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Purification and Partial Characterization of Hepatitis e Antigen (HB_eAg)

H. A. FIELDS,* D. W. BRADLEY, C. L. DAVIS, AND J. E. MAYNARD

Hepatitis Laboratories Division,† Bureau of Epidemiology, Center for Disease Control, Phoenix, Arizona 85014

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Purification of hepatitis e antigen (HB_eAg) from 200 ml of chimpanzee plasma was accomplished by a combination of ion-exchange chromatography on diethylaminoethyl-cellulose followed by gel filtration. High-resolution sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified HB_eAg demonstrated two major polypeptides with estimated molecular weights of 22,000 and 55,000. HB_eAg labeled with ^{125}I showed a high affinity for protein A-conjugated Sepharose CL-4B. The precipitation reaction between HB_eAg and anti-HB_e was inhibited by preincubating the purified antigen with rabbit anti-human immunoglobulin G (IgG). These data show that HB_eAg is associated with a serum fraction with the biophysical and antigenic properties of an immunoglobulin of the IgG class. Sedimentation coefficient analysis of purified HB_eAg resulted in an $s_{20,w}$ value of 11.6 and a molecular weight value of 324,000. These findings, supported by gel filtration and polyacrylamide gradient gel electrophoresis, revealed that HB_eAg has properties analogous to those of a dimer of IgG.

In 1972 Magnius and Espmark (16) identified by immunodiffusion a new and distinct antigen-antibody system in chronic carriers of hepatitis B surface antigen (HB_sAg). This new antigenic determinant, originally designated e antigen, was abbreviated HB_eAg. Magnius and Espmark (16) considered HB_eAg unlikely to be a host constituent of liver cells released by liver damage, since the antigen was not found in cases of viral hepatitis type A. They postulated, however, that HB_eAg was probably specified by the hepatitis B virus (HBV) or produced as a host response to the virus (15). In support of this concept, Trepo et al. (30) failed to find a single case of HB_eAg-positive non-HBV-associated fulminant and chronic hepatitis. In addition, Smith and co-workers (28) were unable to detect HB_eAg or its antibody (anti-HB_e) in patients with posttransfusion hepatitis of non-B origin.

In 1974, Nielson and colleagues (22) found an association between the presence of HB_eAg and indices of persisting or chronic liver disease among carriers of HB_sAg. They suggested that the presence of HB_eAg might have prognostic value in predicting the course of the disease. Some studies appeared to support this idea (17, 19). However, others (5, 29) demonstrated that the simple presence of HB_eAg in a single serum was not a good predictor of future chronicity and that any predisposition to chronicity must be related to long-term persistence of the antigen. Certain studies (4, 17, 28, 30) have also shown that the presence of anti-HB_e might reflect a favorable prognosis.

Maynard et al. (19) showed a high degree of correlation between HB_eAg-positive patients and those patients having high levels of deoxyribonucleic acid polymerase activity. The concept that HB_eAg-positive sera are probably of a higher degree of infectivity than anti-HB_e-positive sera is supported by this study (19) and others (1, 17). Berquist et al. (2) and Schweitzer (25) demonstrated, however, that the presence of anti-HB_e did not necessarily denote the lack of HBV infectivity.

HB_eAg differs from HB_sAg in several biophysical properties. Magnius (14) reported a buoyant density of 1.29 and a sedimentation coefficient of 11.6*s*. In addition, Magnius reported that, during immunoelectrophoresis, HB_eAg migrated in the fast immunoglobulin G (IgG) region. Neurath and Strick recently reported (20) that HB_eAg has the properties of an immunoglobulin and that the e determinant represents an idiotypic determinant. The presence of HB_eAg in unrelated individuals would therefore imply a broad cross-idiotypic specificity for e determinants. In addition, Neurath et al. (21) have reported e determinant sites exposed on the surface of Dane particles and tubular forms of HB_sAg.

Confirmation of these immunochemical obser-

† WHO Collaborating Center for Reference and Research on Viral Hepatitis.

vations seemed necessary. We present a purification procedure based on chromatographic separation techniques and present additional biochemical and immunochemical evidence that HB_eAg is an IgG or is associated with immunoglobulin-purified fractions.

MATERIALS AND METHODS

Reagents. HB_eAg and anti-HB_e material was acquired from two chronic hepatitis B-infected chimpanzees. Both chimpanzees were positive for HB_sAg and anti-HB_c. HB_eAg- and anti-HB_e-containing plasmas were collected by plasmapheresis and stored at −20°C.

Assay for HB_eAg. The center well of rheophoresis plates (Abbott Laboratories, North Chicago, Ill.) was double filled with reference chimpanzee anti-HB_e plasma, and the peripheral wells were filled with either reference chimpanzee HB_eAg or test material. The plates were incubated in a humid chamber at room temperature for a minimum of 48 h before reading. After 72 h of incubation, the plates were washed in saline containing 0.02% NaN_3 over a 3- to 4-day period. The plates were subsequently washed in distilled water for 24 h at room temperature. After washing they were transferred to glass slides and allowed to air dry. The plates were stained in 0.5% Coomassie brilliant blue R-240 (Bio-Rad, Richmond, Calif.), prepared in destain (25% methanol–7% acetic acid–68% distilled water) for 10 min at room temperature. Destaining continued until the background cleared. A photograph of a stained plate is presented in Fig. 1. Two HB_eAg immunoprecipitates were found designated $HB_eAg/1$ (closest to the center well) and $HB_eAg/2$.

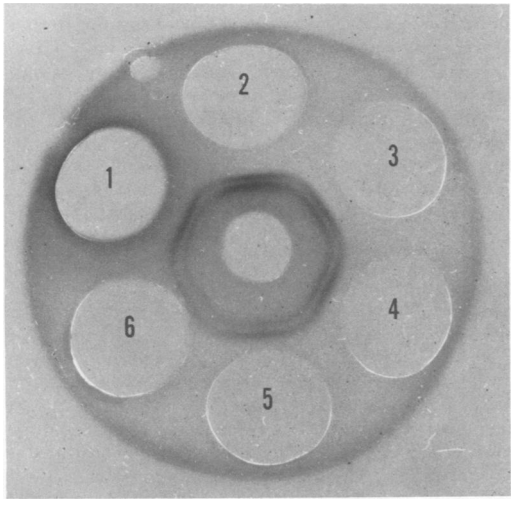


FIG. 1. *Rheophoresis assay for HB_eAg of concentrated pool II from DEAE-cellulose column. Positions 1 and 4 contain reference chimpanzee HB_eAg reagent. Positions 2, 3, 5, and 6 contain increasing twofold dilutions of Hb_eAg. The center well contains reference chimpanzee anti-HB_e reagent. After incubation, the plate was washed, dried, stained, and destained as described.*

