Biochemical Characterization of Australia Antigen

Evidence for Defective Particles of Hepatitis B Virus

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Australia antigen exists in the sera of chronic carriers in several particulate forms, one of which may represent the virion of hepatitis B. This report describes the existence of subpopulations of these 43-nm particles, the Dane particles, on the basis of the staining properties of their internal cores and banding characteristics in cesium chloride (CsCl) density gradients. These data suggested that only a minor proportion of Dane particles contained an intact viral genome and represent the standard infectious virus of hepatitis B. The bulk of the Dane particles appeared to be deficient in viral nucleic acid and, as defective interfering particles, may specifically interfere with the growth of standard virus. Such defective interfering particles could thereby play a role in the persistence of HBV infection in man. (Am J Pathol 81:651–668, 1975)

AUSTRALIA ANTIGEN, now referred to as the hepatitis B surface antigen (HB_sAg), circulates in the sera of chronic carriers as a variety of spherical and filamentous particles which are grouped into three categories based on their morphologic forms: a) the approximately 22-nm spherical particles, b) filaments of various lengths, and c) the 43-nm spherical particles also known as Dane particles. The Dane particle [1] exhibits a complex structure with a lipoprotein outer coat surrounding an internal 27-nm particulate core.[2] All three forms of HB_sAg share common antigens on their surfaces while the core of the Dane particle (HB_cAg) represents a distinct antigenic system.[2-5]

The association of HB_sAg with Type B hepatitis is now well established, and the accumulated evidence indicates that the antigens associated with HB_sAg are specified by the genome of the hepatitis B virus (HBV). The Dane particle represents only a minor component of the total mass of HB_sAg in serum; the bulk of it being composed of the 22 nm and filament forms. There now exists, however, a body of circumstantial but convincing evidence that the Dane particle is the actual virus of Type B hepatitis (HBV), while the remaining forms represent viral coat protein produced in excess by the infected hepatocyte. Such evidence includes the presence of cores and HB_cAg in the nuclei of infected heptocytes,[6-10] the presence

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of DNA polymerase activity in the core [4,11,12] and the isolation of circular double-stranded DNA from the core,[13] and the epidemiologic association of DNA polymerase–containing Dane particles with disease.[14-16]

We describe in this report the existence of subpopulations of Dane particles in the sera of chronic HB_sAg carriers; these populations are defined by the staining properties of their internal cores and different buoyant densities in CsCl. From these observations, we postulate the existence of defective interfering forms of HBV, and, by comparison with other animal virus systems, propose that defective interfering particles may constitute a factor in the persistence of noncytocidal HBV infection in man as represented by the asymptomatic chronic HB_sAg carrier.

Materials and Methods

Source of Dane Particles

Dane particles were obtained from the sera of high-titered asymptomatic carriers of both HB_sAg/adw and HB_sAg/ayw subtypes. The plasma was collected by double-unit plasmapheresis and was stored frozen at −20 C after conversion to serum with $CaCl_2$.

Immune Electron Microscopy

Aliquots of sera were examined by immune electron microscopy (IEM) by a microassay modification of the method described by Almeida, *et al.*;[2] small (0.8 ml) cellulose nitrate centrifuge tubes (Beckman, Inc., Palo Alto, Calif.) were employed for both the incubation and pelleting steps of the procedure. Serum (50 μl) was added to each tube followed by 100 μl of either guinea pig hyperimmune anti-HB_s/ad serum, normal guinea pig serum, or phosphate-buffered saline (PBS). The guinea pig sera were clarified by centrifugation at 40,000 rev/min for 1 hour before use. The mixtures were diluted to 300 μl with PBS, incubated for 1 hour at 37 C, further diluted to 600 μl with PBS, and centrifuged for 30 minutes at 15,000 rev/min in the Spinco Type SW 50.1 rotor. The supernatants were removed, and the tubes drained in an inverted position for 10 to 15 minutes. The pellets were then resuspended in a final volume of 600 μl of PBS and centrifuged as described above. After removal of the supernatant, the drained pellet was resuspended in 50 μl of distilled water and examined by electron microscopy.

Preparation of Dane Particle Concentrates

The Dane particles from selected sera were enriched and partially purified by direct pelleting in the Spinco Type 21 rotor for 4 hours at 20,000 rev/min and 4 C. Each pellet was washed by resuspension to original volume in PBS followed by recentrifugation as described above. The final pellet was resuspended in one-twentieth of its original volume, which was then dispensed in 1-ml aliquots and stored frozen at −70 C until used for experimental studies.

Density Gradient Centrifugation

Isopycnic banding in cesium chloride density gradients employed standard procedures with discontinuous gradients as described in the figure legends. The density of CsCl fractions was determined by refractometry.

Solid-Phase Radioimmunoassays

Microtiter solid-phase radioimmunoassays (SP-RIA) for HB_sAg and HB_cAg were done as previously described.[5,17] A guinea pig hyperimmune serum was used as the source of anti-HB_s and a well-characterized human convalescent serum (Beken) as antibody to HB_cAg.

Electron Microscopy

Dane particle concentrates and precipitates from the IEM studies were negatively stained with 1% phosphotungstic acid (PTA) and examined in an Hitachi HU-11E-1 electron microscope. Sections of human liver and Dane particle pellets were examined after fixation, embedding, and staining by standard procedures as described in the appropriate figure legends.

