Purification of Hepatitis B Antigen Associated Particles: Use of a Reorienting Gradient Rotor (37597)

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Rate zonal sedimentation in density gradients has become a common step in purifying viruses and viral structural components. The introduction of zonal rotors with a rotating seal assembly (1) has considerably increased the scale of this technique as compared with swinging-bucket type rotors. However, some technical difficulties were encountered with rotating seal zonal rotors. Notably, the formation of aerosols may become hazardous if viruses infectious to humans are being centrifuged. In such cases, adequate safety measures have to be introduced which may add to the technical problems and expenses involved in operating these rotors.

In attempts to scale up the separation of the distinct morphological forms of hepatitis B antigen (HBAg) (2), we turned our attention to the SZ-14 reorienting density gradient zonal rotor (Ivan Sorvall, Inc., Newton, CT) which does not require a rotating seal assembly and can be loaded and unloaded at rest. This rotor, which has a sample capacity of approximately 50 ml, has been used so far only for the separation of cells and subcellular particles. Appropriate alterations of techniques, suggested by the operating instructions of the manufacturer of the rotor, permitted establishment of conditions suitable for rate zonal separations of particles having the size of viruses. Separations similar to those obtained in the swinging-bucket rotor SW 25.1 (Beckman Instruments, Palo Alto, CA), which has a sample capacity of about 1 ml/tube, were achieved. The final procedure and its application to the fractionation of HBAg is described here.

Materials and Methods. HBAg was determined by either immunoelectroosmophoresis (IEOP) or by direct solid phase radioimmunoassay (RIA) as described previously (2). HBAg containing sera were fractionated by precipitation with polyethylene glycol 6000 (PEG). The PEG-soluble small spherical HBAg particles about 20 nm in diameter were further purified by affinity chromatography on concanavalin A (Con A)–Sepharose (2).

Rate zonal sedimentation. Centrifugation in the Spinco SW 25.1 rotor was performed essentially as described before (2), except that either 10 to 42% (w/w) sucrose or 10 to 50% (v/v) glycerol gradients in 0.14 *M* NaCl, 0.01 *M* tris(hydroxymethyl)aminomethane, pH 7.2 (Tris) were used. Based on preliminary experiments, the following procedure for rate zonal sedimentation in the rotor SZ-14 was adopted: The core piece of the rotor was inserted into the rotor bowl and 100 ml of light paraffin oil (Soybolt viscosity 125/135; Fisher Scientific Co., Fair Lawn, NJ) were poured into the rotor. Additional steps were performed according to the operating instructions. A linear gradient (1250 ml) of the same composition as given for the SW 25.1 rotor was pumped into the SZ-14 rotor. The Spinco model 141 high capacity gradient pump was used. The loading speed was 40 to 50 ml/min. In some cases, only 1150 ml of gradient were pumped into the rotor followed by additional 100 ml of 50% (v/v) glycerol. The Sorvall RC 2B centrifuge equipped with a manual rate controller was used. The rotor was slowly accelerated to 1000 rpm to reorient the

gradient from axial into radial direction. The rotor was further accelerated to 2500 rpm and the sample (50 ml) was loaded into the annular chamber of the rotor distributor. Under these conditions, the sample was injected by centrifugal force through the layer of oil and deposited in a cylindrical zone between the oil and 10% sucrose (glycerol). The rotor was then further accelerated to operating speed for optimal separation. Deceleration of the rotor and gradient unloading at rest were carried out according to manufacturer's instructions. The Sorvall 49061 peristaltic pump was used to unload the rotor at a speed of 40 to 50 ml/min. Fractions of 50 ml each were collected. Their refractive index was measured by the Abbe refractometer model A (Carl Zeiss, Oberkochen, West Germany) and the concentration of sucrose or glycerol was determined from appropriate tables (3).

Pevicon PSJM membranes (Millipore Corp., Bedford, MA) were used to concentrate the fractions by ultrafiltration.

Electron microscopy. Specimens, corresponding to fractions after rate zonal sedimentation, were deposited on carbon-coated grids. The grids were washed with 1% ammonium acetate, dried and stained with 2% phosphotungstate (pH 6.8 to 7.0). The JEM-100B electron microscope was used. Pictures were taken at a magnification of 40,000 × at 60 kV.

