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DNA Polymerase Associated with Human Hepatitis B Antigen

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DNA polymerase activity was detected in each of eight preparations of concentrated human hepatitis B antigen (HBAg) rich in Dane particles prepared by high-speed centrifugation of antigen-positive human plasma and in none of seven control preparations prepared in the same way from HBAg-negative plasma. The incorporation of ^{3}H-thymidine-methyl-5'-triphosphate into DNA was dependent on four deoxyribonucleoside triphosphates and $MgCl_2$. Treatment of the concentrated HBAg preparations with the nonionic detergent Nonidet P-40 (NP40) more than doubled the enzyme activity. Fractionation of the concentrated HBAg preparation in sucrose density gradients after treatment with NP40 revealed that the enzyme activity appeared within the density range of Dane core antigen but at a slightly higher density than the average for core antigen. The only particles observed by electron microscopy in this region of the gradient were typical 28-nm cores, suggesting that the DNA polymerase activity was associated with a subpopulation of cores. No DNA polymerase activity was found in purified 20-nm HBAg particles. The DNA product of the reaction remained associated with the 110S core and was not susceptible to DNase digestion when associated with the core. Inhibition of the reaction by actinomycin D and daunomycin suggested that the reaction was dependent on a DNA template associated with the core.

Hepatitis B antigen (HBAg) has several characteristics suggesting that it is an antigen of hepatitis B virus. The antigen appears in the serum before the onset of acute serum hepatitis (4, 16, 23, 24, 30) and there is a subsequent specific antibody response (17, 18, 30). The d or y antigenic subtype of HBAg (19) found in secondary cases of hepatitis B is always the same as the subtype of the index case (20, 22) or the original source used in experimental infections (20). This evidence strongly indicates that the d and y determinants are the phenotypic expression of two genotypes of hepatitis B virus and are not determined by factors in the host (20).

There is evidence that the most common structures carrying HBAg determinants in the serum are not complete virus particles. These structures are roughly spherical and average around 20 nm in diameter; however, they are quite heterogeneous varying in size from 16 to 25 nm (2, 3, 10). Purified preparations of the particles are free of detectable nucleic acid (10). These are not characteristic of known small viruses.

Larger and more complex structures which share surface antigens with the 20-nm particles (1, 9) have been observed in some antigen-positive sera. Among these are long filamentous structures of variable length (1-3), and spherical particles 42 nm in diameter with 28-nm electron-dense cores first described by Dane et al. (7). The former are thought to be variant forms of the 20-nm particles (1), and the latter, which are uniform in appearance, have been considered to have a morphology consistent with that of a virus (7).

A recent report (13) has described DNA polymerase activity in crude pellets obtained by high-speed centrifugation of three HBAg-positive sera. The reaction was thought to be dependent on an endogenous RNA template since treatment of the preparations with high concentrations of ribonuclease reduced their

activity. The reaction, however, was found to be stimulated by the addition of poly dAT and not poly rA-oligo dT. We have confirmed the presence of DNA polymerase activity in HBAg preparations concentrated from plasma by centrifugation and not in similarly concentrated material from HBAg-negative plasmas. We find no evidence, however, that the reaction is dependent on RNA. Instead, inhibitor experiments suggest that DNA may be the template for the reaction. The enzyme was found to be associated with the 42-nm Dane particles and a subpopulation of the cores of these structures. No activity was found in highly purified 20-nm particles as previously reported (9). Our findings are consistent with the hypothesis that the 42-nm Dane particles are hepatitis B virions.

MATERIALS AND METHODS

Materials. ^{3}H-Thymidine-methyl-5'-triphosphate (^{3}H-TTP; 45 Ci/mmol) was purchased from New England Nuclear Corp. Nonidet P-40 (NP40) was a gift of the Shell Oil Co. Crystalline bovine pancreatic ribonuclease A free from deoxyribonuclease, electrophoretically pure pancreatic deoxyribonuclease, and highly polymerized calf thymus DNA were purchased from Calbiochem. Poly dAT [12-18] was purchased from Collaborative Research Corp. Poly rA-oligo dT was prepared in a manner described by Weissbach et al. (33). The Ausria kit (11) for radioimmunoassay of HBAg was purchased from Abbott Laboratories. DNA isolated from the serum of patients with systemic lupus erythematosis was provided by H. Holman and was extracted with phenol (5) two times before use. Calf thymus DNA "activated" by limited digestion with exonuclease 3 (26) and ^{3}H-thymidine-labeled ϕX174 were kindly provided by A. Kornberg. ^{3}H-DNA from simian virus 40 (SV40) was a gift from P. Rosenthal. Carrier chicken cell RNA was prepared as previously described (5). Chicken cell ^{3}H-DNA was prepared by incubating chicken embryo fibroblast cultures with ^{3}H-thymidine for 24 h followed by DNA extraction and purification as previously described (28). RSV(RAV-1) ^{3}H-RNA was prepared as previously described (28). RNA from Sendai virus was prepared as previously described (5).

