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Density Gradient Centrifugation in Angle-Head Rotors

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Preparative isopycnic density-gradient centrifugation is ordinarily done in high-speed swinging-bucket rotors such as the Spinco SW 39 and SW 25. In addition to requiring this special equipment, the technique is severely limited by the small number of samples and the low total-sample volume which may be used. In recent studies on methods for isolating small numbers of virus particles from tissues and for separating nucleic acids which differed in density, we required means for centrifuging many samples simultaneously. The possibility of making such separations in ordinary angle-head rotors, which are widely available and have both a large number of tubes and high capacities, was therefore investigated. This application of angle-head rotors depends on reorientation of the density gradient from vertical to horizontal during deceleration, which in effect replaces the mechanical transition of the tubes in the swinging-bucket rotor. This approach appeared feasible since the same basic principle had been applied to the construction of hollow, cylindrical, reorienting gradient rotors (1), in which a density gradient formed at rest is reoriented radially during centrifugation and is subsequently recovered at rest after deceleration. In this paper, data on the isopycnic banding of calf thymus DNA and T3 bacteriophage in CsCl are presented showing: that good resolution can be obtained with angle-head rotors; that fluid translations during deceleration do not cause appreciable mixing of the gradient or sample zone; and that the density gradients formed approximate the calculated compositional density gradients at equilibrium.

MATERIALS AND METHODS

Centrifuges and Rotors. Spinco #40 and #30 rotors were operated at their maximum rated speeds, applying solution density corrections as recommended by the manufacturer in a Spinco model L centrifuge at 25°C. Rotors were allowed to come to rest without braking.

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Unloading and Recording. Tubes were unloaded by insertion of a 15-cm length of 16-gage stainless-steel tubing through the screw hole in the cap to the bottom of the tube. The sample was passed successively through a 0.2-mm ORNL flow cell (2) in a Beckman DB spectrophotometer, a model 34H Milan refractometer (Waters Associates) and a model PA-6 pump (New Brunswick Scientific Co.) into collecting tubes. Connections were made with 0.047-in. i.d. polyethylene tubing. Absorbancy at 260 mμ and refractive index were recorded on a Brown 12-point recorder. The pump rate was 0.5 ml/min for #30 tubes and 0.1 ml/min for #40 tubes.

CsCl Solutions. Saturated CsCl was prepared according to Schildkraut *et al.* (3). One milligram of highly polymerized, calf thymus DNA (Worthington Biochemical Corp.) was dissolved in 21.5 ml of 0.15 *M* NaCl-0.02 *M* Tris, pH 8.5, and added to 78.5 ml of saturated CsCl. Heat-denatured DNA was prepared by heating the DNA-NaCl-Tris solution at 95°C for 15 min and quick cooling in an ice bath.

Bacteriophage. A volume of 0.2 ml of T3 bacteriophage (approximate titer, 10^{13}/ml) was added to 100 ml of 55.5% saturated CsCl.

Density. Densities were calculated from refractive index readings (4).

Calculations. Compositional density gradients at equilibrium were calculated from the β-values published by Ifft *et al.* (4). For these determinations radius versus volume calculations were made, assuming that the centrifuge tubes were cylinders surmounted on one end by a hemisphere with the axis of the cylinder inclined at an angle of 26° to the axis of rotation.

Half-height widths of peaks have been expressed in terms of volume since there is a change in the physical width of the bands during reorientation of the gradient. This is due to the geometry of the tubes and rotor and is apparent from an examination of Fig. 4. The volume at half height is constant. The actual width of the band at speed can be determined by reference to Figs. 2 and 3, where both radius of rotation and gradient volume are used as abscissas. Alternately, for the central portion of the tube where volume is approximately linear with radius, the actual width of a given sample volume at speed with the gradient radially oriented is given very nearly by:

$$w = \frac{(\sin \theta) V}{\pi r^2}$$

where w is the width in cm of a given sample volume V, θ is the angle between the tube axis and the axis of rotation, and r is the tube radius in cm. The sample is generally banded in the central portion of the tube where, for 80% of the tube volume, this relationship holds to a good approximation.