Results. Development of proper conditions for centrifugation. Initial attempts to purify PEG-soluble HBAg in the SZ-14 rotor using 10 to 23% (w/v) sucrose gradients and conditions similar to those used before with the SW 25.1 rotor (2) were unsuccessful. HBAg was recovered in a broad zone in the top 40% of the gradient volume and was not separated from serum proteins. Determination of sucrose in fractions collected after centrifugation revealed that the gradient was maintained only in its middle portion (Fig. 1), suggesting that considerable mixing must have occurred during reorienting. Separate experiments in which a solution of blue dextran (Pharmacia, Uppsala, Sweden) was loaded into the rotor at 2500 rpm revealed that the dye was not evenly deposited on

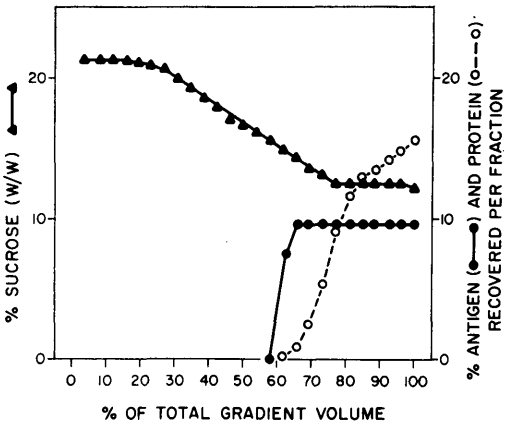


FIG. 1. Attempts to separate PEG-soluble HBAg from residual serum proteins by rate zonal sedimentation at 18,000 rpm for 24 hr in the rotor SZ-14 using a 10 to 23% (w/w) sucrose gradient.

the layer of 10% sucrose but was partly plunged deeper into the gradient by centrifugal force.

In order to improve the conditions for centrifugation, further experiments were carried out with a mixture of influenza virus

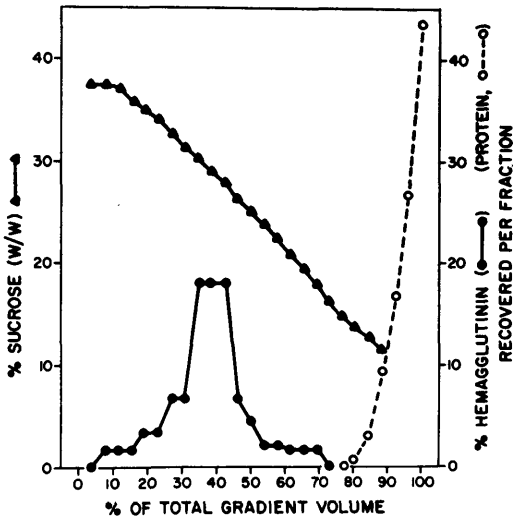


FIG. 2. Separation by rate zonal centrifugation (18,000 rpm for 2.5 hr in the SZ-14 rotor) of influenza virus and bovine serum albumin. Gradient: 10 to 42% (w/w) sucrose in Tris (1250 ml). The sample corresponded to 2 ml of influenza virus vaccine (Merck, Sharp & Dohme) mixed with 48 ml of 5% bovine serum albumin in Tris. The concentration of virus in gradient fractions was measured by hemagglutination titrations.

and bovine serum albumin. The choice of this virus permitted reduction in the time of centrifugation compared to that required for HBAg. The corresponding results are shown in Fig. 2. Similar results were obtained with a 50 times smaller sample by rate zonal sedimentation in the rotor SW 25.1. However, in the latter case the gradient remained linear also in the bottom 12% of the total gradient volume. Thus, mixing in the heavy end of the gradient during reorienting seemed unavoidable (see also Fig. 3). On the other hand, the light end of the gradient did not appear to be affected by reorientation. The stabilization of the light end of the gradient may be ascribed to the paraffin oil overlay. The latter also decelerated the movement of the sample introduced into the rotor and prevented its plunging into the gradient.

Prevention of the formation of water-containing aerosols and their escape into the rotor chamber. Aerosols possibly formed during centrifugation or sample application might escape from the rotor through the narrow gap between the rotor lid and distributor. This gap became sealed by the oil overlay. A sample containing 100 μCi of ^{125}I-labeled γ-globulin in Tris was introduced into the rotor at 2500 rpm. The rotor was filled with 1250 ml of a 10 to 42% (w/v) sucrose gradient and with 100 ml of oil. Some oil leaked into the rotor chamber during application of the sample but leakage of radioactive material was not observed.

