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The Incorporation of Radioactive Uridine into the Hepatitis B Antigen of a Chimpanzee[1] (38986)

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The presence of ribonucleic acid (RNA) in purified preparations of Hepatitis B antigen (Australia antigen, Au, HAA, HB_sAg) was reported by several investigators (1–6). Affinity chromatography provided some evidence that the RNA is a structural part of HB_sAg (5). The purpose of this communication is to provide further evidence by showing the incorporation of radioactive 5′-(^{3}H) uridine into chimpanzee HB_sAg which was purified by precipitation with specific antibody. Purification by antibody precipitation has the advantage over other methods in avoiding drastic procedures which may alter the integrity of the HB_sAg macromolecule and which may alter nucleic acid content. The careful selection of chimpanzee antibody which can be shown not to react with serum proteins of the chimpanzee carrier of HB_sAg has the further advantage of allowing purification in a single step with maximum yield.

Materials and methods. Preliminary experiment. (a) *Incorporation of label.* Twenty-five mCi 5′-(^{3}H)uridine (purchased from C.E.N., Mol, Belgium) in 50 ml of saline was injected intravenously into a male chimpanzee HB_sAg carrier. The chimpanzee was bled at designated times (Fig. 1) to determine the time for maximal incorporation of radioactive uridine into HB_sAg.

(b) *Purification of HB_sAg.* The HB_sAg obtained from 11 ml serum of each bleeding was isolated by precipitation with purified human anti-HB_s (prepared by DEAE cellulose chromatography (7) as described in Fig. 1). The optimal proportion of antigen and antibody was determined by observing the sharpness and position of the precipitin band in immunoelectroprecipitation (IEP) after reacting a constant amount of antigen with increasing amounts of antibody. The washed complex was divided into two equal parts. One part was dissolved in "Aquafor" (New England Nuclear) and radioactivity was measured in a Packard scintillation counter (Model 3380). The other part of each washed complex was extracted with chloroform–phenol buffer (8), and the radioactivity of aqueous phase was measured. To determine whether some radioactivity might have been contributed by chimpanzee serum proteins adhering to the washed antigen–antibody complex, a complex was prepared using chimpanzee plasma obtained before the administration of radioactive uridine (as described in the legend of Fig. 1) and the same human anti-HB_s. The washed complex was dissociated by adding an equal volume of a mixture of formic and acetic acids (equal parts, 0.1 *M*). The acidified complex was centrifuged at 100,000*g* for 3 hr (Spinco SW 50 rotor) through a gradient of 10–30% sucrose made up in the same acid mixture. Ten-drop fractions were collected by bottom puncture and after neutralization with 0.1 *M* NaOH each fraction was tested for serum proteins by immunoelectrophoresis and immunodiffusion.

Purification of labeled HB_sAg after plasmaphoresis. In 8 wk, when sample bleedings of the chimpanzee showed no remaining radioactivity an additional 25 mCi 5′-(^{3}H)-uridine in 50 ml of saline was injected intravenously. After 24 hr, 650 ml of plasma was collected by plasmaphoresis. The plasma was concentrated by centrifugation (Beckman Type 60 rotor) at 160,500*g* for 4 hr. The bottom fifth of each tube was pooled and tested for HB_sAg by IEP. This plasma

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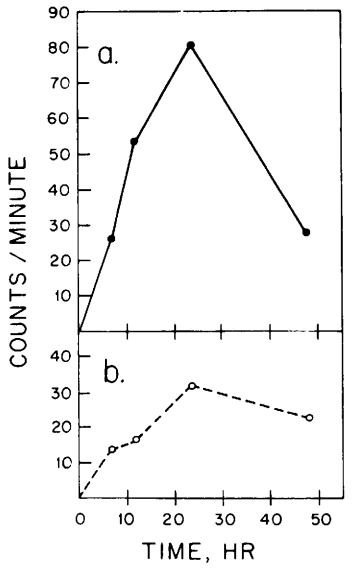

