Hepatitis B Antigens: Separation of Two Populations Differing in Particle Size Distribution (37339)

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Three distinct kinds of particles carrying hepatitis B specific antigenic sites have been distinguished by electron microscopy: spherical particles with diameters of about 20 and 40 nm and tubular forms 20 nm wide (1–5). The smaller spherical particles are usually the most abundant in hepatitis B antigen (HBAg)-containing sera while the 42 nm particles, which are suspected to represent the etiologic agent of hepatitis B, are the least abundant. The distinct morphologic forms of HBAg have been separated from each other by rate zonal sedimentation using antigen preparations which had been partially purified by isopycnic centrifugation (6). However, the instability of 42 nm particles purified by these means suggests that milder purification techniques may be required. A simple method of concentrating and separating the 42 nm spherical particles together with the tubular forms from most of both the smaller spherical particles and of serum proteins is described in this report.

Materials and Methods. HBAg was determined by the following methods: immunoelectroosmophoresis (IEOP) (7); hemagglutination inhibition (HAI) (8) and direct solid phase radioimmunoassay (RIA) (9). The IEOP titer of an HBAg preparation corresponded to the reciprocal of its highest dilution in 10% human serum [diluted with 0.14 *M* NaCl, 0.01 *M* tris(hydroxymethyl) aminomethane (Tris), pH 7.2] at which HBAg was still detectable. The serum used for dilution was free from HBAg and from antibodies against HBAg (HBAb). The relative concentration of HBAg by RIA tests was determined from calibration curves relating radioactive counts to the reciprocal of the dilution of a standard HBAg containing serum. All materials tested by RIA were diluted at least 1:5 in normal human serum.

Precipitation with polyethylene glycol 6000 (PEG). Appropriate volumes of HBAg-containing serum and of PEG (BDH Chemicals Ltd., Poole, England), dissolved in distilled water (30 g/100 ml solution), were mixed and kept at 4° overnight. The precipitates were sedimented by centrifugation at 4500*g* for 20 min. The pellets were resuspended in Tris in 1/10 of the original volume of serum.

Rate zonal sedimentation. One milliliter samples were submitted to rate zonal sedimentation at 15,500 rpm for 15.5 hr in the Spinco rotor SW 25.1. Linear gradients (28 ml) of 10 to 25% sucrose in Tris were used. Fractions of 1 ml each were collected from the bottom of the tubes. For electron microscopy, appropriate fractions were pooled and concentrated by ultrafiltration to 1 ml using a 10 ml cell (Amicon Corp., Lexington, MA) with a Pevicon PSJM025 membrane (Millipore Corp., Bedford, MA). The concentrated samples were layered on top of 1 ml of 25% sucrose in Tris and centrifuged in the Spinco SW 65 rotor at 50,000 rpm for 3 hr. The pellets were resuspended in 0.3 ml of 0.025 *M* phosphate buffer, pH 7.2.

Affinity chromatography. Aliquots (24 ml) of supernatant fluids after precipitation of serum with PEG were chromatographed on a 33 × 1 cm column of concanavalin A (Con A)–Sepharose essentially as described previously (10). Before chromatography, $CaCl_2$ and $MnCl_2$ (both 10^{-3} *M*) were added to the samples. The column was washed with 37 ml of Tris containing 10^{-3} *M* $CaCl_2$ and

10^{-3} *M* $MnCl_2$, followed by the elution buffer (5% methyl-α-D-mannopyranoside in Tris).

Molecular exclusion chromatography. A column (56 × 0.9 cm) of porous glass beads (498 Å pore size, 120 to 200 mesh; Electro Nucleonics, Inc., Fairfield, NJ) was coated with PEG 20,000 (11); washed with 50 ml of 0.5% bovine hemoglobin in 0.14 *M* NaCl, 0.05 *M* tris(hydroxymethyl)aminomethane, 0.005 *M* EDTA (pH 7.5) (TES); followed by TES until the concentration of hemoglobin decreased to 0.005% as monitored spectrophotometrically. Subsequently, the column was washed with 50 ml of 0.5% PEG 300,000 followed by 50 ml of elution buffer (0.005% hemoglobin and 0.02% PEG 20,000 in TES). Samples of 1 ml or less were applied to the column. Fractions of 1 ml each were collected.

Electron microscopy. Specimens were deposited on carbon-coated grids 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. Immune electron microscopy was performed essentially as described by Almeida *et al.* (12).