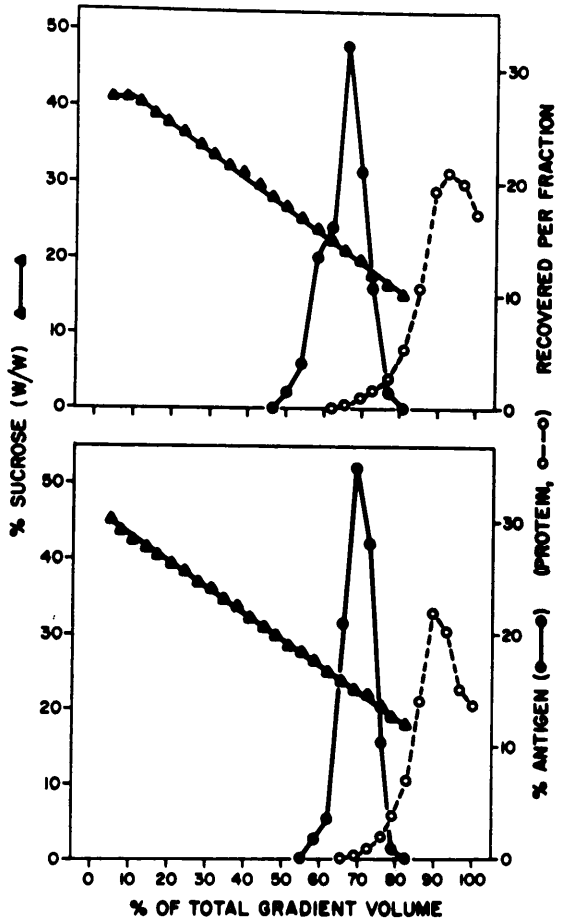


FIG. 3. Comparison of rate zonal sedimentations of PEG-soluble small spherical HBAg particles in rotors SW 25.1 (bottom) and SZ-14 (top). Each centrifugation was performed at 18,000 rpm for 24 hr using 10 to 42% (w/w) sucrose gradients. HBAg was assayed by IEOP.

Separation of HBAg from serum proteins. The efficiency of the separation of serum proteins from PEG-soluble HBAg prepurified on Con A–Sepharose columns by additional rate zonal sedimentation was the same in the SW 25.1 and SZ-14 rotors (Fig. 3). In both cases approximately 0.7% of protein originally present in the serum was recovered in the pooled fractions containing HBAg. The distribution of HBAg and of protein across the gradients harvested from each rotor were very similar. Thus, conditions found suitable for separations in the SW 25.1 rotor may be applied to the SZ-14 rotor with the exception in mind that the maximal speed for the SZ-14 rotor is 19,500 rpm as compared with 25,000 rpm for the rotor SW 25.1.

PEG-precipitated HBAg was also submitted to comparative centrifugations in the two rotors and again the distributions of HBAg and protein across the gradients were similar. Pooled fractions from the SZ-14 rotor, concentrated at least 100-fold, contained in addition to HBAg also impurities detectable by electron microscopy. Therefore, they were recentrifuged in the rotor SW 25.1. Results shown in Fig. 4 indicate that processing of PEG-precipitated HBAg by centrifugation in the SZ-14 rotor increased the purity of HBAg recovered in fractions with detectable protein at least 100-fold. Electron microscopy confirmed that particles differing in size and shape had been separated by the final rate zonal sedimentation step (Fig. 5).

Fractions from the upper 38% of the gradient from the SZ-14 rotor which contained small spherical HBAg particles and the major part of PEG-precipitable serum proteins were not recentrifuged since we were interested mostly in the other morphological forms of HBAg and the small spherical particles were easier to purify from the PEG-soluble portion of HBAg-containing serum in which they were much more abundant (2).

