Proc. Nat. Acad. Sci. USA
Vol. 71, No. 9, pp. 3345–3349, September 1974

Purification of Naked Intranuclear Particles from Human Liver Infected by Hepatitis B Virus

(hepatitis B antigen/DNA polymerase/transmission electron microscopy/immunofluorescence)

SHALOM Z. HIRSCHMAN*, MICHAEL GERBER†‡, AND ESTHER GARFINKEL*

* Division of Infectious Diseases, Department of Medicine, and † Department of Pathology, The Mount Sinai School of Medicine of the City University of New York, New York, N.Y. 10029, and ‡ the Laboratory Service, Veterans Administration Hospital, Bronx, New York 10468.

Communicated by Abraham White, June 13, 1974

ABSTRACT **The isolation and purification of the naked intranuclear particles of a human liver infected with hepatitis B virus is described. The particles had a diameter of 27–28 nm, were hexagonal, and showed the presence of capsomeres and spikes with lengths of 7–10 nm projecting from the surfaces. The particles formed cubic arrays with center-to-center distances of 40–42 nm. The overall particle symmetry was icosahedral. The ultraviolet absorption spectrum of the particles showed a peak at 264 nm with a shoulder at 280 nm. Endogenous DNA polymerase activity was not detected in fractions containing particles. DNA polymerase activity was present when activated DNA or d(A-T)$_n$ was used as exogenous template.**

The first specific biochemical trace of hepatitis B virus (HBV) infection was the discovery of hepatitis B antigen (HBAg) in the serum of infected patients (1). HBAg was observed to be particulate, consisting of two main morphologic forms (2): small round forms 18–20 nm in diameter and tube forms with a diameter of 20 nm, lengths of 50–230 nm, and cross striations with a periodicity of 3 nm (3). Dane *et al.* (4) described larger particles, 40–42 nm in diameter, with a double shell and an inner core resembling a viral nucleoid. These particles also had hepatitis B antigenic determinants on their surfaces. Dane *et al.* (4) suggested that these larger particles may represent the complete virion and that the other morphologic forms of particulate HBAg represented excess coat protein. A new antigen–antibody system in the sera of patients with HBV infection was found by treating pellets prepared from sera particularly rich in Dane particles with 0.5% Tween 80 (polysorbate) in phosphate buffered saline (PBS) to release the internal component of the Dane particle (5). The core of the Dane particle had a diameter of 27 nm and was morphologically similar to rhinoviruses. This particle seemed to be identical to particles observed in hepatocytic nuclei (3, 6, 7) and liver homogenates (8) of patients with HBV infection. Antibody directed against the internal component of the Dane particle, different from antibody to HBAg, was found during acute attacks of hepatitis B and persisted for as long as 3 years (9). However, the postulated identity of the Dane core and hepatocyte intranuclear naked particle has not yet been proved.

Isolation of cores of Dane particles from human serum recently has been described (10). However, these cores were obtained in small quantities by detergent treatment of Dane particles and most of the released cores appeared to be empty. We report the isolation and purification of naked intranuclear particles in high concentration from the liver of a patient with chronic hepatitis B antigenemia.

Abbreviations: HBAg, hepatitis B antigen; HBV, hepatitis B virus; TPMM, 0.05 M Tris·HCl, 40 mM KCl, 10 mM $MgCl_2$, 0.1% 2-mercaptoethanol, pH 7.5 buffer; NP-40, Nonidet-P40.

MATERIALS AND METHODS

Human Liver. A liver weighing approximately 1.7 kg was obtained at necropsy from a patient with chronic hepatitis B antigenemia [type AD (11)] maintained on hemodialysis for renal failure due to chronic glomerulonephritis. No anti-HBAg was detected in the patient's serum by radioimmunoassay. Ultrathin sections of glutaraldehyde-fixed, osmic acid post-fixed and Epon-embedded liver were prepared and examined by electron microscopy using a uranyl acetate-lead citrate stain according to standard techniques (12). Frozen sections of liver were examined by the direct fluorescent antibody technique with fluorescein-conjugated antisera to HBAg, IgG, IgM, IgA and beta-1-C, beta-1-A globulin (13, 14). Human antiserum to HBAg was derived from a patient with hemophilia; the fluorescein-conjugated antibody was shown previously to give both cytoplasmic and nuclear fluorescence with HBV-infected hepatocytes (12). Goat anti-HBAg (kindly supplied by Dr. J. M. Stengle, National Institutes of Health, Bethesda, Md.) also was used; the fluorescein-conjugated antibody gave only cytoplasmic fluorescence with HBV-infected hepatocytes (14). Controls for these tests were as described before (13).