FIG. 1a. Radioactivity of HB_sAg—anti HB_s complex (——); Fig. 1b Radioactivity in the water phase after extraction with chloroform-phenol (---). The serum samples containing HB_sAg (11 ml) were concentrated by centrifugation at 102,000*g* for 4 hr using a Type 65 Beckman rotor. The bottom third of each tube approximately 4 ml, was filtered through Sephadex G-200 (2.5 × 100 cm column), and eluted with 0.01 *M* potassium phosphate buffer pH 7.0 containing 0.15 *M* NaCl. The peak which appeared in the void volume of each sample was collected, concentrated to the original volume (4 ml) and assayed for HB_sAg by IEP. Human anti-HB_s (IgG fraction) was prepared by DEAE cellulose chromatography using 0.01 *M* potassium phosphate buffer pH 8.0 (7). HB_sAg was precipitated at equivalence with this human anti-HB_s fraction, incubated for 1 hr at 37° and then overnight at 4°. The precipitate was centrifuged (Sorvall 2B-rotor SS 34) at 12,000*g* for 1 hr and washed five times with cold 0.15 *M* NaCl. The complex was suspended in 0.4 ml of 0.01 *M* Tris-HCl pH 7.2 and 0.2 ml was used to measure radioactivity. The remainder (0.2 ml) was diluted to 0.5 ml with the Tris-HCl buffer containing 0.5% sodium dodecyl sulphate (SDS) and vigorously shaken for 15 min with an equal volume of chloroform-phenol mixture at room temperature (8). The sample was then centrifuged at 5,000*g* for 15 min at 20° and the water phase collected. The phenol phase with interphase was reextracted with another 0.5 ml Tris-HCl buffer containing 0.5% SDS. The excess phenol in the combined aqueous phases was removed by extraction with diethyl ether and the aqueous phase was concentrated to 0.2 ml. Radioactivity was measured against a buffer blank which was processed in the same manner as the sample.

concentrate was positive by IEP at a dilution of 1:30. Anti-HB_s of chimpanzee origin was fractionated by DEAE cellulose chromatography (7) and the IgG fraction was used to precipitate HB_sAg. The HB_sAg–anti-HB_s complex was precipitated at equivalence using the procedure previously outlined. The precipitate was washed by centrifugation five times with cold 0.15 *M* NaCl and resuspended in 0.1 *M* Tris–HCl buffer, pH 7.2.

Thin layer chromatography. Nucleic acid was extracted from HB_sAg–anti-HB_s complex with buffered phenol, precipitated with alcohol and dissolved in distilled water. For hydrolysis of nucleic acids 3 *N* KOH (sufficient to produce a final concentration of 0.3 *N*) was added and the mixture incubated for 18 hr at 37°. This was neutralized with 4 *N* perchloric acid and chromatographed on "Polygram cel 300" PEI/UV_{254} (Brinkman Instrument, Inc., Westbury, NY). Each nucleotide was cut out and extracted from the chromatogram with 0.12 *M* potassium phosphate buffer, pH 7.0. A section of the chromatogram opposite each identified nucleotide was extracted as a control. Concentrated extracts were rechromatographed, eluted and reconcentrated three times to eliminate tailing as a consequence of salt concentration.

Results. The highest amount of radioactivity was found in the washed complex from the 24 hr bleeding. After chloroform phenol buffer extraction of this complex, the aqueous phase was found to contain the highest amount of radioactivity (Fig. 1).

The procedure used for the purification of HB_sAg (formation of an antigen–antibody complex with washing) did not result in the precipitation of chimpanzee serum proteins. Neither was there evidence that the complex adsorbed chimpanzee serum proteins. After dissociation of the washed complex by formic-acetic acids and separation on a sucrose gradient we detected by immunoelectrophoresis and immunodiffusion only HB_sAg and anti-HB_sAg immune globulin (IgG) in fractions.

Under the electron microscope 20 nm but no "Dane" 42 nm particles were seen in a

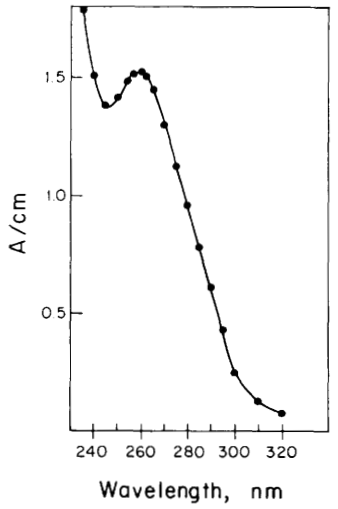

FIG. 2. Absorption curve of RNA isolated from chimpanzee HB_sAg–anti-HB_s complex. RNA (chloroform–buffered–phenol extracted and alcohol precipitated twice) was dissolved in 1 ml of water and read in a Beckman 2000 spectrophotometer.