Discussion. Reorienting gradient rotors, first described by Anderson *et al.* (4), were used in the industrial preparation of vaccines using continuous sample flow combined with isopycnic banding (5, 6). The results

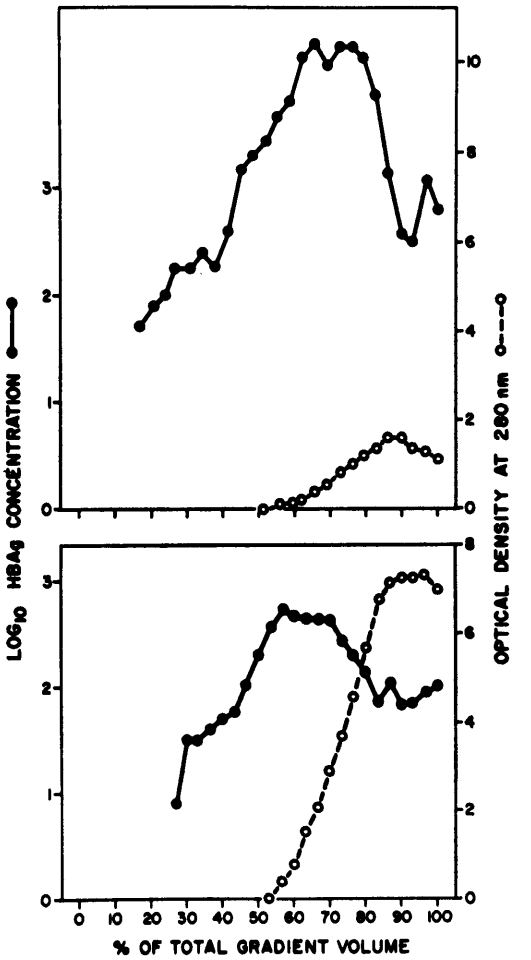
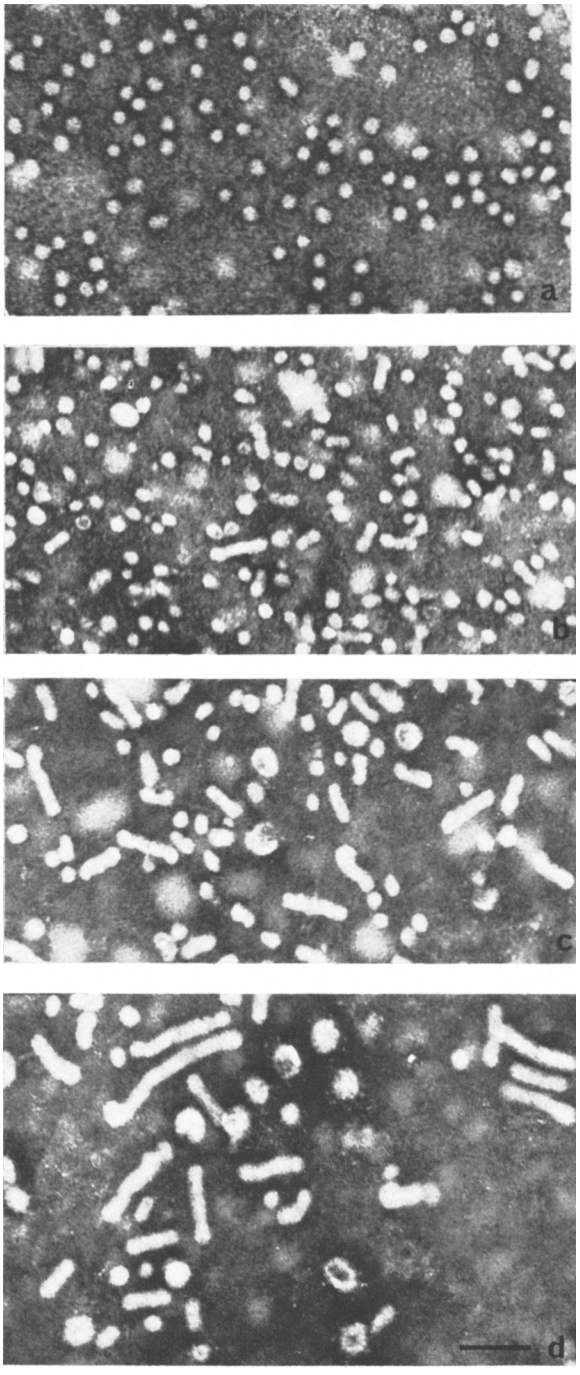


