to 3000 rpm according to Schedule A-3 and that speed was

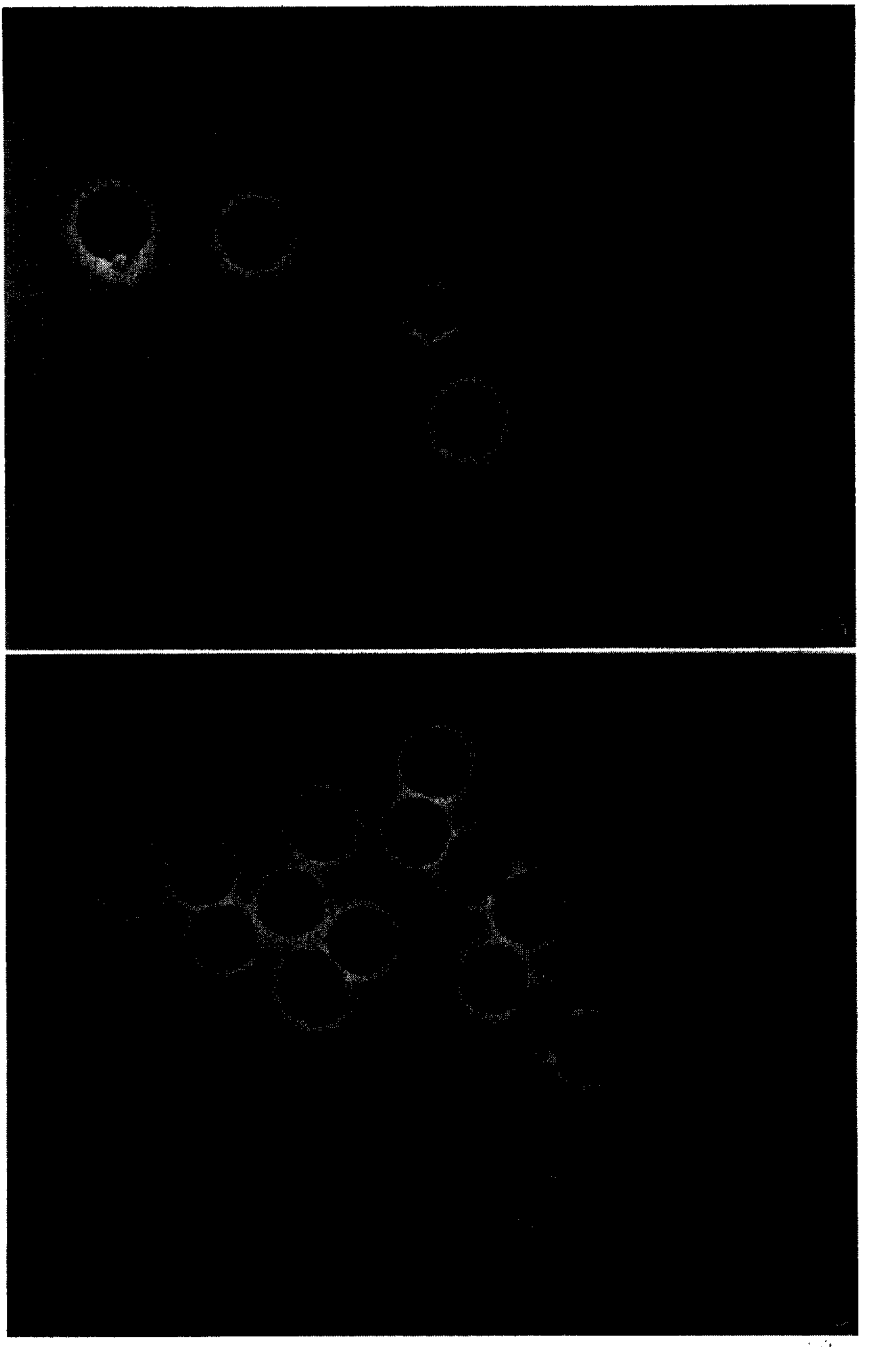

FIG. 10. Nuclei isolated by (A) isopycnic banding and (B) on sedimentation-rate basis.

maintained for 15 min. The rotor was then decelerated according to Schedule D-2. The gradient was pumped from the bottom of the rotor with a Sigmamotor peristaltic pump. The nuclei were recovered at about 55% w/w sucrose and were much cleaner than those isolated by isopycnic banding (Fig. 10B).