Method of Purification of Intranuclear Particles. The general approach to the purification of the intranuclear particles was first to isolate the hepatocytic nuclei intact and then to release the naked particles from the nuclei for further purification. Hepatocytic nuclei were isolated by modification of the procedure given by Chauveau *et al.* (15). Samples (20–30 g) of liver were blotted dry with filter paper and finely minced while being kept on ice. The minced liver tissue was washed several times with cold saline containing gentamicin (50 μg/ml) in a large test tube until the wash was clear. The liver tissue then was suspended in a mixture of 5 ml of 0.5 M sucrose and 40 ml of 0.25 M sucrose–3.3 mM $MgCl_2$ and homogenized in a Virtis homogenizer at the medium setting for 6 min. The homogenate was filtered through four layers of pre-wetted cheese cloth and centrifuged at 1000 $\times$ *g* for 10 min. The pellet obtained after centrifugation was suspended in 10 ml of 0.25 M sucrose–3 mM $MgCl_2$. The pellet was further homogenized with 15 up-and-down strokes of a Dounce homogenizer with

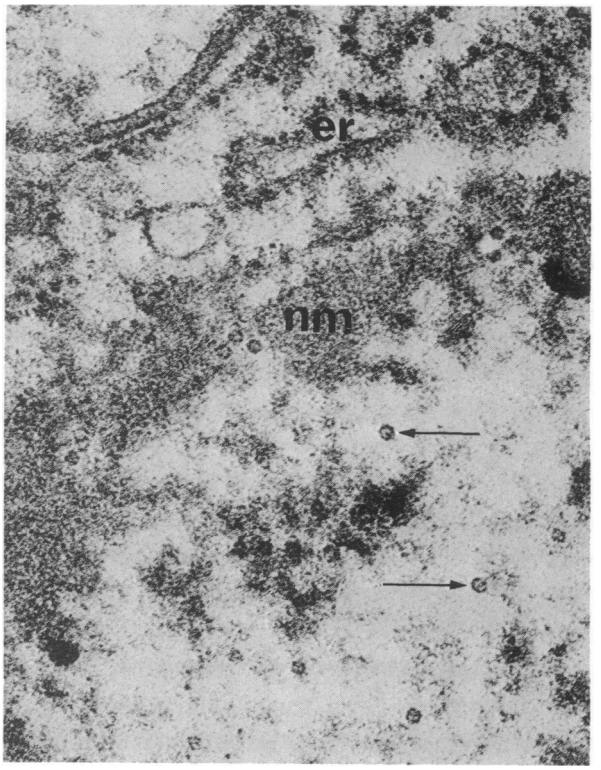

FIG. 1. Ultrathin section of liver stained with uranyl acetate and lead citrate showing presence of naked particles in the hepatocytic nucleus and absence of particles in the cytoplasm. *nm*, nuclear membrane; *er*, endoplasmic reticulum. The *arrows* point to naked intranuclear particles (×89,000).

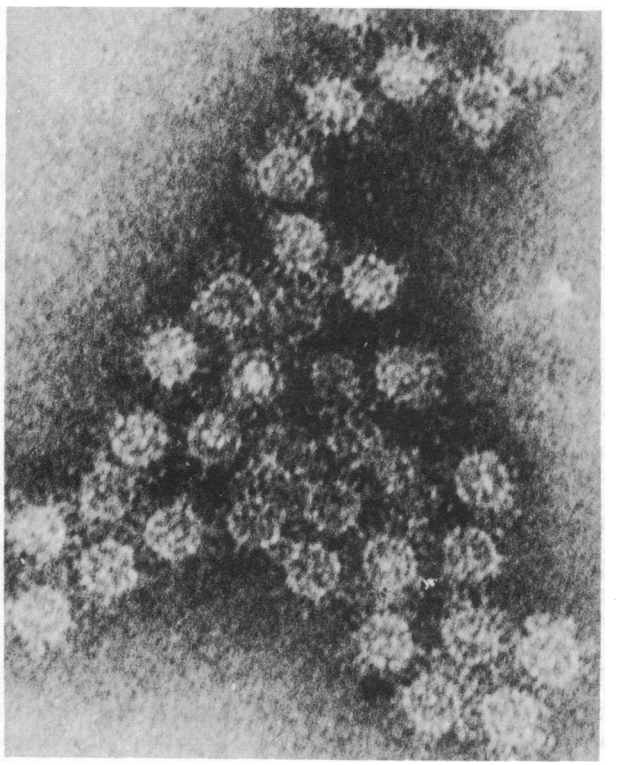

FIG. 2. Morphology of naked intranuclear particles. The particles were suspended in TPMM buffer and stained with 2% phosphotungstic acid at pH 7.2. Particles are aligned in an array with center-to-center distances of 40–42 nm (×243,000).