Purification of HB_eAg. A 200-ml portion of chimpanzee HB_eAg-positive plasma, which had a titer of ≥1:8 for HB_eAg as determined by rheophoresis, was thawed. The plasma was clarified by centrifugation at 10,000 × *g* for 30 min at 5°C. The supernatant was converted to serum by the addition of 0.8 ml of thrombin, topical (stock Y-2073-1, Parke, Davis & Co., Detroit, Mich.), containing 1,000 U.S. units of thrombin and 0.004 M $CaCl_2$. The suspension was allowed to stand overnight at 4°C. The clot was dispersed by a sharp instrument and centrifuged at 10,000 × *g* for 30 min at 5°C. An equal volume of saturated $(NH_4)_2SO_4$ was added slowly to the supernatant at 4°C to render the serum albumin poor. The pH was maintained at around neutrality by the addition of dilute acid or base. The precipitate was stirred for 1 h at 4°C and subsequently centrifuged at 10,000 × *g* for 30 min at 5°C. A 100-ml volume of 0.01 M phosphate buffer (pH 7.8) (PB) was added to the precipitate which was resuspended by gentle mixing. The suspension was dialyzed against 20 volumes of PB with at least five changes over a period of not less than 48 h. After dialysis the retentate was centrifuged at 10,000 × *g* for 30 min at 5°C to remove the small amount of precipitate which occurred during dialysis.

DEAE-cellulose chromatography. A 2.6-by-90-cm column of diethylaminoethyl (DEAE)-cellulose (DE52, Whatman, Clifton, N.J.) was prepared according to the manufacturer's instructions. The column was equilibrated with PB until the conductivity and pH of the effluent was the same as the affluent. After equilibration, 100 ml of dialyzed preparation was applied to the column and the material was eluted (250 ml/h) in a stepwise fashion using a peristaltic pump and the buffer schedule indicated in Table 1. The fractionation was monitored by an ISCO (Lincoln, Neb.) UA-5 monitor at 280 nm. Each optical density peak was pooled and concentrated to 100 ml with an Amicon (Lexington, Mass.) TCF10 ultrafiltration unit equipped with an Amicon YM10 membrane. Each concentrated pool was analyzed for HB_eAg activity (rheophoresis), HB_sAg activity by radioimmunoassay

TABLE 1. *DEAE-cellulose phosphate buffer elution schedule*

Buffer no.	pH	NaCl (M)	Vol (ml)
1	7.8	0	500
2	7.8	0.02	300
3	7.0	0.025	200
4	7.0	0.03	200
5	7.0	0.035	200
6	7.0	0.04	200
7	7.0	0.045	200
8	7.0	0.05	250
9	6.5	0.06	250
10	6.5	0.07	250
11	6.5	0.08	250
12	6.5	0.09	250
13	6.5	0.10	200
14	6.5	0.12	200
15	6.5	0.15	200
16	6.5	0.20	400
17	4.3	1.00	500

(Ausria II, Abbott Laboratories), and protein concentration (12). In addition, each pool was analyzed on neutral polyacrylamide gradient gel electrophoresis (PGGE).

Gel filtration chromatography. A 2.6-by-90-cm column of AcA22 (LKB, Pleasant Hill, Calif.) was poured and equilibrated with PB containing 0.02 M NaCl and 0.025% NaN_3 (PBNN). Concentrated pool II from the DEAE-cellulose fractionation of a $(NH_4)_2SO_4$-precipitated HB_eAg-positive serum was applied to the column and eluted with PBNN at a flow rate of 13 ml/h by using a peristaltic pump. The effluent was followed at 280 nm with an ISCO UA-5 monitor. Appropriate fractions were assayed by rheophoresis for HB_eAg, IgG, and transferrin. HB_sAg was assayed by radioimmunoassay. Fractions demonstrating HB_eAg activity were pooled and concentrated to 3 ml by ultrafiltration using a 47-mm stirred cell (Millipore Corp., Bedford, Mass.) equipped with a Millipore PTGC membrane. The concentrate was applied to a 2.6-by-90-cm column of Sephacryl S-200 (Pharmacia, Piscataway, N.J.) equilibrated with PBNN. Column elution was achieved by using a peristaltic pump at a flow rate of 19 ml/h. The effluent was followed at 280 nm with an ISCO UA-5 monitor. Each fraction (3 ml) was assayed for HB_eAg, IgG, and transferrin by rheophoresis.

Immunoelectrophoresis. Immunoelectrophoresis (IEP) was performed in a LKB Multiphor unit. Samples (10 μl) were subjected to electrophoresis at 4 V/cm in barbital buffer at 5°C. After 75 min the plates were removed from the chamber, and the troughs were cut out. Each trough was filled with antisera as designated (Behring Diagnostics, Somerville, N.J.) and incubated for 48 h in a humid environment at room temperature. After incubation the immunoplates were washed, pressed, dried, stained with 0.5% Coomassie brilliant blue R-250 for 10 min at room temperature, and destained.

PGGE. Qualitative analysis of plasma proteins was determined by PGGE. Gradient gels (4 to 30%) were purchased from Pharmacia, and electrophoresis was carried out by the manufacturer's directions using the Pharmacia GE-4 electrophoresis apparatus and the Pharmacia EPS 500/400 power supply. After electrophoresis the slabs were removed and fixed for 1 h at room temperature. The fixing solution consisted of 47.0 g of trichloroacetic acid, 17.0 g of sulfosalicylic acid, 150 ml of methanol, and 350 ml of distilled water. The gel was then stained for 2 h at room temperature. The staining solutions consisted of 1.25 g of Coomassie brilliant blue R-250, 227 ml of methanol, 227 ml of distilled water, and 46 ml of glacial acetic acid. Insoluble material was removed by filtration through a Whatman no. 1 filter paper. The gels were destained in a solution containing 30% methanol, 10% acetic acid, and 60% distilled water until the background cleared. The gels were then photographed with a Polaroid MP-3 camera equipped with type 55 film.

Analytical PAGE. Analysis of polypeptides by polyacrylamide gel electrophoresis (PAGE) using sodium dodecyl sulfate (SDS), mercaptoethanol, and urea was performed as previously described (3, 6) except for the following modifications. Instead of cylindrical gels, the gel was cast in a vertical slab gel apparatus purchased from Hoefer Scientific Instruments (San Francisco, Calif.). All reagents were purchased from Bio-Rad. The separating gel was prepared with an acrylamide gel strength of 10%, maintaining a bisacrylamide-acrylamide ratio of 0.8:30. The separating gel was cast within 1 cm of the well formers, and after polymerization the stacking gel was poured. The electrode buffer contained 0.025 M tris(hydroxymethyl)aminomethane-hydrochloride, 0.5 M urea, 0.1% SDS, and 0.192 M glycine, and was adjusted to pH 8.5. Immediately prior to application of the sample to the stacking gel, the sample was solubilized by boiling for 2 min in a solution containing 0.5 M urea, 2% SDS, and 2% mercaptoethanol. Bovine serum albumin, ovalbumin, chymotrypsinogen, and deoxyribonuclease (Sigma Chemical Co., St. Louis, Mo.) were prepared in 1-mg/ml concentrations and were used as molecular weight markers. Electrophoresis was carried out at 15 mA/slab until the bromophenol blue marker approached the end of the slab. After electrophoresis the gels were fixed, stained, destained, and photographed as described above for PGGE. Estimates of molecular weights were determined by plotting the relative migration of known molecular weight standards versus the logarithm of their molecular weights, as described by Weber and Osborn (31).