Results. Supernatant fluids after precipitating various HBAg-containing sera with PEG (final concn, 5.5%) contained 70 to 97% of HBAg originally present. Rate zonal sedimentation of the supernatant fluids and of the redissolved pellets obtained from the same serum revealed considerable differences in the size distribution of HBAg specific particles between the two preparations (Fig. 1). Anti-

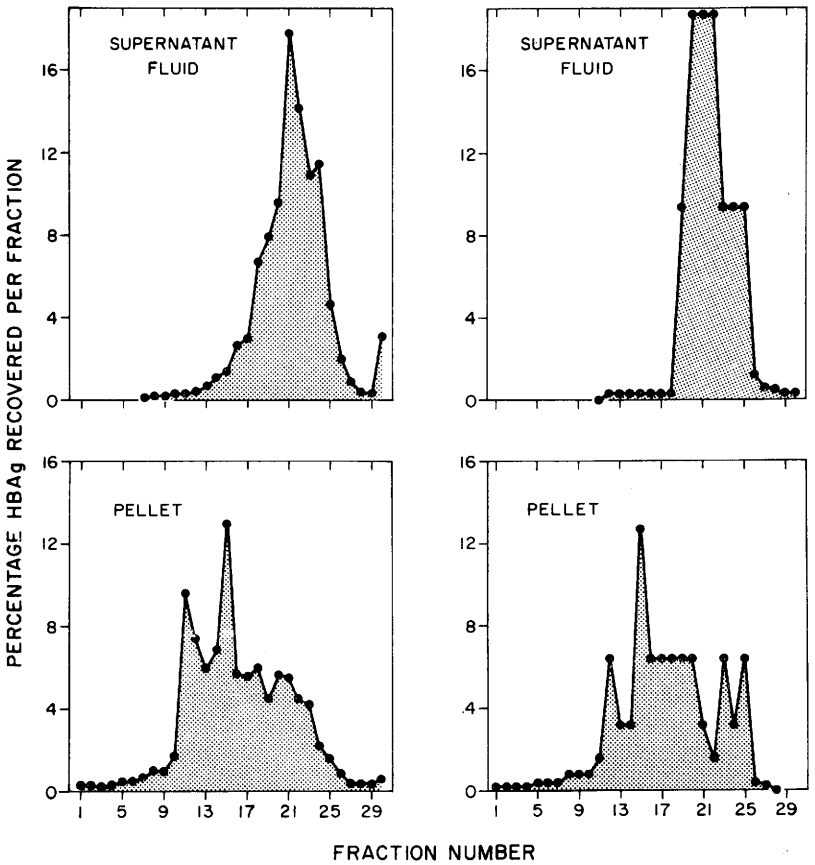
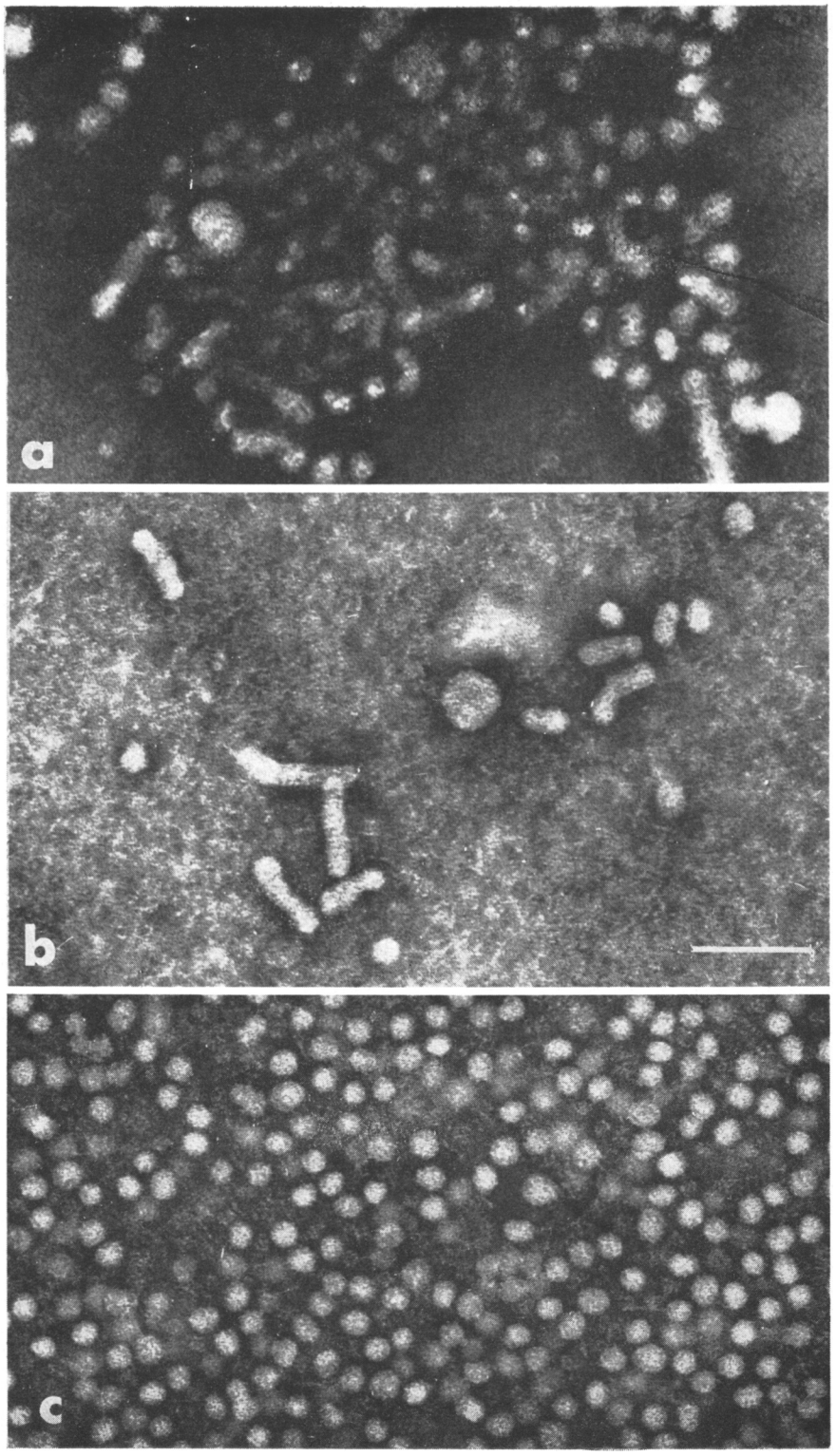