0.025 ml sample of complex produced from chimpanzee plasma and chimpanzee anti-HB_s; the HB_sAg in this complex was equivalent to that contained in 0.65 ml of concentrated plasma. 0.1 ml of complex, equivalent to that contained in 2.6 ml of concentrated chimpanzee plasma, contained 294 cpm; 0.3 ml of complex, equivalent to the HB_sAg contained in 7.8 ml of concentrated plasma, was extracted with chloroform–phenol (see Fig. 1) (8). Material precipitated from the aqueous phase on addition of 2 vol of 100% ethanol containing 2% potassium acetate was dissolved in water. An absorption spectrum of this material is shown in Fig. 2.

Chromatography of hydrolyzed nucleic acid revealed four nucleotides—guanosine, uridine, adenosine and cytidine phosphates (Fig. 3). Each sample was measured for radioactivity (Table I). The extracted nucleotide sample identified as uridine contained a significantly large amount of radioactivity when compared with the controls and other nucleotides.

Discussion. Several investigators have suggested that the particles of HB_sAg are coat proteins covering the "Dane" particle (10, 11). The "Dane" particle has been considered the leading candidate for the Hepatitis B virus. Kaplan *et al.* (12, 13) showed an association of DNA polymerase with the 28 nm cores of the "Dane" particle and Robinson *et al.* (14) produced evidence that the cores contain double stranded circular DNA with a molecular weight of approximately 1.6×10^8. For DNA polymerase to be associated with endogenous DNA template is striking indeed since none have been reported in other viruses. Evidence that DNA polymerase is specific for the "Dane" core is weak and was postulated as possibly host material nonspecifically adhering to "Dane" particles and "Dane" cores (15). The significance of DNA polymerase in cases of Hepatitis B has also been questioned by Loeb *et al.* (17) who reported DNA polymerase in several normal sera and in only one out of five preparations of purified HB_sAg.

Our results show the presence of RNA in purified chimpanzee HB_sAg. RNA was extracted with buffered phenol and precipitated with ethanol and perchloric acid (see addendum). In addition to these chemical characteristics the extracted material produced a peak of absorption at 260 nm and was shown to be composed of four nucleotides after KOH degradation and separation on thin layer chromatography. Our method of purification, precipitating HB_sAg with specific antibody, avoided the possible loss of nucleic acids by density gradient sedimentation in cesium salts. Dissociation of the washed antigen–antibody complex by acid and separation on sucrose gradients produced no evidence of serum proteins other than anti-HB_s immunoglobulin. While this does not prove freedom from adsorbed host RNA purification by conventional methods (cesium chloride gradients) also does not eliminate RNA from isolated HB_sAg fractions (4). In addition (see addendum), RNAase treatment would have eliminated traces of adsorbed RNA. RNA could have been protected from enzyme degradation only by the external lipoprotein coat on the HB_sAg macromolecule.

These data are not necessarily incom-

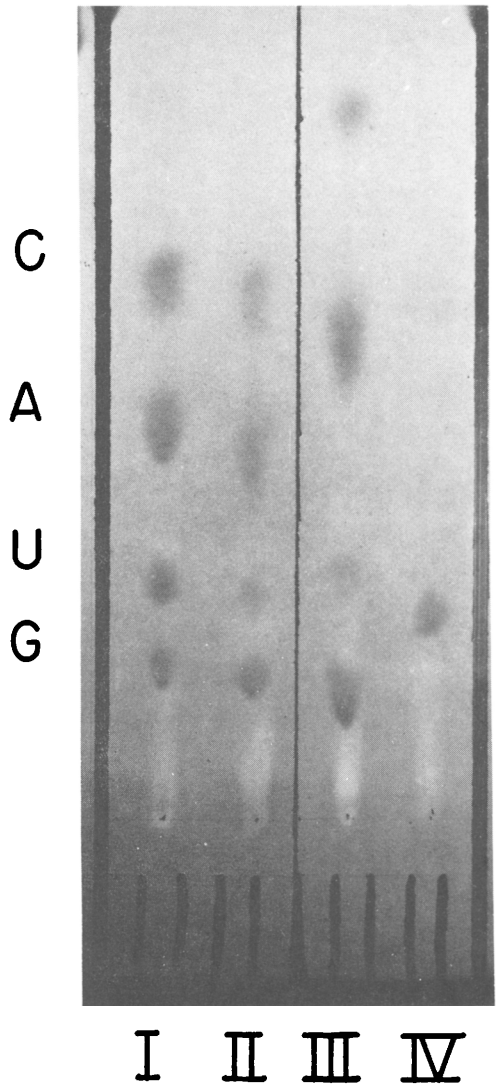

FIG. 3. Thin-layer chromatography of hydrolyzed RNA from chimpanzee HB_sAg on polygram cel 300 PEI/uv_{254} [photographed under uv light].