Iodination. Iodination of purified HB_eAg with ^{125}I was carried out by the chloramine-T method of Greenwood et al. (8), as modified by Hollinger et al. (9). Purified preparations of HB_eAg (20 μg) were iodinated for 30 s and desalted on Sephadex G-200.

Protein A-Sepharose CL-4B chromatography. A 1.5-by-3.3-cm column of protein A conjugated to Sepharose CL-4B was prepared by the manufacturer's instructions (Pharmacia). A 0.5-ml volume of purified ^{125}I-labeled HB_eAg was applied to the column bed and allowed to incubate at room temperature for 10 min. The column was washed with PB, and 0.6-ml fractions were collected. Each fraction was assessed by gamma spectrometry for total counts per minute. When the counts per minute approached background level, the column was eluted with 0.2 M $NaHCO_3$ buffer (pH 10.9) containing 0.5 M NaCl. The percentage of counts per minute washed and eluted from the column was determined. An equal volume of eluted labeled HB_eAg was mixed with HB_eAg-positive plasma and subjected to radio-agar gel diffusion. After 72 h of incubation the gels were washed, stained, destained, and exposed to NS-2T X-ray film (Kodak). After a 24-h exposure the film was developed.

Sedimentation coefficient. The method of McEwen (13) was used in estimating the sedimentation coefficient of purified HB_eAg. A 10 to 40% (wt/wt) linear sucrose gradient was overlaid with 0.5 ml of purified HB_eAg and centrifuged in an SW50 rotor at 35,000 rpm at 5°C for 18 h. The gradient was fractionated from the top in 0.2-ml fractions by an ISCO model 640 density gradient fractionator equipped with an ISCO UA-4 monitor to record the optical density (at 280 nm; OD_{280}) profile. Each fraction was assayed for percent sucrose by determining refractive indices and for HB_eAg activity by rheophoresis.

Immunodiffusion analysis. Purified HB_eAg was reacted against chimpanzee anti-HB_e and rabbit anti-human IgG in a three-well arrangement by use of

rheophoresis plates. The immunoprecipitates were washed, stained, and destained as described above.

Immunoabsorption analysis. Purified HB$_e$Ag was mixed with equal volumes of either chimpanzee anti-HB$_e$ or rabbit anti-human IgG (Behring Diagnostics). The mixture was allowed to incubate at 37°C for 1 h and then overnight at 4°C. After incubation the mixture was centrifuged at 14,000 × *g* for 30 min, and the supernatants were assayed for HB$_e$Ag activity by rheophoresis.

RESULTS

Fractionation of plasma on DEAE-cellulose. Fractionation of plasma on DEAE-cellulose using the buffer schedule listed in Table 1 was previously reported by Oh and Sanders (23). The fractionation of chimpanzee HB$_e$Ag-positive plasma made albumin poor by precipitation with equal volumes of saturated ammonium sulfate is presented in Fig. 2. A total of 5.75 g of protein was applied to the column and fractionated into 19 optical density peaks representing an 82% recovery of total protein. Each peak was pooled and concentrated to the original sample volume (100 ml) by ultrafiltration. Each pool was assayed for HB$_e$Ag/1, HB$_e$Ag/2, HB$_s$Ag, and milligrams of protein per milliliter. In addition, each pool was analyzed by immunoelectrophoresis against rabbit anti-normal human serum and by PGGE. Figure 2 shows that HB$_e$Ag/1 was found in the first 17 pools. HB$_e$Ag/2 appeared in pools II through XVII. Pool II contained both HB$_e$Ag/1 and HB$_e$Ag/2 at a rheophoresis titer of 1:16. HB$_s$Ag was found

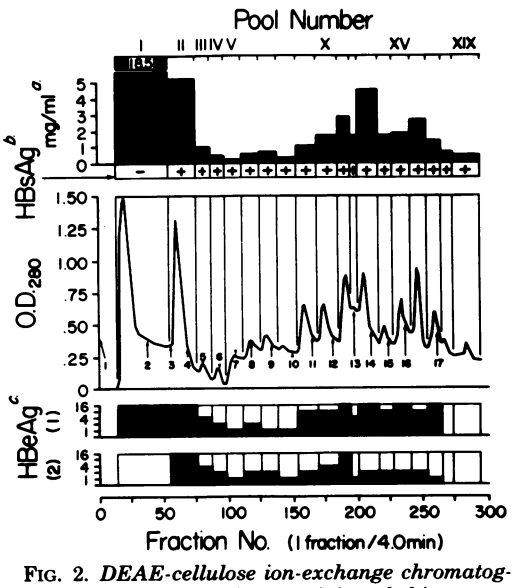


FIG. 2. *DEAE-cellulose ion-exchange chromatography of ammonium sulfate-precipitated chimpanzee HB$_e$Ag-positive plasma.*

present in every pool except pool I where HB$_s$Ag-negative sera were used as controls. However, when PBNN was used as the negative control, the counts per minute obtained were almost twice as high as the negative sera. Using this value as the negative control, pool II was found negative for HB$_s$Ag. There was little correlation between the appearance of HB$_e$Ag and HB$_s$Ag. For example, both pools XVIII and XIX had HB$_s$Ag activity but did not have HB$_e$Ag activity. HB$_e$Ag/1 and HB$_e$Ag/2 were not found in constant relative proportions. For example, pool II had equal proportions of HB$_e$Ag/1 and HB$_e$Ag/2, whereas pool XVII had an HB$_e$Ag/1 titer of 1:16 and an HB$_e$Ag/2 titer of undilute.

IEP of each pool using rabbit anti-normal human serum is presented in Fig. 3. Pool I contained one major IgG immunoprecipitate and perhaps one other. IEP of pool II showed four immunoprecipitin lines. Pools III through IX had very similar patterns, demonstrating plasma proteins possessing a more positive charge than the very complex patterns found in pools X to XIX.