Results

Immune Electron Microscopy

Plasma units from chronic HB_sAg carriers were selected for high titer (1:200 to 1:800 by complement fixation assay[18]) and antigenic subtype (*adw* or *ayw*) and screened by IEM as possible source material for Dane particle purification. The precipitates obtained from the reaction of HB_sAg with specific antibody were examined by electron microscopy after negative staining. Figure 1 demonstrates the association of specific antibody with all three forms of HB_sAg and cross-linking between the forms based on their common *a* determinant. Pellet fractions from control tubes with test serum and normal guinea pig serum or PBS contained single Dane particles, filaments, and 22-nm forms without demonstrable antibody, and, in many cases, immune aggregates of various sizes. An unusually large immune aggregate of 22-nm forms is shown in Figure 2 and we interpret these aggregates to represent circulating immune complexes. When 12 *adw* and 12 *ayw* units were examined by this technique, no significant differences were observed with regard to subtype in the proportion of units containing either Dane particles or circulating immune complexes (Table 1). Dane particles were observed in 83% of the 24 units, while 71% of those units had evidence of circulating immune complexes.

Based on this point study, it appears that most, if not all, asymptomatic

Table 1—Immune Electron Microscopy of Serum From Asymptomatic Chronic Carriers of HB_sAg

Subtype	Dane particle positive	Immune complex positive
HB_sAg/*adw*	11/12 (92%)	9/12 (75%)
HB_sAg/*ayw*	9/12 (75%)	8/12 (67%)
Total	20/24 (83%)	17/24 (71%)

carriers of HB_sAg circulate virions in their sera, regardless of subtype. Furthermore, even with high concentrations of circulating antigen, these carriers demonstrated a functional, if ineffectual, humoral immune response.

Preparation of Dane Particle Concentrates

Sera selected by IEM to contain high concentrations of Dane particles were concentrated and partially purified by direct pelleting under moderate centrifugal conditions. These conditions removed most of the 22-nm forms and normal serum proteins but pelleted greater than 95% of the Dane particles. Twentyfold concentrates from such sera were examined by electron microscopy after negative staining. Dane particles represented the predominant particulate form in most preparations, although variable quantities of filaments, 22-nm forms, and host material were also present. The Dane particles measured 43.6 nm $\pm$ 2.7 nm in diameter, in good agreement with published values.[1,2,19] Analysis of core diameters in such preparations showed a considerable variation with an average value of 24.3 nm $\pm$ 4.0 nm; this measurement is lower than the published values of 27 nm.[2,19,20]

Subpopulations of Dane Particles Based on the Staining Properties of Cores

Dane particle concentrates were examined in thin sections as described in the legend for Figure 3. Electron micrographs (Figure 3) revealed that the Dane particles could be classified into several categories, based on the staining properties of their internal cores: *full cores*, *partially full cores*, or *empty cores*. Quantitative evaluation of six Dane particle–rich preparations (Table 2) indicated that particles with full and empty cores represented only a minor proportion of the total Dane particle population of any preparation, the bulk of which were classified as being partially full. In six preparations the percentage of Dane particles with full cores ranged from 3.4 to 13.0%, with an average value of 7.2%.

Table 2—Distribution of Dane Particles According to Staining Properties of Cores

Serum	Dane particle cores			Total
	Full	Partial	Empty	
1	49 (13.0%)	279 (73.8%)	50 (13.2%)	378
2	28 (11.3%)	179 (72.2%)	41 (16.5%)	248
3	8 (11.3%)	61 (85.9%)	2 (2.8%)	71
4	6 (5.8%)	92 (88.4%)	6 (5.8%)	104
5	41 (4.9%)	728 (87.6%)	62 (7.5%)	831
6	14 (3.4%)	374 (91.2%)	22 (5.4%)	410
Total	146 (7.2%)	1,713 (83.9%)	183 (8.9%)	2,042

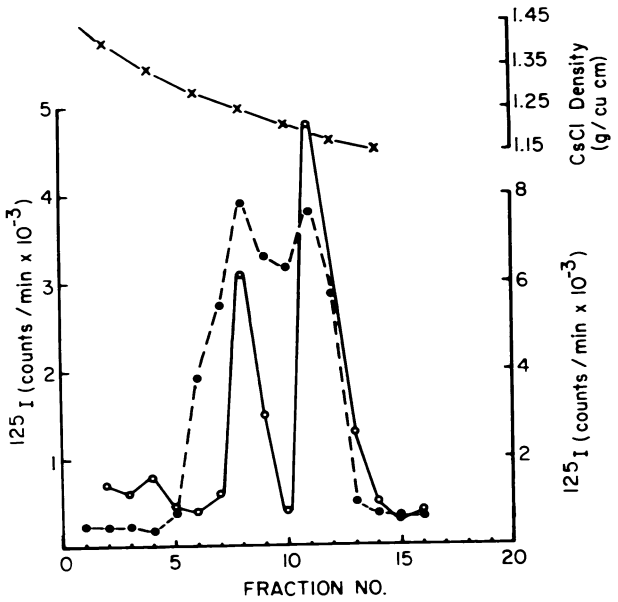
As suggested by Jokelainen *et al.*,[19] the staining intensity of Dane particle cores with uranyl acetate was due to the presence of nucleic acid within the core. Robinson *et al.*[13] reported the direct isolation of circular double-stranded DNA from core particles. If the Dane particle is indeed the virus of hepatitis B, these observations suggest that only a minor population of the Dane particles which circulate in the sera of chronic carriers contain the complete HBV genome. Furthermore, the ratio of partially full to full particles ranged from 6:1 to 27:1. Since the Dane particles with partially full cores probably represent deletion mutants of HBV, they might play a role in the pathogenesis of hepatitis B disease by the mechanism of interference.[21]