The effluent stream from the rotor was monitored at 260 mμ by a Gilford modified Beckman DU spectrophotometer equipped with a 0.2 cm flow cell. The effluent was collected in 20 ml fractions and the w/w % sucrose determined refractometrically.

Portions of each fraction were taken for total protein and DNA analysis. Total protein was determined by a modification of the Lowry method (27). A 10 ml sample of each fraction was diluted to 20 ml, and then spun 1 hr at 2000 rpm to pellet the cellular material from the sucrose. After a Schneider extraction (28), the DNA in each pellet was determined by the method described by Burton (29). Figure 11 shows the absorption

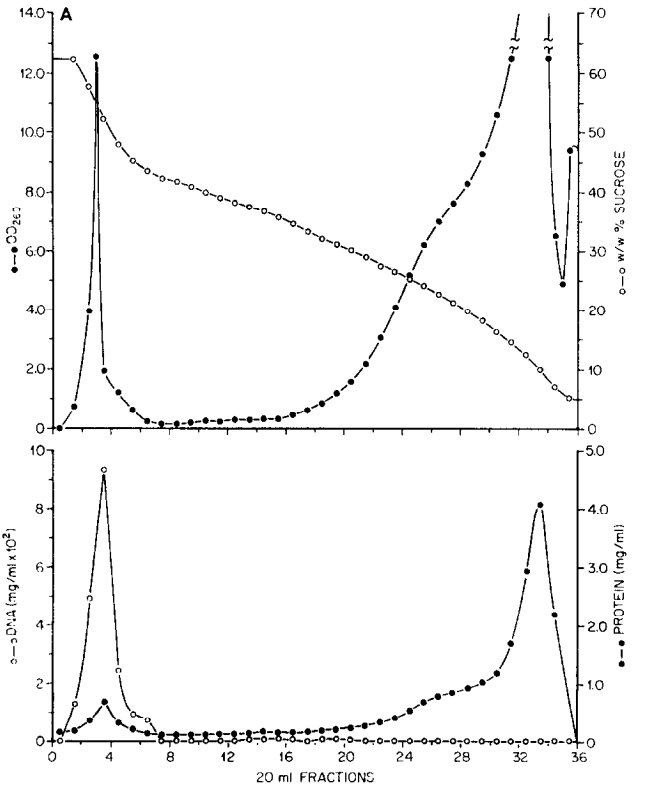

FIG. 11. Absorbance profile of (A) nuclear isolation from rat liver homogenate and (B) of DNA and total protein from the separation shown in A.

at 260 mμ of the effluent stream from the rotor and the protein and DNA concentrations of the fractions.

DISCUSSION

The A-XVI rotor provides a means of making centrifugal separations of whole cells and the larger subcellular components on liter-sized gradients. The simple construction and the fact that it is loaded and unloaded statically contribute to the ease of operation. Because the rotor is at rest, the need for dynamic fluid line seals is eliminated and it is possible to pump the rotor contents out directly from the bottom of the rotor or to displace the contents by pumping a dense solution to the bottom of the rotor.

Sucrose gradients may be reoriented without serious degradation with proper control of acceleration and deceleration in the A-XVI rotor. The smoothness of acceleration is probably more important than the rate of acceleration, particularly at speeds below 800 rpm. At higher speeds, reorientation of the gradient is complete and the gradient is relatively stable. It appears that in the A-XVI rotor two septa are necessary to prevent appreciable destruction of the gradient shape by shear stress and Coriolis forces, but more than two septa show little improvement.

Nuclei from rat liver were successfully isolated in the A-XVI on a sedimentation-rate basis. The nuclei were recovered at a density of about 1.26 gm/cc or 55% w/w sucrose, whereas isopycnic banding would require a density of about 1.34 gm/cc or 68% w/w sucrose. Nuclei isolated by this method appear to be cleaner than those isolated isopycnically and the method has the advantage of being of short duration. Thus, the nuclei may spend a shorter time exposed to hypertonic sucrose solutions before being recovered.

Future work with this system will involve the isolation of smaller cell components; and, in some cases, isopycnic rather than sedimentation-rate separations may be desirable. A higher-speed version with a less massive flywheel and a more carefully controlled drive system are presently under development.