PGGE (Fig. 4) of each pool demonstrated that pool I had IgG as its major component with a trace amount of transferrin. IgG did not electrophorese to its limiting porosity in this system as evidenced by the smeared, stained globulin covering the top half of the slab. Pool II had at least five plasma proteins, including a major smear of protein which may have been IgG and fibrinogen. The tentative identification of plasma proteins by PGGE has been made by their relative migration compared to whole plasma. The presence of fibrinogen in pool II was confirmed by IEP using specific rabbit anti-fibrinogen. Pools III through VIII had very similar profiles. Each successive pool became increasingly more complex in the number of plasma proteins present, with pool XVI being the most complex. Pools XVII through XIX had fewer numbers of plasma proteins. Thus, the IEP and the PGGE data were very similar, indicating that the pools became increasingly more complex with regard to the number of plasma proteins found in each pool. Because of the high titer of HB$_e$Ag found in pool II and the relatively few numbers of plasma proteins present by IEP and PGGE, pool II was used for further purification of HB$_e$Ag.

Fractionation of serum by DEAE-cellulose. Chimpanzee-positive HB$_e$Ag plasma was converted to serum (to remove fibrinogen), made albumin poor, and chromatographed on DEAE-cellulose by the buffer schedule presented in Table 1. Fractionation was continued, however, only until the third optical density

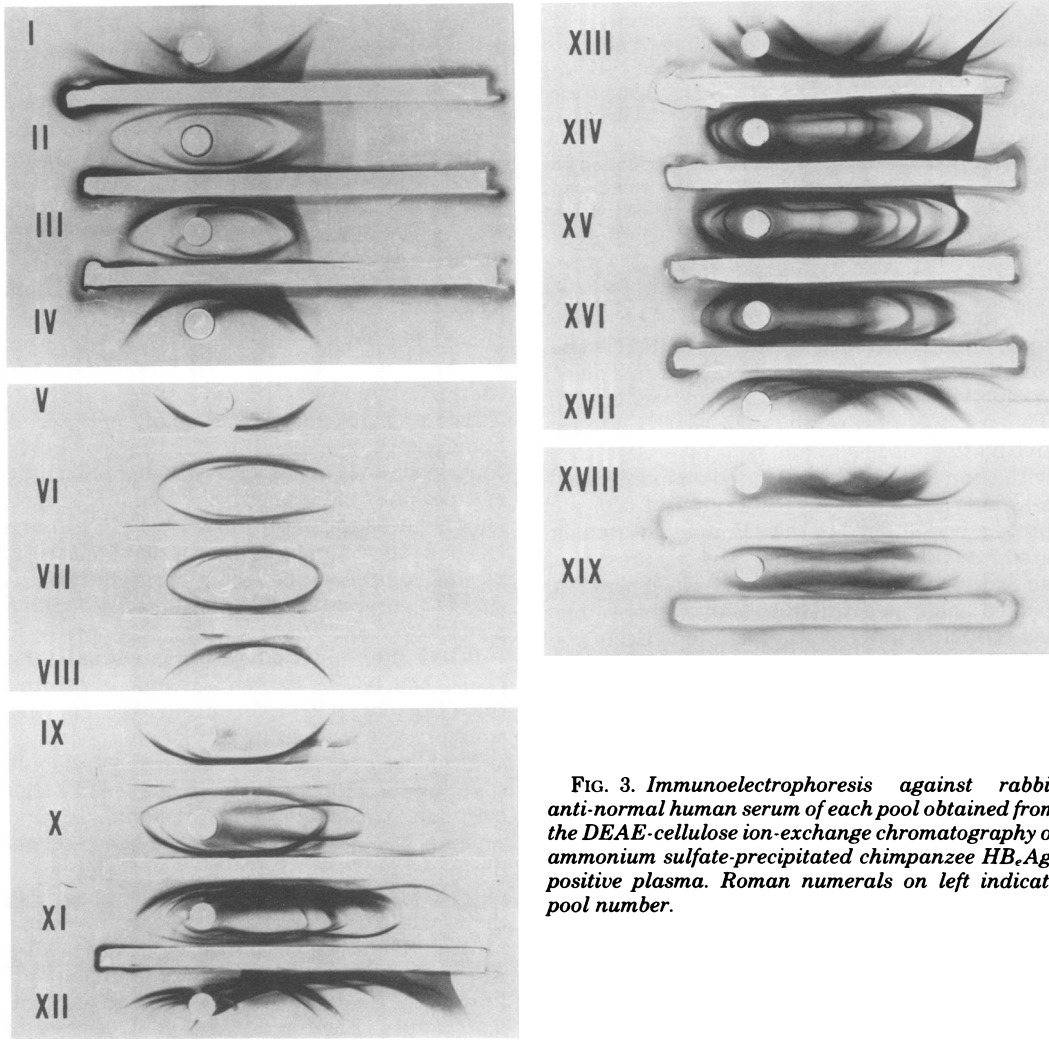
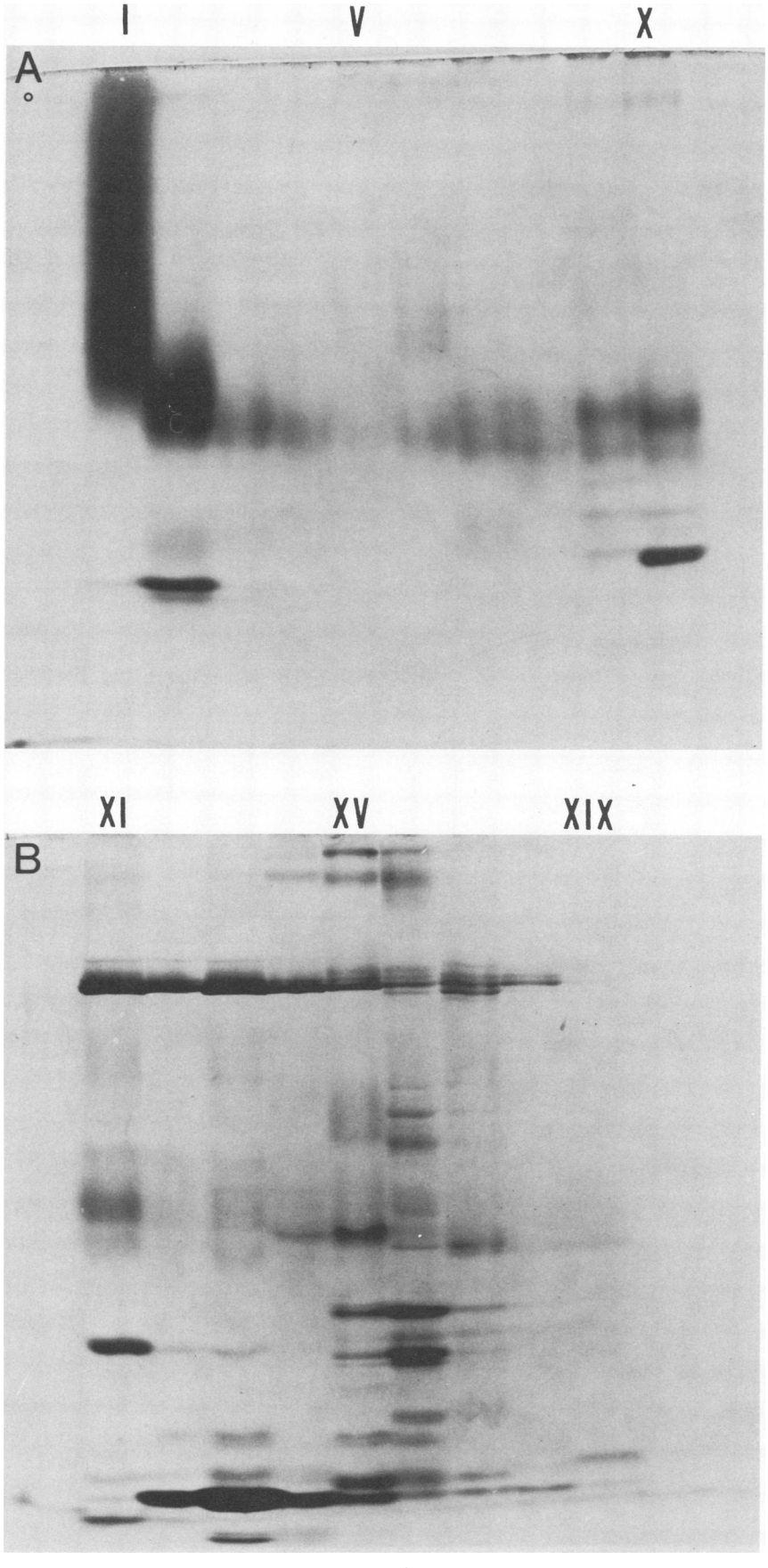