Density Populations of Dane Particles

Our routine procedures for the purification of HB_sAg from serum involves two successive bandings in CsCl followed by a rate zonal step in sucrose.[22-25] In the rate zonal step, Dane particles and filaments are recovered near the cushion, well separated from the predominant 22-nm form. If Dane particles consist of populations with different amounts of nucleic acid, they should be separable by virtue of their buoyant densities in suitable density gradients. However, our normal purification procedure would select against a higher density population. A plasmapheresis unit containing Dane particles was divided equally; half was subjected to our routine purification procedure and the other half was used to prepare a twentyfold concentrate of Dane particles by pelleting as described above. When the fractions from the rate zonal step which contained the Dane particle forms were rebanded in CsCl, the Dane particles banded together with the filaments in a sharp zone at 1.20 g/cu cm with no evidence of density heterogeneity. Isopycnic banding of the Dane particle concentrate, however, revealed two populations of particles at 1.19 and 1.25 g/cu cm, respectively (Text-figure 1). Dane particles in this gradient were defined as particulate structures containing both HB_sAg and HB_cAg reactivity by the microtiter solid phase radioimmunoassays.[5,17]

Figure 4 shows a similar gradient with another Dane particle concentrate in which two light-scattering bands were observed at 1.20 and 1.25 g/cu cm, respectively. These bands were isolated and examined by electron microscopy after negative staining with 1% PTA. The 1.20 g/cu cm band (Figure 5A) contained both filaments and Dane particles while the higher density (1.25 g/cu cm) band was composed of Dane particles essentially free of other HB_sAg forms (Figure 5B); a micrograph of this fraction at lower magnification (Figure 6) better demonstrates the composition of this fraction.

The demonstration of density subpopulations of Dane particles appears



TEXT-FIGURE 1—Isopycnic banding of a twentyfold concentrate of Dane particles in a CsCl density gradient. Sample (1 ml) was layered onto an initially discontinuous density gradient (1.1 to 1.5 g cu cm) and centrifuged for 5 hours at 35,000 rev min in an SW Rotor at 4 C. Gradient fractions (0.75 ml) were collected by bottom puncture and assayed for CsCl density (X) by refractometry and HB_sAg (*solid circles*) and HB_cAg (*open circles*) by solid-phase radioimmunoassays.

to verify the prediction of this result which was based on uranyl acetate staining of Dane particle cores. While a direct quantitative association between these observations has not yet been established, the differences in buoyant density of Dane particles probably reflects the nucleic acid content of their cores.

Does the High Density Subpopulation of Dane Particles Contain the HB_sAg-Specific DNA Polymerase Activity

Analysis of eight Dane particle–rich, twentyfold concentrates for DNA polymerase activity,[11] HB_cAg content,[5] and high-density Dane particles revealed a close association between enzyme activity and concentration of high-density Dane particles, the HB_cAg content being more variable.[26] Several preparations with high concentrations of Dane particles and HB_cAg had no detectable high density band and very little polymerase activity. These observations indicated that the endogenous DNA polymerase activity might be associated only with the heavy Dane subpopulation of particles. An earlier report by Kaplan *et al.*[11] using sucrose density gradients suggested such an association. A direct experimental evaluation of this hypothesis is now possible and currently underway in our laboratories using the higher resolving CsCl gradients.

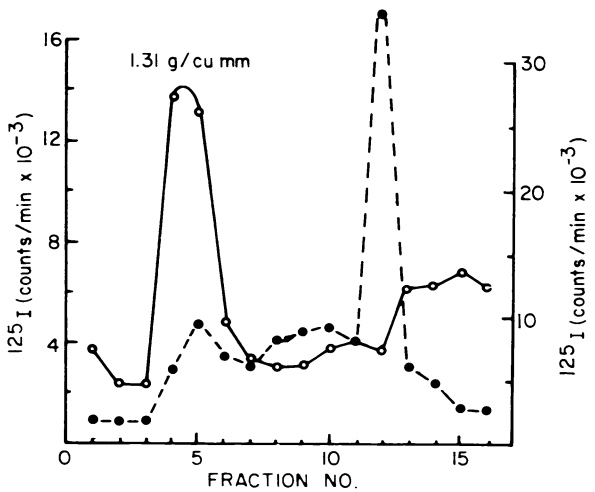
Isolation of Dane Particle Cores

The isolation of Dane particle cores in sufficient quantity for biochemical analysis using serum as a source has been hampered by the scarcity of

Dane particle–rich serum. Text-figure 2 shows the results of one of our early attempts to isolate core particles by isopycnic banding in CsCl of a 1% NP 40–treated Dane particle concentrate. HB_cAg from serum banded with a buoyant density of 1.31 g/cu cm in CsCl, in good agreement with the results of Lipman *et al.*[27] Electron microscopic examination of the peak of HB_cAg fractions, however, revealed aggregated core particles bound together by a soluble protein. Therefore, the 1.31 g/cu cm value did not necessarily represent the actual HB_cAg density due to variable amounts of adsorbed protein. Moritsugu *et al.*[12] recently demonstrated two density populations of DNA polymerase-positive cores; the high-density population banded at 1.35 to 1.36 g/cu cm in CsCl and the low density population between 1.28 to 1.32 g/cu cm. These authors showed that the low-density population was due to the presence of antibody to HB_c on the particles, which is the probable explanation for the protein "tags" described above; they also presented evidence for a population of DNA polymerase-negative cores within the heterogeneous low density population.