SUMMARY

The A-XVI rotor is a simply constructed and easily operated device for making centrifugal separation of whole cells and the larger formed subcellular particles from homogenates. Nuclei were successfully isolated on a sedimentation-rate basis.

Studies were made on the effects of reorientation on sucrose gradients. It was shown that, with proper control of acceleration, deceleration, and

internal configuration, sucrose gradients can be reoriented without serious degradation.

REFERENCES

1. ANDERSON, N. G., *in* "The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis" (N. G. Anderson, ed.), *J. Natl. Cancer Inst. Monograph* **21,** 9 (1966).
2. ANDERSON, N. G., *Science* **154,** 103 (1966).
3. ANDERSON, N. G., WATERS, D. A., FISHER, W. D., CLINE, G. B., NUNLEY, C. E., ELROD, L. H., AND RANKIN, C. T., JR., *Anal. Biochem.* **21,** 235 (1967).
4. ANDERSON, N. G., PRICE, C. A., FISHER, W. D., CANNING, R. E., AND BURGER, C. L., *Anal. Biochem.* **7,** 1 (1964).
5. ANDERSON, N. G., AND BARRINGER, H. P., *The Joint NIH-AEC Zonal Centrifuge Development Program Semiannual Report,* ORNL-3415 Special, p. 51 (1962).
6. ANDERSON, N. G., CANNING, R. E., AND RANKIN, C. T., JR., *The Joint NIH-AEC Zonal Centrifuge Development Program Semiannual Report,* ORNL-3415 Special, p. 49 (1962).
7. PRICE, C. A., AND BOSTIAN, G., *The Joint NIH-AEC Zonal Centrifuge Development Program Semiannual Report,* ORNL-3656 Special, p. 15 (1963).
8. ANDERSON, N. G., AND RUTENBERG, E., *Anal. Biochem.* **21,** 259 (1967).
9. HSU, H. W., *Biophys. J.* **9,** 173 (1969).
10. BERMAN, A. S., *in* "The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis" (N. G. Anderson, ed.), *J. Natl. Cancer Inst. Monograph* **21,** 41 (1966).
11. BLOBEL, G., AND POTTER, VAN R., *Science* **154,** 1662 (1966).
12. GRIGGS, H. M., AND DIJKSTRA, J., *Brit. J. Cancer* **21,** 198 (1967).
13. MAGGIO, R., SIEKEVITZ, P., AND PALADE, G. E., *J. Cell Biol.* **18,** 267 (1963).
14. GADOWSKI, P. D., AND STEINER, J. W., *J. Cell Biol.* **37,** 147 (1968).
15. WILBUR, K. M., AND ANDERSON, N. G., *Exptl. Cell Res.* **2,** 47 (1951).
16. ALBRECHT, C. F., *Exptl. Cell Res.* **49,** 373 (1968).
17. ALBRIGHT, J. F., AND ANDERSON, N. G., *Exptl. Cell Res.* **15,** 271 (1958).
18. ANDERSON, N. G., *Science* **121,** 775 (1955).
19. ANDERSON, N. G., AND BURGER, C. L., *Science* **136,** 646 (1962).
20. ELROD, L. H., AND ANDERSON, N. G., *J. Tenn. Acad. Sci.* **42,** 54 (1967).
21. FALZONE, J. A., BARROWS, C. H., AND YIENGST, M. J., *Exptl. Cell Res.* **26,** 552 (1962).
22. FISHER, R. F., HOLBROOK, D. J., JR., AND IRVIN, J. L., *J. Cell Biol.* **17,** 231 (1963).
23. FISHER, W. D., AND CLINE, G. B., *Biochim. Biophys. Acta* **68,** 640 (1963).
24. KATO, T., AND KUROKAWA, M., *J. Cell Biol.* **32,** 649 (1967).
25. ROODYN, D. B., *Biochem. J.* **64,** 361 (1956).
26. JOHNSON, I. R., MATHIAS, A. P., PENNINGTON, F., AND RIDGE, D., *Biochem. J.* **109,** 127 (1968).
27. ELROD, L. H., *The Molecular Anatomy of Cells and Tissues (The MAN Program)* Annual Report, ORNL-4171 Special, p. 123 (1967).
28. SCHNEIDER, W. C., *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. III, p. 680. Academic Press, New York, 1957.
29. BURTON, K., *Biochem. J.* **62,** 315 (1956).