FIG. 3. *Immunoelectrophoresis against rabbit anti-normal human serum of each pool obtained from the DEAE-cellulose ion-exchange chromatography of ammonium sulfate-precipitated chimpanzee HB_eAg-positive plasma. Roman numerals on left indicate pool number.*

peak eluted (Fig. 2). The optical density height of the second peak was lower due to the removal of fibrinogen. Pool II was made as shown in the figure. The pool was concentrated to approximately 15 ml and analyzed by IEP against various rabbit anti-human plasma proteins (Fig. 5) and by PGGE (see Fig. 8). Of the plasma proteins assayed, only IgG, transferrin, plasminogen, and β_2-glycoprotein were detected. However, PGGE demonstrated the presence of two additional plasma proteins.

Gel filtration. Pool II was concentrated to approximately 15 ml by ultrafiltration and applied to a 2.6-by-90-cm column of AcA22 (Fig. 6). Every other fraction was assayed for HB_eAg, IgG, and transferrin. HB_eAg activity eluted at the beginning of the optical density profile. The bulk of the protein material, as evidenced by the optical density tracing, was comprised of IgG and transferrin. Fractions 104 through 122 were pooled and concentrated to 3 ml by ultrafiltration. The concentrate was applied to a 2.6-by-90-cm column of Sephacryl S-200 (Fig. 7). Each fraction was assayed for HB_eAg, IgG, and transferrin. Two optical density peaks were obtained; the first peak was comprised of HB_eAg and IgG, whereas the second peak was comprised of only IgG. HB_eAg peaked at fraction 49, and IgG

FIG. 4. *Polyacrylamide gradient gel electrophoresis of pools obtained from the DEAE-cellulose ion-exchange chromatography of ammonium sulfate-precipitated chimpanzee HB_eAg-positive plasma. (A) Pools I through X; (B) pools XI through XIX.*


I
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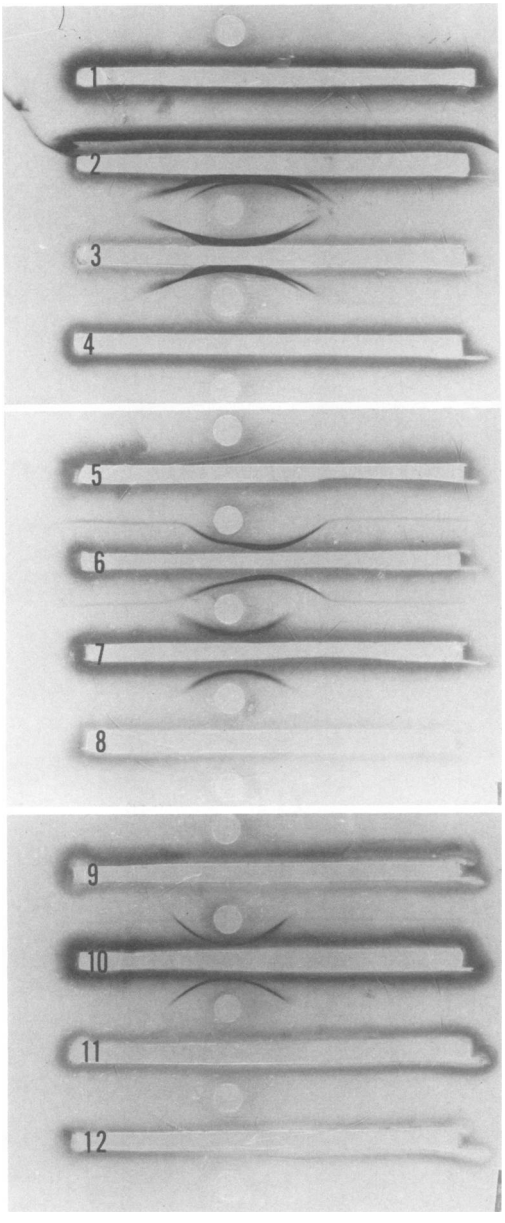

FIG. 5. *Immunoelectrophoresis against various anti-plasma proteins of concentrated pool II obtained from the DEAE-cellulose ion-exchange chromatography of ammonium sulfate-precipitated chimpanzee HB_eAg-positive serum. (1) Chimpanzee anti-HB_e; (2) rabbit anti-normal human serum; (3) rabbit anti-human IgG; (4) rabbit anti-human IgM; (5) rabbit anti-human IgA; (6) rabbit anti-human transferrin; (7) rabbit anti-human plasminogen; (8) rabbit anti-human fibrinogen; (9) rabbit anti-human C3/C3C; (10) rabbit anti-human β_2-glycoprotein I; (11) rabbit anti-human hemopexin; (12) rabbit anti-human β-lipoprotein.*

peaked at fraction 55. No transferrin was detected. Fractions 48, 49, and 55 were analyzed by PGGE (Fig. 8), and fractions 49 and 55 were analyzed by IEP (Fig. 9).