a tight-fitting Teflon pestle. The homogenate was underlayed with 10 ml of 0.34 M sucrose–3 mM $MgCl_2$ and centrifuged at 1500 × *g* for 15 min. The pellet obtained after centrifugation was suspended with a Dounce homogenizer, using a loose-fitting glass pestle, in approximately 9 volumes of 2.4 M sucrose–3 mM $MgCl_2$ and centrifuged at 39,000 × *g* for 2 hr. This nuclear pellet was suspended in 10 ml of 0.25 M sucrose–3 mM $MgCl_2$ and centrifuged at 1000 × *g* for 10 min. The pellet then was washed with 10 ml of 0.05 M Tris·HCl, 40 mM KCl, 0.1% 2-mercaptoethanol, 10 mM $MgCl_2$, pH 7.5, buffer (TPMM buffer) and centrifuged at 1000 × *g* for 10 min. The nuclear pellet was resuspended in 6 ml of the same buffer and sonicated with a Bronwill Biosink III sonifier at 63 W/cm². The total sonication time was 90 sec with 30 sec of sonication followed by 30 sec of cooling. Small aliquots were removed after each sonication period and examined by phase microscopy to follow the breakage of nuclei. The sonicate was made up to 10 ml with TPMM buffer and centrifuged at 10,000 × *g* for 15 min. The pellet was discarded and the supernate was centrifuged at 39,000 × *g* for 4 hr. The resulting pellet was taken up in TPMM buffer and cesium chloride was added to a density of 1.32 g/ml as determined from the refractive index of the solution. Centrifugation was then carried out at 100,000 × *g* for 24 hr in a Beckman L 65B ultracentrifuge at 4°. The centrifuge tube was pierced and fractions were collected by drop counting. All the supernatant fluids and the low-speed pellets discarded during the preparation were studied by electron microscopy. The volumes given above were adjusted upwards for larger scale preparations.

Electron Microscopy. The CsCl gradient fractions were dialyzed exhaustively against TPMM buffer and examined under the electron microscope on collodion-coated copper grids after negative staining with 2% phosphotungstic acid at pH 7.2 (16).

Immunoabsorption Studies. Fluorescein-conjugated human antiserum to HBAg was absorbed with purified round and tube forms of HBAg (surface antigen) (16) or dialyzed CsCl fractions containing naked particles at a serum to antigen preparation ratio of 10:1 (v/v) at 37° for 1 hr. This was followed by overnight incubation at 4° and centrifugation for 15 min at 1500 × *g*. Antiserum treated with phosphate-buffered saline (PBS, 0.01 M phosphate–0.14 M NaCl, pH 7) was used as control. The absorbed sera were tested on the patient's liver and on an HBV-infected liver known to show both cytoplasmic and nuclear fluorescence with the original fluorescein-conjugated antiserum (HBV standard liver).

Ultraviolet Spectrophotometry. Ultraviolet absorption spectra were determined with a Beckman Acta-V spectrophotometer using 100-μl microcells and microapertures.

Assays for DNA and RNA Polymerases. Assays for endogenous DNA and RNA polymerase activities were performed on all CsCl gradient fractions as previously described except that the concentrations of $MgCl_2$ and Nonidet-P40 (NP-40) in the final reaction mixture were 10 mM and 0.1%, respectively (17, 18). Assays for DNA polymerase activity were carried out at pH 7.5 and 37°. RNA polymerase activity was measured at pH 7.5 and both 32° and 37°. Calf-thymus DNA was activated by the method of Schlabach *et al.* (19) and was

added to a concentration of 20 μg/0.1 ml of final reaction mixture. The synthetic templates $(rA)_n \cdot (dT)_n$, $(rA)_n \cdot (dT)_{10}$, $(dA)_n \cdot (dT)_{10}$, and $d(A\text{-}T)_n$, purchased from Calbiochem, were added to a concentration of 0.1 A_{260} units per 0.1 ml of final reaction mixture. $(rA)_n$ purchased from Miles Laboratories was added to a final concentration of 20 μg/0.1 ml. [^{3}H]dTTP and [^{3}H]UTP were purchased from New England Nuclear Corp. Protein concentrations were determined by the method of Lowry *et al.* (20).