For the #40 rotor this reduces to:

$$w = 0.25V$$

and for the #30 rotor this becomes:

$$w = 0.089V$$

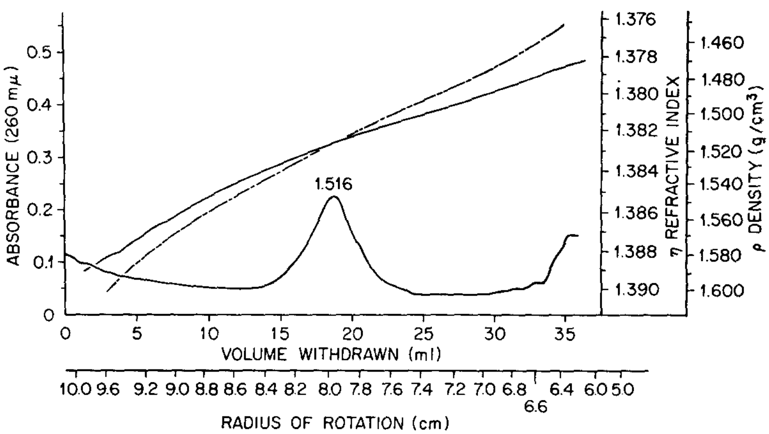

FIG. 1. T3 bacteriophage banded in CsCl by 30-hr centrifugation at 26,000 rpm. Upper solid curve, gradient; dashed curve, theoretical gradient; lower curve, optical density at 260 mμ.

RESULTS

Figure 1 is typical of the results obtained with T3 bacteriophage in the #30 rotor. After a 30-hr centrifugation at 26,000 rpm, the phage have banded at a density of 1.51 with a half-height volume of 3.5 ml. The experimentally determined gradient approaches the calculated compositional density gradient at equilibrium (dashed line). The tube was sampled from the bottom and zero volume corresponded to the maximum radius of rotation and the maximum density.

Figure 2 compares the banding of T3 in the #40 rotor after 30-hr centrifugation at 33,000 rpm to an identical tube recentrifuged for 5 min. The reorientation of the fluid column has not appreciably disturbed the gradient column or sample zone. The phage are banded at $\rho = 1.51$ and the half-height volume is 0.4 ml. The experimentally determined density gradient corresponds fairly well to the theoretical gradient.

The banding of heat-denatured calf thymus DNA in the #40 rotor is shown in Fig. 3. Renatured DNA is seen as a shoulder on the back side of

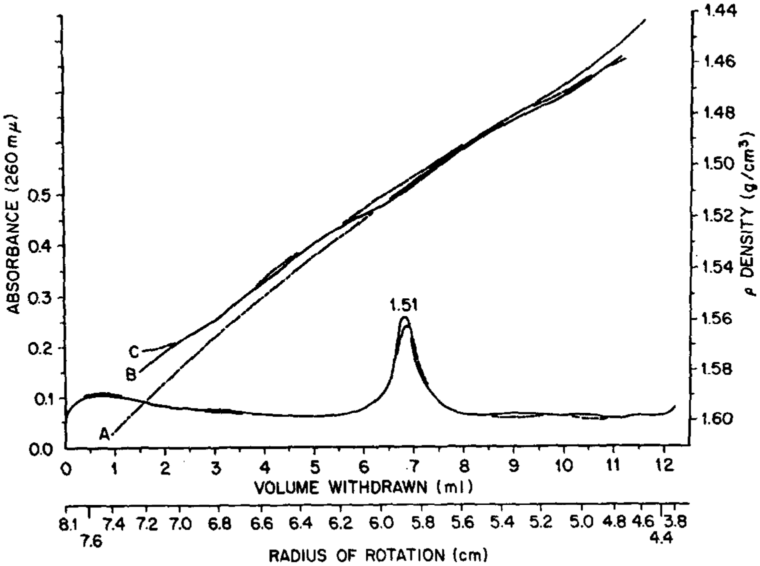

FIG. 2. T3 bacteriophage banded in a #40 rotor by 30-hr centrifugation at 33,000 rpm (solid lines) and after recentrifuging for 5 min (dashed lines). Calculated density gradient (–·–·–).

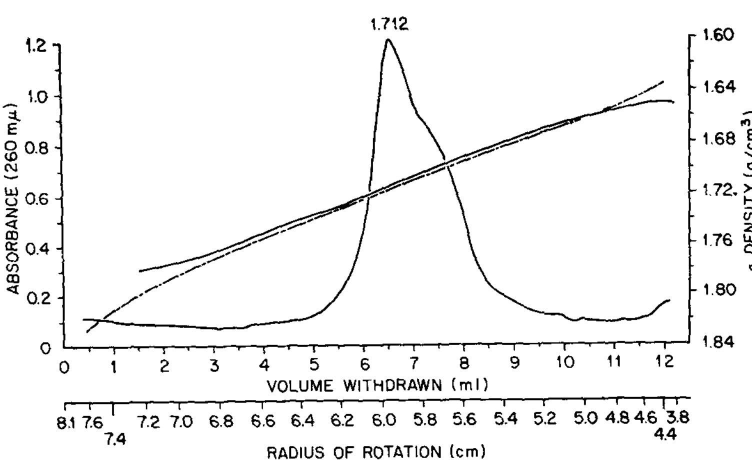

FIG. 3. Heat-denatured DNA banded in CsCl in the #40 rotor by 45-hr centrifugation at 33,000 rpm. Lower solid line, optical density at 260 mμ; upper solid line, gradient; upper dashed line, calculated compositional density gradient.