PGGE demonstrated that only one broad protein band was observed in each fraction. The protein stained in fractions 48 and 49 had a higher relative molecular weight than the protein stained in fraction 55. The broad bands are typical for IgG species. It is important to note that each fraction contained only one protein, and fractions 48 and 49 were not contaminated by the material found in fraction 55, indicating that these populations had been resolved.

IEP of fraction 49 demonstrated the presence of IgG and a trace of HB_eAg. IEP of HB_eAg required about 10 times more antigen for detection than rheophoresis (data not shown). IEP of fraction 55 only demonstrated the presence of IgG. Thus, HB_eAg appeared in the same fraction as a higher-molecular-weight IgG species free from all other detectable plasma proteins.

SDS-PAGE. Fraction 48 and fraction 55 were subjected to high-resolution slab gel SDS-PAGE. A photograph of the stained slabs is presented in Fig. 10. The major stained bands in fraction 48 and fraction 55 correspond in molecular weight to the heavy and light chains of commercially available standard IgG (Pentex Inc., Kankakee, Ill.), 55,000 and 25,000, respectively. The other minor bands present were found in both fractions 48 and 55 and are either artifactual or represent additional breakdown polypeptides common to both the first and second optical density peaks.

Sepharose CL-4B protein A. Twenty micrograms of protein from fraction 49 were iodinated, desalted, and chromatographed through a 1.5-by-3.3-cm column of Sepharose CL-4B conjugated with protein A as described in Materials and Methods. Ninety-three percent of the total effluent counts was eluted with 0.2 M $NaHCO_3$ (pH 10.9). The eluted material was mixed with equal volumes of HB_eAg-positive plasma and subjected to radio-agar gel diffusion. After development of the X-ray film, an autoradiographic line appeared which was coincident with the HB_eAg–anti-HB_e–stained immunoprecipitate and when superimposed showed lines of identity with controls.

Sedimentation coefficients. Purified HB_eAg obtained from fraction 48 after chromatography through Sephacryl S-200 was sedimented in a Beckman SW50 rotor, fractionated, and assayed as described in Materials and Methods. The optical density profile demonstrated a gaussian distribution (data not shown) indicative of a homogeneous population. Purified

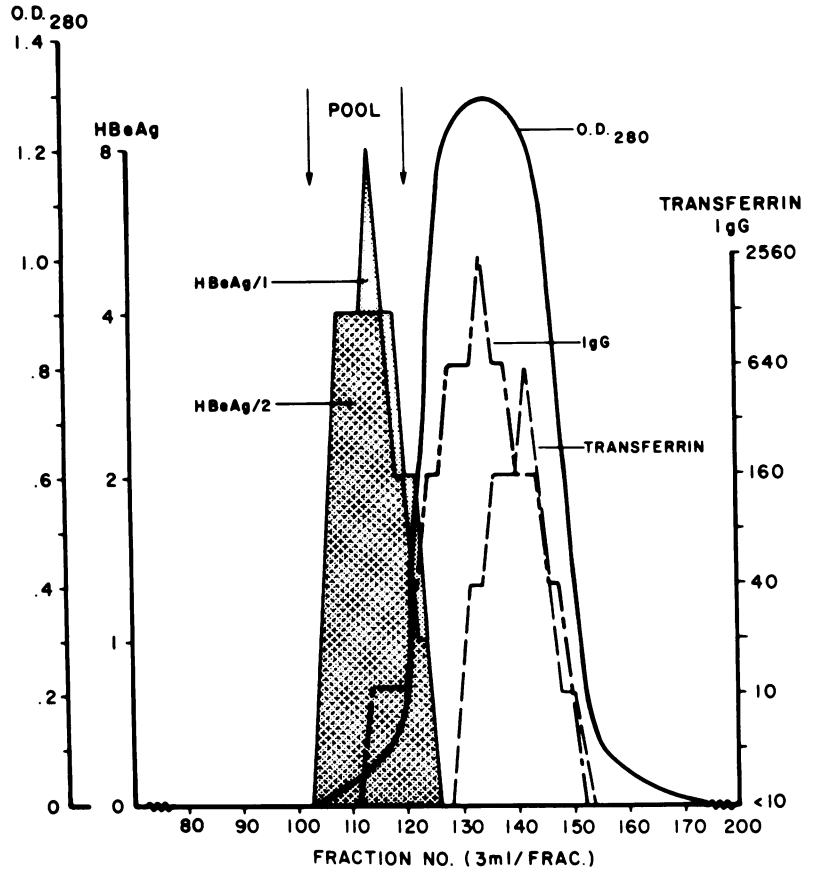

FIG. 6. *Gel filtration on AcA22 of concentrated pool II from DEAE-cellulose ion-exchange chromatography of ammonium sulfate-precipitated HB$_e$Ag-positive serum.*

HB$_e$Ag sedimented to a sucrose concentration of 21.6%, which corresponds to an average sedimentation coefficient of 11.6*s*.

Immunodiffusion analysis. In a three-well pattern using rheophoresis plates, purified HB$_e$Ag (fraction 48) gave uninterpretable lines due to the presence of a very large immunoprecipitate between rabbit anti-human IgG and chimpanzee anti-HB$_e$.

Immunoabsorption analysis. Purified HB$_e$Ag was incubated with chimpanzee anti-HB$_e$ and rabbit anti-human IgG. After incubation, HB$_e$Ag activity was assayed by rheophoresis. Undilute chimpanzee anti-HB$_e$ removed all of the HB$_e$Ag activity. A 1:10 dilution of rabbit anti-human IgG reduced the HB$_e$Ag titer from 1:8 in the control to undilute. Using more anti-IgG removed all of the purified HB$_e$Ag; however, enough residual anti-IgG remained after absorption to produce an anti-IgG:IgG immunoprecipitate. A 1:10 dilution eliminated this problem.

DISCUSSION

Neurath and Strick (20) reported that HB$_e$Ag has properties of an immunoglobulin, predominantly of the IgG$_4$ subclass. They also speculated that HB$_e$Ag is an idiotypic determinant formed in response to HBV infection which blocks the host's immune surveillance mechanisms by which virus proliferation is stopped. We present confirmational evidence that HB$_e$Ag exists as a dimer of IgG or is associated with an IgG complex. However, the nature of the determinant site remains speculative.