RESULTS

Human Liver. Light microscopy of the liver showed chronic aggressive hepatitis. Electron microscopic examination showed 24-nm noncoated particles diffusely spread, without clumping, throughout the hepatocytic nuclei (Fig. 1); no naked particles were found in the cytoplasm. Particulate forms characteristic of HBAg (surface antigen) or Dane particles were not observed in the hepatocytes. Incubation of frozen sections from several blocks of the patient's liver with fluorescein-conjugated human antiserum to HBAg yielded moderately strong granular fluorescence in 30–50% of the hepatocytic nuclei and in the cytoplasm of few scattered hepatocytes. Incubation with fluorescein-conjugated goat anti-HBAg showed cytoplasmic fluorescence of rare hepatocytes. No immunoglobulins or beta-1-C, beta-1-A globulin were detected in the hepatocytes.

Purification of Intranuclear Particles. The naked particles were not observed in the supernatant fluids and low-speed pellets discarded during the preparation. Examination of the final nuclear pellet by electron microscopy showed the nuclear membranes to be intact, with the particles not only diffusely spread throughout the nuclei but also present in clumps.

High-speed centrifugation following sonication of the nuclear preparation yielded a thick gelatinous tan pellet that contained many typical particles in arrays (*vide infra*). This pellet could be dissolved only with great difficulty. Thus, it was decided to employ a sucrose cushion for this pelleting or to omit the high-speed centrifugation following sonication, and to band the particles directly in CsCl or sucrose following the low-speed centrifugation after sonication and then to further purify the particles by rebanding on CsCl. The yield of naked particles was increased by omitting the high-speed centrifugation of the sonicate.

Morphology of Purified Intranuclear Particles. The naked intranuclear particles were obtained in fractions of CsCl density gradients with a density of 1.30 g/ml. Examination of the fractions dialyzed against TPMM buffer showed the particles to have a diameter of 27–28 nm, and to be hexagonal in shape (Fig. 2). Most of the particles showed the presence of capsomeres and spikes projecting from the particle surfaces. The spikes had a length of 7–10 nm and a terminal knob. The particles formed cubic arrays with center-to-center distances of 40–42 nm (Fig. 2). Thus, the particles demonstrated icosahedral symmetry. The particles were unstable and after being thawed several times following storage at −20° appeared to disintegrate into the component capsomeres. No HBAg (surface antigen) was detected by radioimmunoassay in fractions containing naked particles. A very small amount of HBAg (surface antigen) was detected at a density of 1.22 g/ml.

Immunoabsorption Studies. Abolition of the cytoplasmic fluorescence of the patient's and the HBV standard livers without reduction in nuclear fluorescence was achieved by absorbing the fluorescein-conjugated human antiserum to HBAg three times with purified round and tube forms of HBAg (surface antigen). The nuclear fluorescence of the HBV standard liver was markedly diminished by absorbing the fluorescein-conjugated human antiserum (0.2 ml) three times with 2 μg of protein each time of fractions containing typical naked particles.

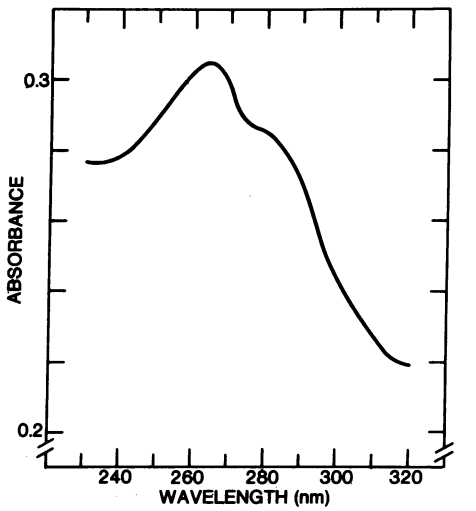

FIG. 3. Ultraviolet absorption spectrum of naked intranuclear particles. Particles representing 12 μg of protein in 100 μl of TPMM buffer were used to record the spectrum.

Ultraviolet Absorption Spectra. The ultraviolet absorption spectrum of the particles demonstrated a peak at 264 nm with a shoulder at 280 nm and a trough at 230 nm (Fig. 3).