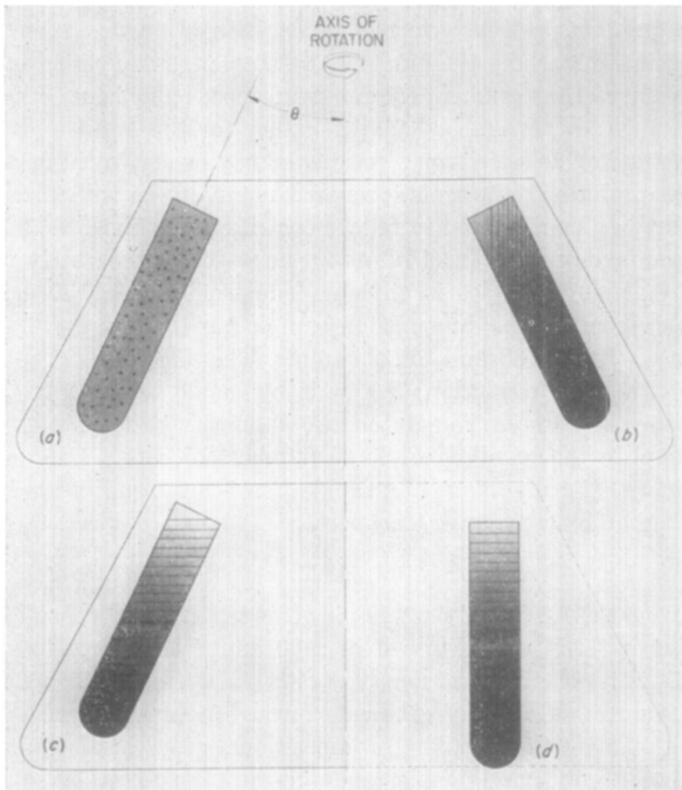

FIG. 4. Stages in isopycnic separation of particles in an angle-head rotor: (a) starting condition with sample in CsCl, (b) gradient (indicated by shading) formed radially with sample banded near center of tube, (c) rotor at rest with gradient reoriented, and (d) tube removed from rotor for sampling.

the peak. The value for buoyant density of denatured DNA agrees with published value obtained in the analytical ultracentrifuge by Doty *et al.* (5). The density gradient obtained after 45 hr of centrifugation approaches very nearly the theoretical gradient. Native DNA was also run and banded at $\rho = 1.699$, as expected.

DISCUSSION

The basic principle involved in the use of angle-head rotors for isopycnic density-gradient separations is shown in Fig. 4. The gradient formed radially during centrifugation is reoriented as the rotor slows and comes to rest. Several stages in the process are represented in Fig. 4. The transition occurs smoothly with little mixing. This is shown by the data in Fig. 2, where a gradient previously formed by centrifugation was recentrifuged briefly without disturbing the gradient or sample band. Further evidence for the stability of the gradient column is the close agreement between the experimental gradients and calculated values for the gradient columns. Preformed, as well as self-formed, gradients may be employed for separations. Such preformed gradients, prepared manually or with mechanical gradient engines, can be used to reduce centrifugation times to 2 or 3 hr.

The advantage of angle-head rotors for large-scale preparative work or for handling many samples is obvious. The fixed-angle #30 and #40 rotors hold 4 times the amount of sample as their swinging-bucket counterparts, the SW 39 and SW 25 rotors. This increase in capacity is obtained with good resolution as shown by small half-height volumes obtained in this study. The automatic monitoring devices employed further simplify the handling of large numbers of samples. Angle-head rotors also offer the advantage of a rapid approach to equilibrium because of redistribution by convective transport.

SUMMARY

By utilizing DNA and T3 bacteriophage as test materials, good isopycnic banding in CsCl was obtained in #30 and #40 Spinco fixed-angle rotors. Values for the buoyant densities of the materials tested agree with published values and density gradients formed after prolonged centrifugation approximate the calculated compositional gradient at equilibrium. Where a large number of samples or large-sample volumes are required, fixed-angle rotors may be used for isopycnic density centrifugation instead of swinging-bucket rotors.

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