This study presents a method for purifying HB$_e$Ag from plasma based on ion-exchange chromatography followed by gel filtration chromatography. Ion exchange on DEAE-cellulose demonstrated that HB$_e$Ag was found in many optical density peaks (Fig. 2). This suggested that HB$_e$Ag is heterogeneous with respect to charge or is associated with other plasma proteins. The second optical density peak contained the highest concentration of HB$_e$Ag/1 and

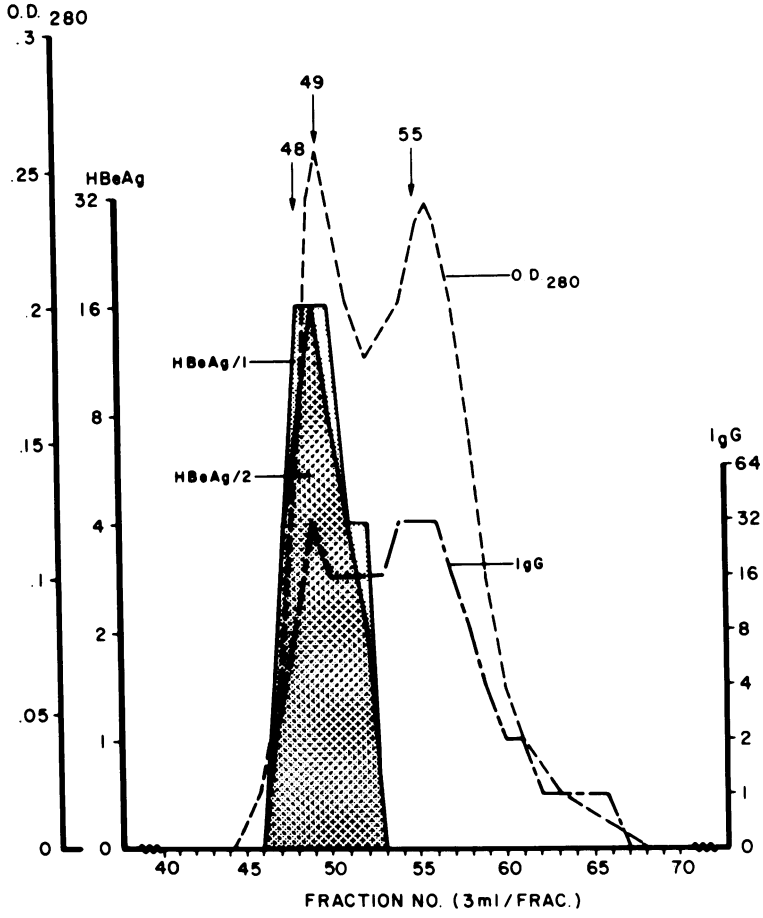

FIG. 7. *Gel filtration on Sephacryl S-200 of pooled HB$_e$Ag-positive fractions from gel filtration on AcA22.*

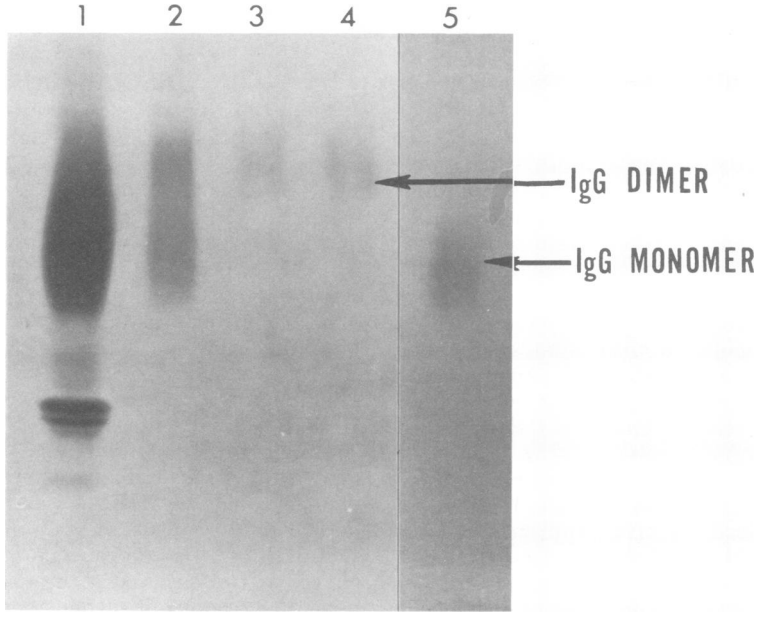

FIG. 8. *Polyacrylamide gradient gel electrophoresis at various steps of HB$_e$Ag purification from chimpanzee HB$_e$Ag-positive serum. (1) Concentrated pool II after DEAE-cellulose fractionation; (2) concentrated pool after AcA22 fractionation; (3) fraction 48 after Sephacryl S-200 fractionation; (4) fraction 49 after Sephacryl S-200 fractionation; (5) fraction 55 after Sephacryl S-200 fractionation.*

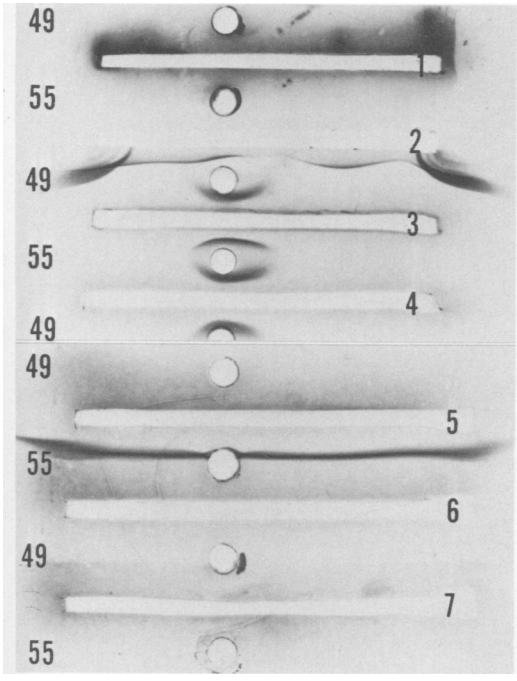

FIG. 9. *Immunoelectrophoresis against various anti-plasma proteins of selected fractions from Sephacryl S-200 fractionation. Numbers on left indicate fraction numbers. (1) Chimpanzee anti-HB_e; (2) blank; (3) rabbit anti-normal human serum; (4) rabbit anti-human IgG; (5) rabbit anti-human transferrin; (6) rabbit anti-human β_2-glycoprotein I; (7) rabbit anti-human plasminogen.*

HB_eAg/2 and the fewest number of plasma proteins (Fig. 4). HB_sAg appeared also to elute in the second peak; however, when PBNN was used as the negative control, the second peak became negative for HB_sAg. This second peak was pooled, concentrated, and chromatographed on AcA22 which separated HB_eAg from the bulk of the other plasma proteins present. HB_eAg-positive fractions were pooled, concentrated, and rechromatographed on Sephacryl S-200 which further removed any monomeric species of IgG from HB_eAg. Recycling gel filtration chromatography with other media (AcA34, LKB; A0.5 M, Bio-Rad) has proven equally effective.