Liver appears to represent an excellent source of HB_cAg. Figure 7 is an electron micrograph of a liver obtained at autopsy which showed strong nuclear fluorescence for HB_cAg. Core particles which appear to be mostly empty were observed in high concentration in the nucleus; this field also reveals a Dane particle with a full core in the cytoplasm of the same cell.

Cores have been isolated from liver under conditions in which the problems of coexistent antibody to HB_cAg were avoided. Barker *et al.*[20] isolated cores with a buoyant density range in CsCl of 1.30 to 1.33 g cu cm from the liver of an immunosuppressed chimpanzee; the animal



TEXT-FIGURE 2—Isopycnic banding of NP 40-treated Dane particle concentrate in CsCl. Sample was treated with 1% NP 40 for 1 hour at 37 C, layered onto a discontinuous 1.1 to 1.5 g cu cm CsCl gradient, and centrifuged for 16 hours at 35,000 rev/min and 4 C. Fractions (0.75 ml) were collected by bottom puncture an assayed by solid-phase radio-immunoassays for HB_SAg (*solid circles*) and HB_CAg (*open circles*).

was devoid of circulating antibody to HB_cAg. Hirschman *et al.*[10] reported a similar range of buoyant densities for cores isolated from purified nuclei of human livers and also found these cores to be free of detectable endogenous DNA polymerase activity.

The cumulative data suggest the existence of at least two density populations of HB_cAg, one with a density of about 1.36 g/cu cm in CsCl which contains the endogenous DNA polymerase and another heterogeneous (1.28 to 1.33 g/cu cm) population without DNA polymerase activity. We might speculate that the heavy core population is derived from the high density Dane particles and corresponds to the full cores observed in the stained sections of Dane particle concentrates. Likewise, the heterogeneous low density population of cores may correspond to the light Dane particle population and contain partially full or empty Dane particle cores or both.

Discussion

The 43-nm Dane particle consists of a lipoprotein coat surrounding an approximately 27-nm core and most probably represents the virion of hepatitis B. The 22-nm form of HB_sAg, however, is the predominant form circulating in the sera of chronic carriers and probably represents viral coat protein produced in excess by the infected hepatocyte. While the 22-nm form does not contain viral DNA, it does share antigenic determinants with the outer coat of the Dane particle. As purified from serum and other forms of HB_sAg, the 22-nm form can be considered for use as a subviral vaccine. Indeed, preliminary experiments in seronegative chimpanzees established that the 22-nm form was not infectious, stimulated antibody to HB_sAg, and protected against live HBV challenge.[28] The large excess of viral surface antigen represented by the 22-nm spherical and filament forms undoubtedly affects the course of disease due to competition for receptor sites on susceptible cells and interaction with the various immune effector systems of the host. Disease caused by the extrahepatic deposition of immune complexes, for example, appears to be well documented.[29-31] The observations presented in this paper, however, also suggest another mechanism of interference based on the existence of deletion mutants or defective interfering particles of HBV in the serum of chronic carriers.

Defective interfering particles probably occur in every animal virus system and their defect lies in the loss of some of the viral nucleic acid.[21,32,34] On the basis of this study, the bulk (84%) of the Dane particles from sera contained partially full cores and may represent defective interfering particles. The apparent lack of polymerase activity in such particles could be due to a defect in the endogenous template. The mechanisms

of defective interference are not yet known. Although present as a minor component, Dane particles with full cores probably contain the entire viral genome and thereby represent the standard infectious virus of hepatitis B. By definition, defective interfering particles are noninfectious and specifically interfere with the growth of standard virus. For this reason, attempts to cultivate HBV *in vitro* should be made with preparations of heavy Dane particles or with sera containing a high proportion of heavy particles. Since endogenous DNA polymerase activity appears to be associated only with the heavy Dane population, the ratio of enzyme activity to HB_cAg concentration may be a useful indicator in this regard.

Hepatitis B virus infection is generally accepted to be noncytocidal, the pathology being mediated by the host immune response. Edgington and Chisari [35] proposed that the pathogenesis of the disease is dependent upon a balance between suppressive and cytopathic immune responses, suppression of all or part of the viral gene function in the infected cell being due possibly to humoral antibody as reported for other viruses.[36,37] In many virus systems however, defective interfering particles are required in order to establish persistent noncytocidal infection both *in vivo* and *in vitro*.[33] Neither interferon nor antibody appear to be involved in this phenomenon. Rather, interference is regarded as an intracellular event, the molecular basis of which might vary between different virus systems. As discussed by Holland *et al.*,[33] the evolutionary advantage of DI particles may lie in the protection of the host from the lethal effects of excessive replication in a target organ and the provision of a mechanism for virus persistence in a noncytocidal state. From these considerations, we propose that the initiation of persistent HBV infection in the asymptomatic chronic carrier results from those intracellular mechanisms involved in the production and enrichment of defective interfering particles.