Assays for DNA and RNA Polymerases. Assay of all fractions obtained from CsCl density gradients of the particle preparations showed no detectable endogenous DNA or RNA polymerase activities even with 25 μg of protein of fractions containing high concentrations of typical naked particles in the reaction mixtures. No DNA-dependent RNA polymerase activity was found with native or activated calf-thymus DNA, or $d(A\text{-}T)_n$ as templates. However, when activated calf-thymus DNA or $d(A\text{-}T)_n$ was used as exogenous template, DNA synthesizing activity was detected in fractions containing typical naked particles (Fig. 4). The products of these reactions were digested by DNase but not by RNase (each 100 μg/ml). No DNA polymerase activity was detected with native calf-thymus DNA, $(rA)_n \cdot (dT)_n$, $(rA)_n \cdot (dT)_{10}$, $(dA)_n \cdot (dT)_{10}$, or $(rA)_n$ as exogenous templates (Table 1). No RNA polymerase activity was detected with $(rA)_n$ as template.

DISCUSSION

The morphology of the naked intranuclear particle found in our purified preparations was similar to that found by Huang and Groh (8) by direct electron microscopic examination of Pronase-treated liver homogenates and also resembled cores prepared from serum Dane particles (10). The purification procedure described here required neither detergent nor protease treatment. Precipitation by antibody was not necessary to concentrate artificially the particles for electron microscopic examination. The density of the particles was similar to that described for the intranuclear particles found in chimpanzee livers infected with HBV (9) and for cores of

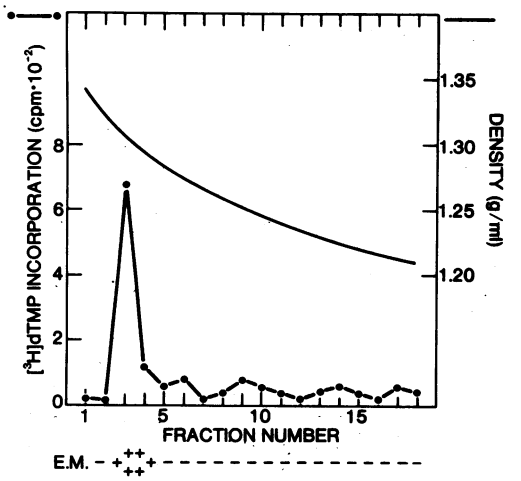

FIG. 4. DNA polymerase activity in fractions of CsCl density gradient of a preparation of naked particles from 25 g of liver. The fractions from the gradient were dialyzed against TPMM buffer. A reaction mixture (0.1 ml) contained 2.5 μg of protein, 2.5 μCi of [³H]dTTP (specific activity, 20 Ci/mmole), and 20 μg of activated DNA. Incubation time was 3 hr at 37°. *E.M.* = results of electron microscopic study of the fractions for the presence of particles. − = no naked particles; + = naked particles extremely rare; $^{++}_{++}$ = high concentration of naked particles and presence of typical arrays. The particles in this preparation were first pelleted by high speed ultracentrifugation and then banded in a CsCl gradient.

Dane particles isolated from human serum (10). The purified particle with its projecting spikes, hexagonal shape, and icosahedral symmetry resembles other mammalian viruses in these properties. The absorption by the particles of the antibody-mediating hepatocytic nuclear fluorescence suggests that such fluorescence of HBV-infected hepatocytes is due to the presence of the particles. Indeed, it was previously shown by indirect immuno-electron microscopy that the antibody used in these studies bound to naked intranuclear particles (12).

DNA polymerase of low activity had been detected in pellets of HBAg prepared from the sera of patients with hepatitis (17). The DNA-synthesizing activity was stimulated by $d(A\text{-}T)_n$ but not by $(rA)_n \cdot (dT)_n$ or $(rA)_n \cdot (rU)_n$ (17). The endogenous DNA polymerase was inhibited by intercalating agents such as ethidium bromide but not by rifampicin congeners known to inhibit reverse transcriptases (RNA-dependent DNA polymerases) of oncornaviruses (18). It was subsequently shown that the DNA polymerase was not associated with purified small round particles of HBAg (21, 22). Kaplan *et al.* (23) selected patients for the presence of appreciable numbers of Dane particles in their sera and found significant endogenous DNA polymerase activity in the HBAg-containing pellets prepared from these sera. Ultracentrifugation of the pellets on sucrose density gradients seemed to demonstrate the association of the DNA polymerase activity with Dane cores, although the peak DNA polymerase activity preceded the peak Dane core antigen by several fractions. The DNA polymerase could not be stimulated by exogenous templates such as $(rA)_n \cdot (dT)_n$, $d(A\text{-}T)_n$ and activated DNA. The report did not definitely establish that DNA polymerase is an integral part of the Dane core. Indeed, Loeb *et al.* (24) described DNA polymerase activity in normal human sera that also could not be stimulated by exogenous templates.