Standards

G = Guanosine phosphate
U = Uridine phosphate
A = Adenosine phosphate
I = 3′-nucleotides, 10 μg each
III = 5′-nucleotides, 10 μg each
IV = 3′-uridine phosphate, 10 μg

Sample

C = Cytidine phosphate
II = RNA from chimpanzee HB_sAg

The RNA sample was hydrolyzed in 0.3 *N* KOH for 18 hr at 37° and was neutralized with 4 *N* perchloric acid. Insoluble salts were removed by centrifugation. Multiple application of the sample were applied by touching the chromatogram with a 10 μl disposable pipette containing the sample and drying with a portable hair dryer. The chromatogram was washed for 10 min in absolute methanol. Resolution was accomplished by the procedure of Randerath (9) where the first solvent (1 *M* acetic acid) is allowed to migrate to 10 cm above the starting line and the second solvent (0.3 *M* lithium chloride) to 18 cm. Nucleotides from the chromatogram were extracted with 0.12 *M* potassium phosphate buffer, pH 7.0. The pooled extracted nucleotides were chromatographed twice and concentrated. This was then rechromatographed and the figure above represents this final separation.

TABLE I. RADIOACTIVITY OF NUCLEOTIDES FROM RNA–CHIMPANZEE HB_sAg.

	Count/min[a]
Guanosine phosphate	2.3
Uridine phosphate	150.5
Adenosine phosphate	9.1
Cytidine phosphate	6.3

[a] Background of 30.6 cpm, measured in control sample was subtracted.

patible with the hypothesis that the "Dane" particle is the virus of Hepatitis B. If the DNA polymerase were RNA dependent ("reverse transcriptase"), there could conceivably be cooperation between the DNA polymerase, the RNA from the 20 nm particle and the DNA from "Dane" cores. On the other hand, the "Dane" particle could be construed as the true virus of Hepatitis B and the RNA of the surface as messenger or priming host RNA (18). As an alternative hypothesis, the small 20 nm particle might be considered a separate RNA virus, part of a yet unknown helper virus or a "Viroid" similar to those infectious agents described by Zuckerman (19) and Diener (20). The participation of "Dane" particles with such RNA entities might be required for infectivity. The relationship between the two kinds of particles associated with Hepatitis B and an elucidation of the role of DNA polymerase may shed light on the etiology of this disease and explain the mechanism of infection.

Summary. Radioactive (^3H) uridine was

incorporated into RNA isolated from the HB_sAg of a chimpanzee carrier. HB_sAg was purified by precipitation as an immune complex with the IgG fraction of chimpanzee anti-HB_s. RNA was extracted from the washed complex with buffered phenol precipitated with alcohol and four nucleotides were identified by thin layer chromatography after alkali degradation.

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Addendum. After this work was prepared for publication we became aware of the possibility that radioactive uridine could have been incorporated into uridine diphosphoglucose and not RNA. We have since repeated the procedure for the isolation of nucleic acid described in methods but included two additional steps. (1) The chimpanzee plasma was treated with 25 μg/ml of RNAse (4000 units/mg, Worthington, Freehold, NJ) and incubated for 15 min at 37°. A control containing ^{14}C labeled RNA was shown to be completely degraded under the same conditions of treatment. The treated plasma was then extracted with buffered phenol as described in the Materials and methods section. (2) The alcohol precipitated and redissolved RNA was then precipitated with perchloric acid (final concentration 0.2 *N*). The perchloric acid precipitate, adhering to a fiberglass filter, contained the same radioactive count as the KOH hydrolyzed material originally described under Materials and methods. Uridine diphosphoglucose is not precipitable under these conditions.

To further exclude the possibility that the material we were dealing with was not RNA an aliquot of ethanol precipitated material (approx one-fifth of the total yield described in methods) was dissolved in distilled water and treated with 80 μg of RNAse for 15 min at 37°. Perchloric acid was added to a final concentration of 0.2 *N*. There was no visible precipitate. The supernatant was neutralized with 3 *N* KOH and the precipitate of potassium perchlorate discarded by centrifugation. The supernatant contained approximately 90% of the radioactivity of the sample prior to RNAse treatment. This indicated that our tritiated uridine was indeed incorporated into RNA and converted by RNAse to acid soluble nucleotide.

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