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[*Illustrations follow*]

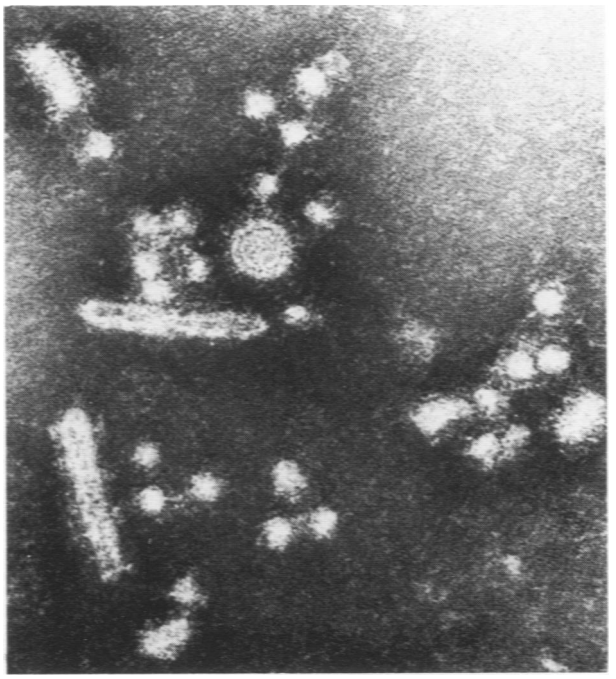

Figure 1—Electron micrograph of immune complex between HB_sAg/ayw serum and guinea pig anti-HB_s/ad. (Negatively stained with 1% PTA, × 180,000).

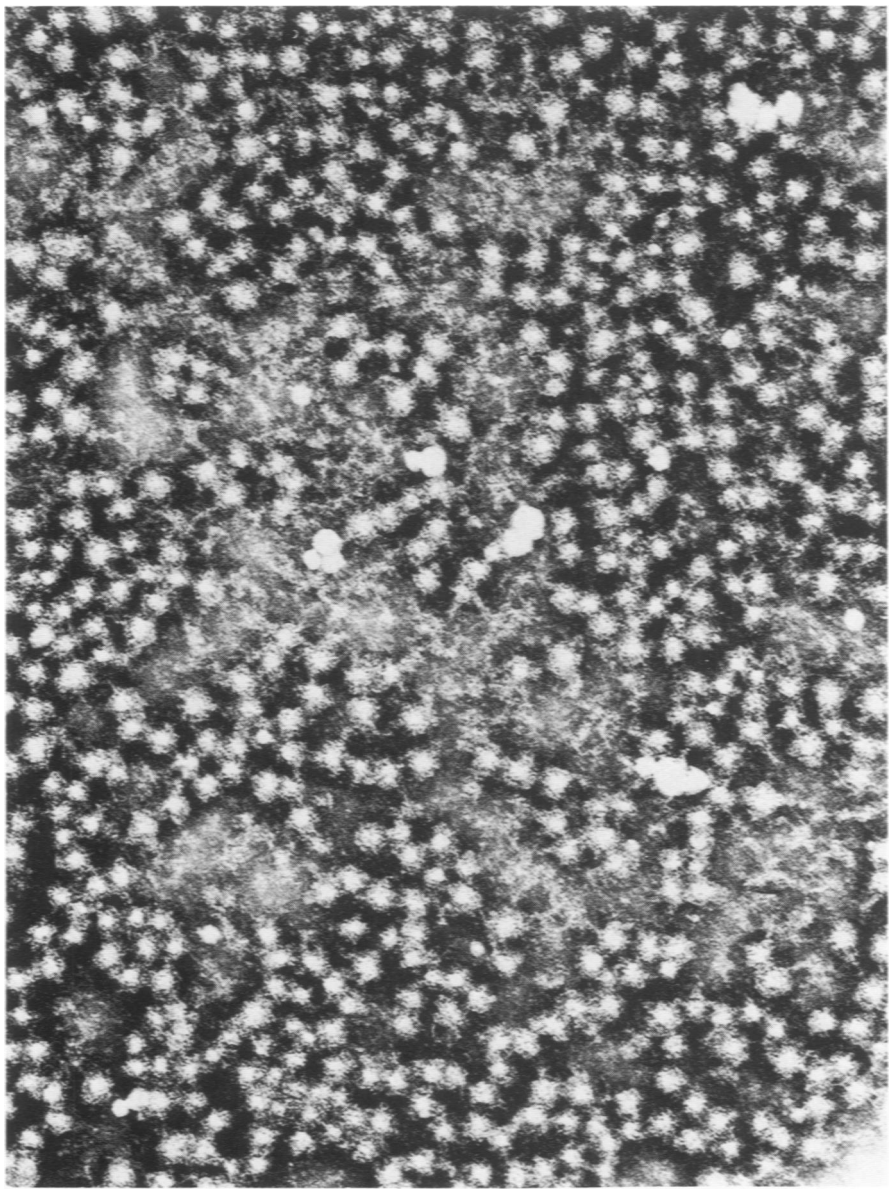

Figure 2—Electron micrograph of circulating immune complex in serum of HB_sAg carrier (Negatively stained with 1% PTA, × 180,000).