FIG. 1. Distribution of HBAg particles in fractions after zonal sedimentation of resuspended pellets and supernatant fluids obtained after adding PEG (final concn, 5.5%) to HBAg-containing serum. HBAg was determined either by RIA (left panels) or by HAI (right panels) tests. Fraction 1 = bottom of gradient.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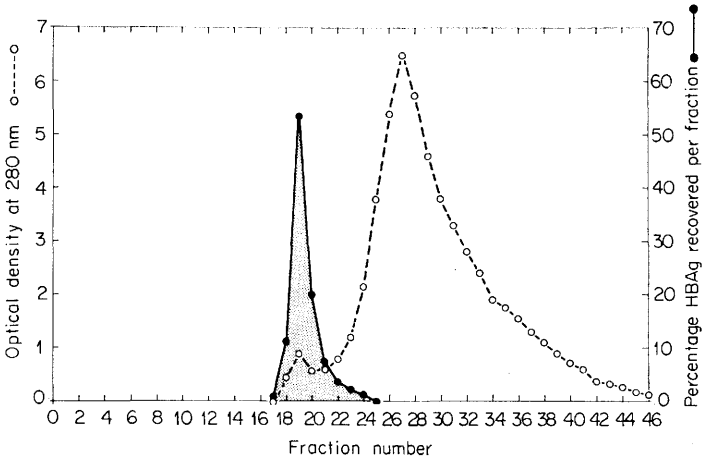

FIG. 3. Molecular exclusion chromatography of HBAg-containing serum (1 ml) on a column (56 × 0.9 cm) of porous glass beads. Fractions of 1 ml were collected. HBAg was assayed by IEOP. The optical density of fractions was read in cuvettes 0.25 cm wide; the readings were multiplied 4×, and plotted on the ordinate.

gen–antibody complexes formed after addition of anti-HBAg serum to the redissolved pellets contained 42 nm diameter spherical particles, tubular forms and 20 nm spherical particles (Fig. 2a). Only the latter particles were observed in antigen–antibody complexes formed after addition of the same antiserum to the supernatant fluids.

Further experiments were done to confirm that the sedimentation rates of HBAg particles in the resuspended pellets and supernatant fluids were predominantly related to their distinct morphologic features and not to the formation of aggregates in the pellets. Four and one half milliliters of 30% PEG were added to 20 ml of an HBAg-containing serum. The precipitate containing approximately 22% of protein originally present in the serum was dissolved in 2 ml of Tris and chromatographed on a column of porous glass under conditions given for Fig. 3. The fractions corresponding to the void volume of the column, containing all PEG-precipitable HBAg and approximately 5% of the protein originally present in the serum, were pooled, concentrated by ultrafiltration to 1 ml and centrifuged under conditions given for Fig. 1. The supernatant fluid was chromatographed on Con A–Sepharose (Fig. 4). Fractions 40 to 56, containing 91% of PEG-soluble HBAg and 6.4% of protein originally present in the serum, were pooled, concentrated and chromatographed on porous glass. The latter procedure resulted in additional 5-fold purification of HBAg. The sedimentation rate profiles of the two partially purified HBAg preparations were similar to those shown in Fig. 1. Fractions 1 to 16, derived from rate zonal sedimentation of PEG-pelleted material, were pooled, concentrated and investigated under the electron microscope. Tubular forms, 42 nm diameter and occasional 20 nm diam-