Evidence which suggests that HB_eAg exists as a dimer of IgG is as follows: (i) SDS-PAGE demonstrated the presence of polypeptides which have the same relative migration as heavy and light chains of IgG; (ii) Sepharose CL-4B-conjugated protein A had an extremely high affinity for purified HB_eAg; (iii) immunoabsorption with rabbit anti-human IgG resulted in the removal of purified HB_eAg; (iv) PGGE of purified HB_eAg resulted in a characteristic electrophoretic pattern of IgG; (v) IEP of purified HB_eAg resulted in an immunoprecipitate in the fast gamma region; (vi) sedimentation coefficient analysis and gel filtration chromatography demonstrated that HB_eAg is larger than the monomeric form of IgG. These results provided evidence that HB_eAg exists as or is associated with a dimer of IgG. It should be noted, however, that ion-exchange chromatography may have selected out IgG dimers (pool II) which are associated with HB_eAg.

High-resolution slab gel SDS-PAGE demonstrated the presence of polypeptides corresponding to heavy and light chains of purified IgG. In addition, several other polypeptides were resolved. These other polypeptides, however, were resolved in both fractions 48 and 55. Since HB_eAg was not detected in fraction 55, and fraction 48 represented high HB_eAg activity, these polypeptides probably are not associated with HB_eAg. The nature of these minor peptides awaits further analysis.

Protein A reacts with the Fc region of IgG from several species (7, 10, 26) and is able to bind two or more molecules of IgG per molecule of protein A (27). Kronvall and Williams (11) found that the reaction is specific for IgG subclasses 1, 2, and 4. This property of protein A results in a specific affinity chromatographic procedure for the isolation of IgG molecules. Because purified HB_eAg bound to and eluted from a protein A–Sepharose CL-4B column, it must share properties with the Fc portion of IgG subclass 1, 2, or 4. This result provided additional supportive evidence that HB_eAg is or is associated with an IgG.

Immunoabsorption studies of purified HB_eAg with chimpanzee anti-HB_e and rabbit anti-human IgG demonstrated that both antisera removed HB_eAg activity. This strongly suggests that the HB_eAg determinant and the IgG determinant reside on the same molecule or complex of molecules. We are attempting to prepare monospecific anti-HB_e in rabbits to be used in an immunodiffusion study to establish measurement of identity.

Electrophoresis of purified HB_eAg in gradient gels of polyacrylamide (PGGE; Fig. 8) demonstrated a smear of stainable protein characteristic of IgG (K. Acuff, Isolabs, Akron, Ohio, personal communication). In addition, this smear migrated slightly less into the gel than the monomeric form of IgG (fraction 55). The smear on PGGE is probably the result of the pH of the buffer not being high enough to drive the molecules towards the anode (18).

Sedimentation coefficient analysis of purified HB_eAg resulted in an $s_{20,w}$ value of 11.6. A linear relationship on a logarithmic scale was found

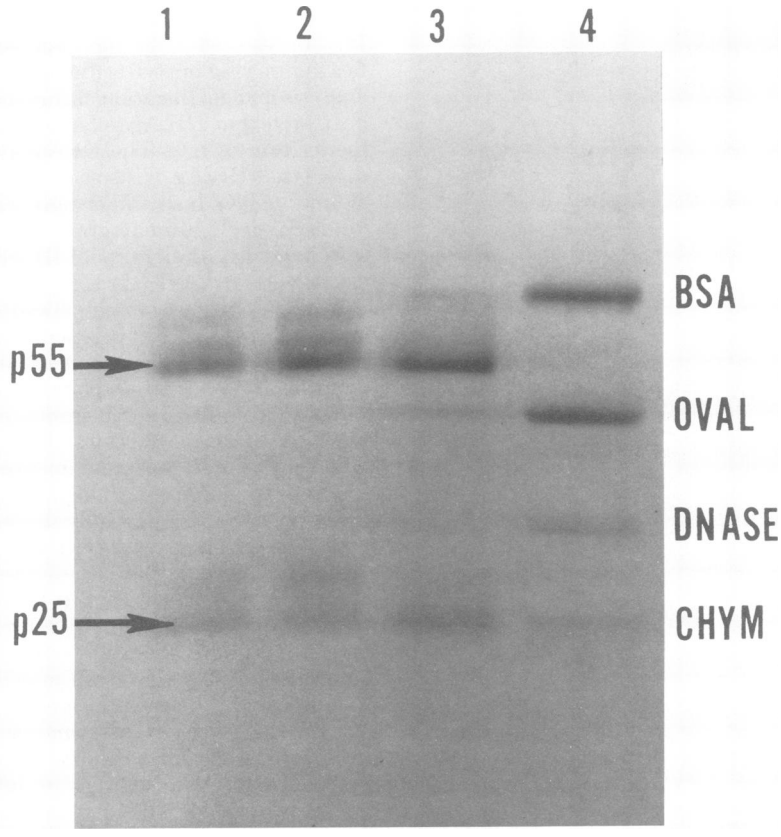

FIG. 10. *SDS-PAGE of selected fractions from Sephacryl S-200 fractionation. (1) Fraction 48; (2) fraction 55; (3) human IgG fraction II (Pentex, Inc.); (4) molecular weight standards: BSA, bovine serum albumin; OVAL, ovalbumins; DNASE, deoxyribonuclease; CHYM, chymotrypsinogen.*

between $s_{20,w}$ and the molecular weight of phage (24). An empirical equation relating $s_{20,w}$ and molecular weight was derived: $s_{20,w} = 1.114 \times 10^{-3} \times MW^{0.729}$, where MW is the molecular weight. This relationship also holds true for IgG with an $s_{20,w}$ value of 6.9. Substituting 11.6 for the $s_{20,w}$ value of HB_eAg, a molecular weight of 324,000 was obtained. This correlates with its relative elution position from the Sephacryl S-200 gel filtration column.

We have presented in this study a purification method for HB_eAg and a partial characterization. The antigen can be purified relatively easily by conventional chromatographic means. Partial characterization strongly suggests that the antigen is associated with a dimer of IgG. However, considering the heterogeneity of the antigen on ion-exchange chromatography, we may have selected out only a population of molecules possessing the HB_eAg determinant. The data are also consistent with the interpretation that HB_eAg is strongly associated with IgG and that an additional polypeptide was not demonstrated by PAGE analysis because of sensitivity limits. The correct interpretation must await the development of antisera which would permit demonstration of immunological identity between HB_eAg and diameric IgG. Further analysis of the other optical density peaks from ion-exchange chromatography may also answer this question. In addition, experiments are continuing to further define the nature of the determinant site and ultimately its relationship to the chronic carriage state.

ADDENDUM

After this manuscript was submitted for publication, Vyas et al. (Science **198**:1068–1070, 1977) reported that HB_eAg was associated with an abnormal serum lactate dehydrogenase isozyme-5 which they termed LDH-5ex. We are presently investigating this relationship.

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