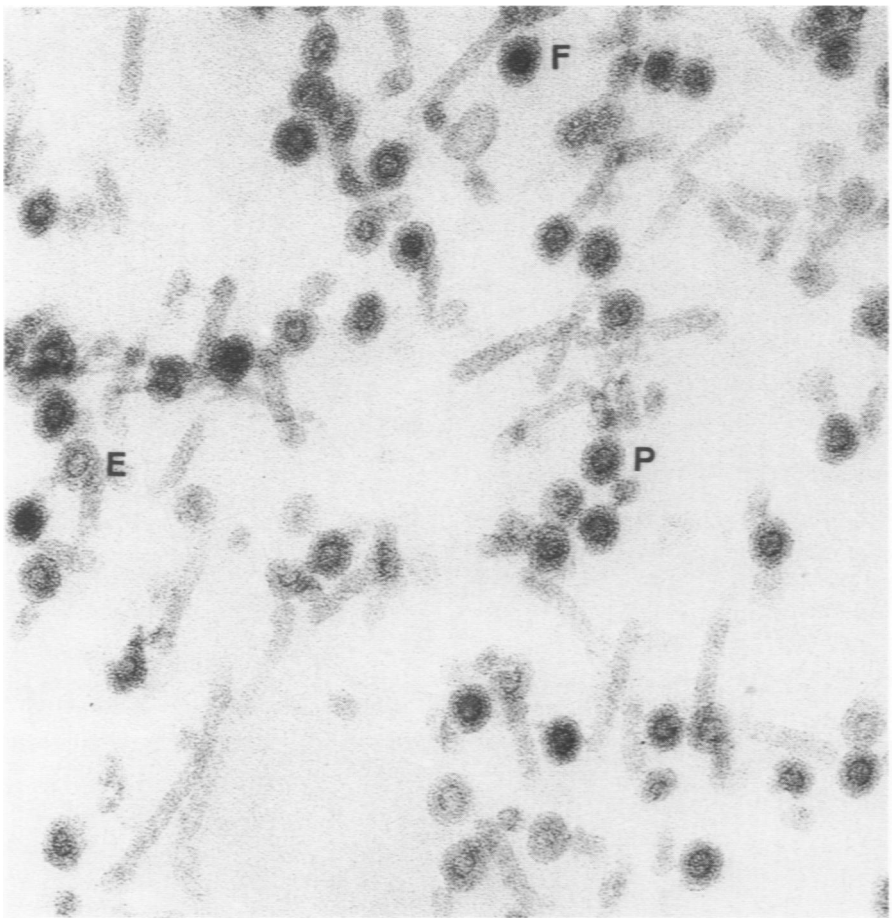

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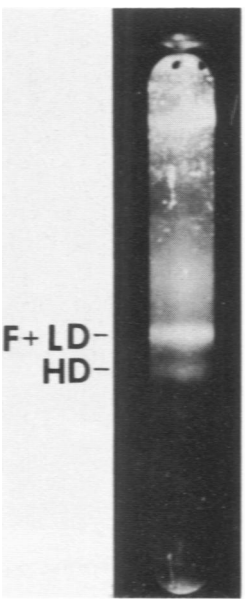
Figure 3—Electron micrograph of Dane particle concentrate showing full (*F*), partially full (*P*), and empty (*E*) cores. A twentyfold concentration of Dane particles was fixed in 1% glutaraldehyde and postfixed in 1% osmium tetroxide, stained *en bloc* with uranyl acetate, dehydrated through an alcohol series of propylene oxide, and embedded in Epon 812; thin sections were stained with lead citrate and examined in a Hitachi Hu-11E-1, × 150,000). **Figure 4**—Isopycnic banding of a twentyfold concentration of Dane particles in CsCl. A 2-ml sample was layered onto a continuous CsCl density gradient (111 to 1.4 g/cu cm) and centrifuged for 5 hours at 35,000 rev/min and 4 C in the SW 41 rotor. Two light-scattering bands were observed at 1.20 and 1.25 g/cu cm, and their composition is shown in Figure 5. *F + LD* = filaments and light Dane particles found in the 1.20 g/cu cm band (Figure 5A) and *HD* = heavy Dane particles observed in the 1.25 g/cu cm fraction (Figure 5B).