FIG. 2a. HBAg–antibody complexes formed after addition of anti-HBAg human serum to a resuspended pellet obtained after precipitating HBAg-containing serum with PEG. (b) Particles precipitated from HBAg-containing serum by PEG. The particles were partially purified by molecular exclusion chromatography and then submitted to rate zonal sedimentation and recovered from the region of the gradient corresponding to fractions 1 to 16 in Fig. 1. (c) Particles soluble in 5.5% PEG, partially purified by affinity chromatography and molecular exclusion chromatography and then submitted to rate zonal centrifugation and recovered from the region of the gradient corresponding to fractions 18 to 25 in Fig. 1.

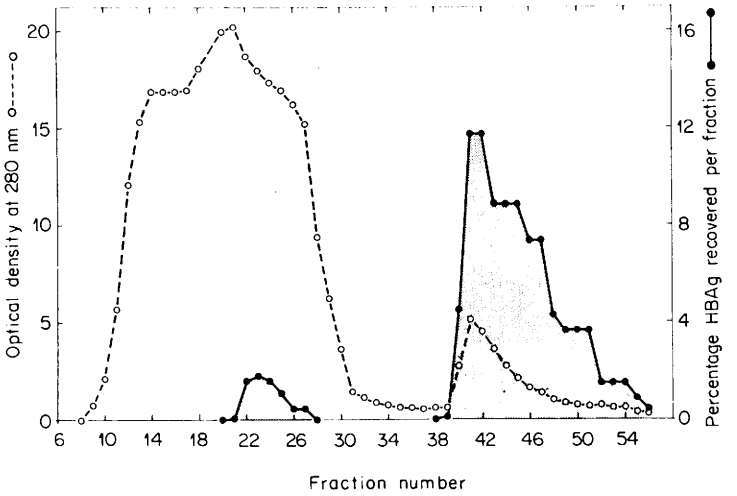

FIG. 4. Affinity chromatography of the supernatant fluid (24 ml) obtained by precipitating HBAg-containing serum with PEG (5.5%). A 33 × 1 cm column of Con A–Sepharose was used. Fractions of 2 ml were collected. HBAg was assayed by IEOP. For measurements of optical density, the fractions were diluted with Tris and the readings were multiplied by the appropriate dilution factor, and plotted on the ordinate.

eter particles were observed (Fig. 2b). Fractions 1 to 16 derived from rate zonal sedimentation of HBAg soluble in PEG, contained only a small number of 20 nm spherical particles. The latter were very abundant in pooled fractions 18 to 25 (Fig. 2c). Experiments with another serum gave similar results. In these experiments, fractions after rate zonal sedimentation were pooled in groups of four and each group was investigated by electron microscopy. Partial separation of tubular forms from the 42 nm particles was observed in agreement with results of Bond and Hall (6).

Both *ad* and *ay* subtypes of HBAg (13) were separated by precipitation with PEG into two populations having distinct particle size distributions.

Decreasing the concentration of PEG below 5.5% did not result in more selective precipitation of faster sedimenting HBAg particles and diminished the proportion of HBAg recovered in the pellet.

Discussion. HBAg was quantitatively precipitated from serum at pH 4.6 at a PEG concentration of 8% (14). Our observations that the 42 nm diameter particles and the tubular forms of HBAg were apparently completely precipitated at neutral pH with 5.5% PEG are in agreement with the finding that, in general, larger particles are less soluble in PEG solutions than smaller ones and that the precipitation is more selective for larger particles (15, 16). Precipitation of a minor portion of the 20 nm spherical particles may, perhaps, be attributed to differences in the chemical composition of the soluble and precipitated particles. This possibility will be considered in future studies.

Approximately 20% of serum proteins were precipiated under the same condiitons as were the 42 nm particles and the tubular forms of HBAg. According to data of Chesebro and Svehag (16), the precipitated proteins should contain predominantly the low density α_2- and β_2-lipoproteins and IgM. The precipitation of α_2-lipoproteins and IgM, which both interact with Con A (17), was of great advantage for further purification of the smaller spherical PEG-soluble HBAg particles by affinity chromatography, since the apparent capacity of Con A–Sepharose columns to bind HBAg was increased 2- to 3-fold.

The partial separation by precipitation with PEG of the distinct morphological forms of HBAg is expected to facilitate their further purification and characterization.

Summary. Addition of polyethylene glycol

6000 to sera containing hepatitis B antigen (HBAg) at neutral pH resulted in the precipitation of the 42 nm diameter spherical particles and of the tubular forms of HBAg with most of the 20 nm diameter spherical particles remaining in solution. This procedure may serve as a first step in the purification of the distinct morphologic forms of HBAg.

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