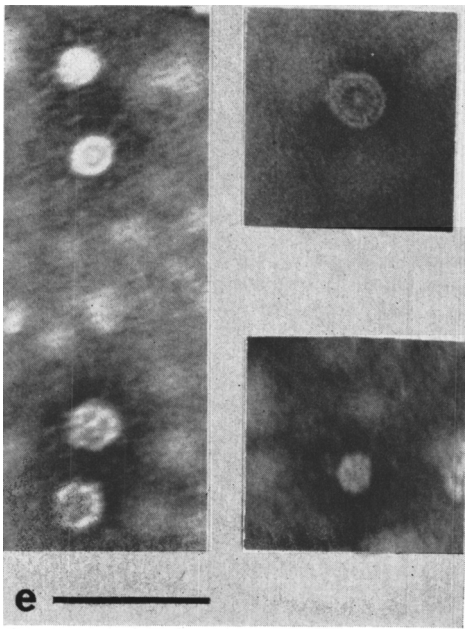
FIG. 4. Rate zonal sedimentation of HBAg particles precipitated from serum by PEG and subsequently resuspended in Tris in 1/5 of the volume of serum. Centrifugation was performed in the SW 25.1 rotor at 15,000 rpm for 15.5 hr in a 10 to 50% (v/v) glycerol gradient in Tris. The following samples were centrifuged: 1 ml of the resuspended pellet (bottom) and a pool of fractions, concentrated to 1 ml, corresponding to 53.9 to 61.6% of the gradient (measured from the heavy end) after centrifuging 40 ml of the resuspended pellet in the SZ-14 rotor for 15.5 hr at 15,000 rpm in 1150 ml of a 10 to 50% (v/v) glycerol gradient layered on 100 ml of 50% glycerol. The pooled fractions contained 0.25% of protein originally present in the serum and 40% of HBAg precipitated with PEG, corresponding to 6% of HBAg initially present in the serum (top). HBAg was assayed by RIA.

presented here show that a commercially available reorienting gradient rotor may be



applied to large-scale laboratory rate zonal sedimentation of virus particles.

The distinct morphological forms of HBAg were separated, at least partly, by rate zonal centrifugation (7). The separation of the majority of the smaller spherical HBAg particles from filamentous forms and larger spherical particles by precipitation with PEG before rate zonal centrifugation (2) favors isolation of the larger more complex particles



associated with HBAg. Zonal sedimentation in the SZ-14 rotor further facilitates this goal by increasing the scale of separations while minimizing the hazard of exposure of laboratory personnel to aerosols containing the infectious agent of hepatitis B.

Particles endowed with surface projections were observed in some fractions after rate zonal sedimentation. The exact nature of these particles remains to be elucidated. It is possible that the surface projections correspond to antibodies attached to the particles.

The dimensions of the particles as measured on the electron micrographs may have been influenced by the presence of glycerol in the samples. This would mostly apply to particles shown in Fig. 5e.

Summary. The SZ-14 reorienting density gradient rotor (Ivan Sorvall, Inc., Newton, CT) having a sample capacity of approximately 50 ml may be used for the purification of viruses under appropriate conditions which are described. The procedure has been successfully applied to the purification of the diverse particle types associated with hepatitis B antigen under conditions minimizing the danger of laboratory contamination during centrifugation.

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FIG. 5. Particles in fractions obtained after consecutive rate zonal sedimentations in rotors SZ-14 and SW 25.1. (a to d) Particles recovered from the following portions of the gradient corresponding to Fig. 4, top: (a) spherical particles 17 to 25 nm in diameter from 72.4 to 75.9% of the gradient; (b) spherical particles of similar dimensions as in (a) some of which seem penetrated by negative stain, and filaments 20 nm wide and up to 90 nm long from 62.1 to 65.6% of the gradient; (c) 25 and 40 nm spherical particles. Some of the latter are apparently endowed with surface projections. Filaments up to 25 nm wide and 100 nm long, from 51.8 to 55.2% of the gradient; (d) spherical particles up to 50 nm in diameter, some of which have surface projections, and filaments up to 28 nm wide and 230 nm long, from 38 to 41.4% of the gradient; (e) spherical particles 25 to 42 nm in diameter some having an inner core and a hexagonal or pentagonal outline. Some of the particles have surface projections. These particles were obtained from pooled fractions corresponding to 19.3 to 50% of the gradient from the SZ-14 rotor (see text to Fig. 4, top). The fractions contained approximately 0.75% of HBAg, detectable by RIA, initially present in the serum. They were concentrated to 1 ml and recentrifuged in the rotor SW 25.1 under conditions given for Fig. 4. Fractions corresponding to 19.3 to 50% of the gradient were pooled and concentrated to 1.5 ml. Filaments were much less frequent in this preparation than spherical particles. Bar length = 100 nm.

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