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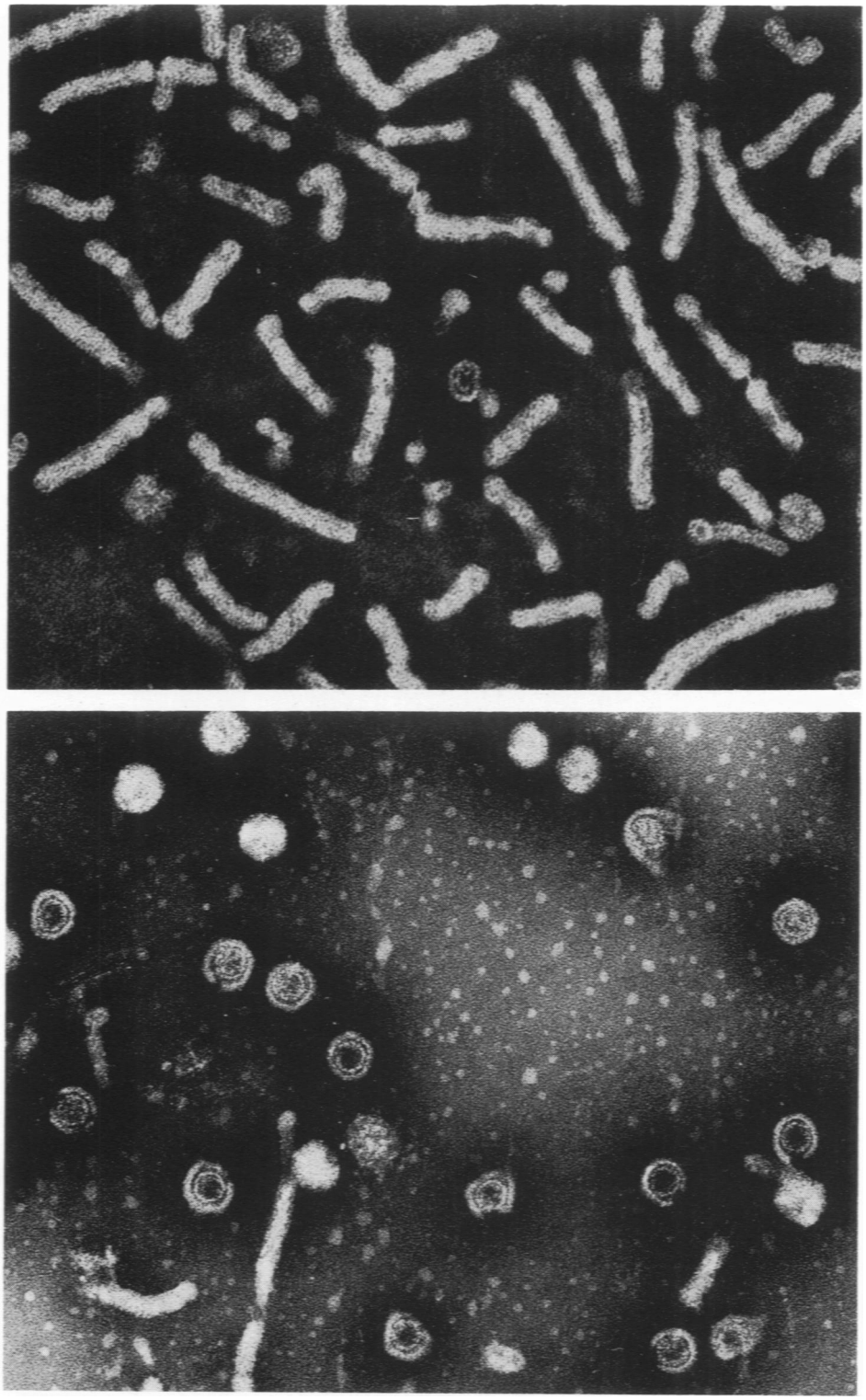

Figure 5—Electron micrographs of fractions from CsCl density gradient (Figure 4). **A**—Filaments and Dane particles from the 1.20 g/cu cm fraction. **B**—Dane particles from the 1.25 g/cu cm fraction. (Negatively stained with 1% PTA, × 150,000).