TABLE 1. *Stimulation by exogenous templates of DNA polymerase activity associated with naked intranuclear particles**

Template	Concentration (per 0.1 ml)	[³H]dTMP incorporation (pmole/mg of protein per hr)
No exogenous template	—	0
Activated calf-thymus DNA	20 μg	2
Native calf-thymus DNA	20 μg	0
$d(A\text{-}T)_n$	0.1 A_{260} unit	1.5
$(rA)_n \cdot (dT)_n$	0.1 A_{260} unit	0
$(rA)_n \cdot (dT)_{10}$	0.1 A_{260} unit	0
$(dA)_n \cdot (dT)_{10}$	0.1 A_{260} unit	0
$(rA)_n$	20 μg	0.02

* Each reaction contained 2.5 μg of protein of fraction containing high concentration of naked particles with typical arrays. The reaction mixture contained 0.05 M Tris·HCl, 40 mM KCl, 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM each of dATP, dCTP, and dGTP, 2.5 μCi of [³H]dTTP, and 0.1% NP-40 at pH 7.5. The specific activity of the [³H]dTTP was 20 Ci/mmole. The reaction mixtures were incubated at 37° for 3 hr. Two control reactions were run with each experiment: (*a*) the complete reaction mixture immediately terminated and not incubated, and (*b*) the reaction mixture without naked particles incubated for 3 hr. The cpm of these controls averaged 192 ± 50 and were subtracted as background from the assays.

No endogenous DNA polymerase activity was detected after repeated attempts in the fractions containing typical naked particles in the studies reported here. Yet the ultraviolet absorption spectrum of the particles, with a peak at 264 nm and a shoulder at 280 nm, strongly suggested that the particles isolated contained both nucleic acid and protein. Indeed, double-stranded DNA has been isolated from the particles.§ It is possible that the absence of detectable endogenous DNA polymerase activity in the preparations of intranuclear particles in the experiments reported here may have been a result of insufficient concentrations of particles in the reaction mixtures or to some shearing of the endogenous nucleic acid, perhaps by the sonication or action of hepatic nucleases, during the purification procedure.§ However, it may be that the DNA polymerase found in sera of patients with hepatitis B infection (17, 23) is attached to the HBV particle during late-nuclear or cytoplasmic maturation. By means of the exogenous templates activated DNA and $d(A\text{-}T)_n$, DNA polymerase activity was detected only in fractions containing naked particles. Both of these templates are known to stimulate mammalian DNA polymerases (19, 25). Again, these data did not definitely establish that DNA polymerase is an integral part of the naked particle. The enzyme will have to be isolated and shown to be virus specific. Stimulation of the DNA polymerase activity by activated DNA and $d(A\text{-}T)_n$ but not by known stimulators of reverse transcriptases, such as $(rA)_n \cdot (dT)_n$ and $(rA)_n \cdot (dT)_{10}$ (26), seemed to support the suggestion of Kaplan *et al.* (23) that the template for the DNA polymerase is DNA.

The ability to prepare concentrated purified preparations of the intranuclear particles should allow one to answer the fol-

§ Hirschman, S. Z., Gerber, M. A., and Garfinkel, E., "DNA purified from the naked intranuclear particles of hepatitis B virus infected human liver," *Nature*, in press.

lowing questions: (*1*) is this naked particle infectious either by itself or with a coat of hepatitis B antigen surface protein; (*2*) does this naked particle contain a virus specific nucleic acid polymerase; (*3*) what is the nature of the nucleic acid contained in the particle; (*4*) what are the number and nature of the capsomeres and the spikes projecting from the particles. Furthermore, working with such concentrated preparations, one may now attempt replication of this particle in tissue culture.

The authors are grateful to Dr. Richard Rosenfield and Florence Schultz for performing the radioimmunoassays for HBAg and anti-HBAg. This study was supported by Grant AI11472 from the U.S. Public Health Service. This is the first of a series of studies pertaining to the naked intranuclear particles of HBV-infected human liver.

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