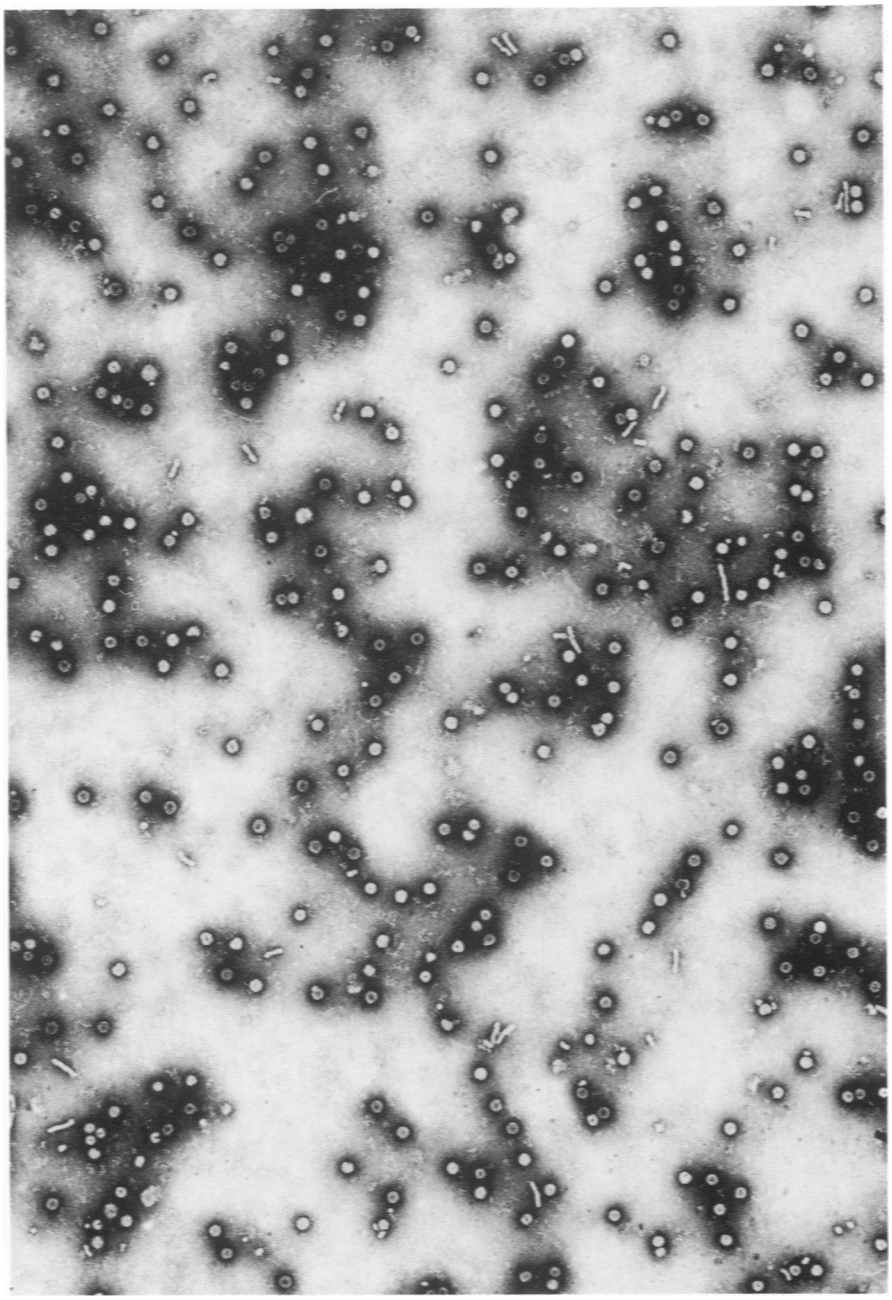

Figure 6—Electron micrograph of 1.25 g/cu cm (HD) band shown in Figure 4 at a low magnification to show the composition of the fraction (Negatively stained with 1% PTA, × 30,000).

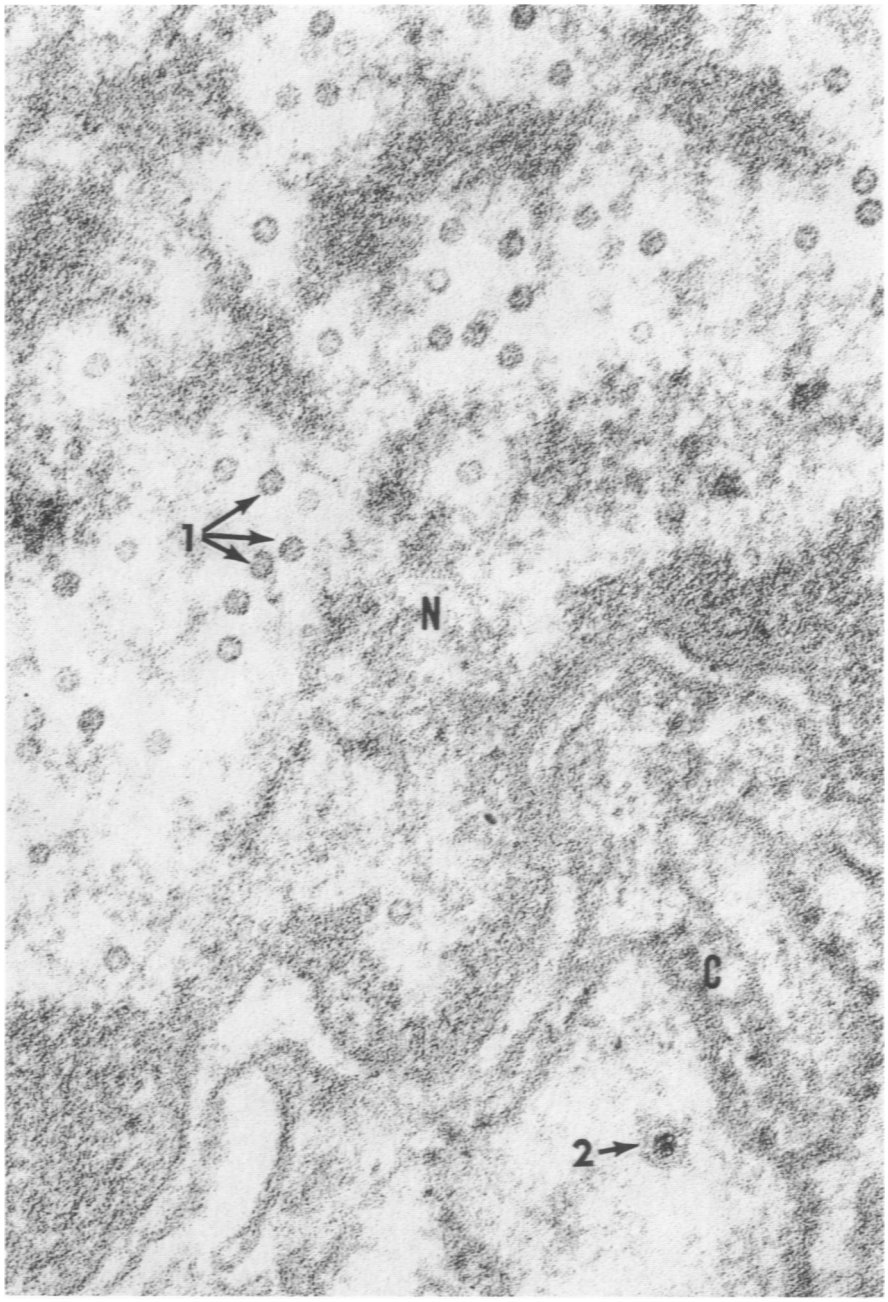

Figure 7—Electron micrograph of human liver obtained at autopsy. Liver was fixed with 2% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated through an alcohol series to propylene oxide, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined in an Hitachi Hu-11E-1. The micrograph shows cores (*1*) within the nucleus (*N*) and a Dane particle (*2*) in the cytoplasm (*C*). (× 150,000)