Plasma specimens and antigen preparations. Plasmas from 60 chronic HBAg carriers were examined by electron microscopy (8) for the presence of Dane particles (7) and several specimens with relatively high concentrations of such particles were chosen for further study. These plasmas, designated 495528, 595239, 595839, 630198a&b, 672497, 729094, 767932, and 806001, all had high HBAg titers by complement fixation (see Table 4). Plasmas 630198a and 630198b were different concentrated preparations from the same plasma. Plasmas from normal subjects had no detectable HBAg by radioimmunoassay. These plasma are designated 639, 643, 680, 684, 691, 841, and AH. Plasma pellets were prepared by centrifugation of the plasma in a Spinco Type 21 rotor at 20,000 rpm for 4 h at 4 C in a Spinco Type L ultracentrifuge. Each pellet was resuspended to the original volume in phosphate-buffered saline (pH 7.4) and recentrifuged as described above. The resulting second pellet was resuspended in phosphate-buffered saline at 1/20 the original volume and stored at −70 C. Such concentrated HBAg or material from normal plasma will be referred to sometimes as enzyme preparations. The final preparations from all HBAg-positive plasmas contained high titers of HBAg and protein concentrations between 0.9 and 1.2 mg of protein per ml determined by the method of Lowry et al. (21). Highly purified 20-nm HBAg particles were prepared by a four-step procedure previously described (9). The two purified preparations used are designated Ytr (0.37 mg of protein per ml) which was subtype ayw and DD (0.70 mg of protein per ml) which was subtype adw. Purified Rous sarcoma virus (RSV[RAV-1]) was prepared as previously described (14) and the final preparation had an absorbancy at 280 nm (A_{280}) of 21 and A_{280}/A_{260} of 1.05. A solid-phase radioimmunoassay (11) was used for HBAg determinations. A similar radioimmunoassay (R. H. Purcell et al., manuscript submitted for publication) was performed to detect the antigen of Dane cores by using a human convalescent serum which has been shown to aggregate the cores from Dane particles and not other particulate structures in HBAg-positive sera (1).

Phenol extraction of the DNA polymerase product. The reaction mixture was treated for 2 h at 37 C with Pronase at 1 mg per ml in the presence of 0.1% sodium dodecyl sulfate (SDS). It was then adjusted to a volume of 20 ml with a solution containing 0.01 M Tris-hydrochloride, pH 7.4, 0.1 M NaCl, 0.001 M EDTA, 1% SDS, and 0.05% mercaptoethanol, and 200 μg of chicken cell RNA was added as carrier. The solution was then extracted three times at 60 C with phenol saturated with buffer consisting of 0.1 M Tris-hydrochloride, pH 7.4, 0.1 M NaCl, and 0.005 M EDTA. The nucleic acid in the aqueous phase was then precipitated with two volumes of ethanol (5) and the precipitate was redissolved in buffer consisting of 0.01 M Tris-hydrochloride, pH 7.5, 0.10 M NaCl, and 0.001 M EDTA.

Assay for radioactivity. Trichloroacetic acid-precipitable radioactivity was determined by scintillation counting after collecting and washing precipitates on glass fiber filters (5) or after spotting samples on Whatman no. 3 paper disks and washing as previously described (27).

Electron microscopy. The method for uranyl acetate staining and examination of preparations in an electron microscope has been described (10).

RESULTS

DNA polymerase activity in concentrated HBAg preparations. HBAg preparations, concentrated 20-fold from plasmas of chronic antigen carriers by simple centrifugation and resuspension, as described in Materials and Methods, were found to catalyze the incorporation of ^{3}H-TTP into a trichloroacetic acid-insoluble product when incubated with the nonionic detergent NP40 and four deoxynucleoside triphosphates. Table 1 shows the results of

an experiment with two concentrated HBAg preparations (630198b and 806001). The incorporation of ^3H can be seen to increase with time and preparation 630198b had more activity per milligram of protein than preparation 806001. Similarly concentrated material from antigen-negative plasma (AH) and highly purified 20-nm HBAg particles (Ytr and DD) failed to show any activity. Figure 1 shows the time course of a typical reaction where ^3H incorporation continued for the 4-h duration of an experiment using preparation 630198b. The rate of incorporation for this crude enzyme preparation was equivalent to about 10 pmol of TTP per mg of protein per h. Unconcentrated Dane particle-rich plasma was found to have an enzyme activity per unit volume about 5 to 10% of that in the 20-fold concentrated preparations.

Table 2 shows some of the reaction requirements. Four deoxynucleoside triphosphates were required for optimal activity, suggesting that the reaction product is DNA. The reaction dependence was greater for dGTP than for dATP and dCTP. No incoporation was observed in the absence of $MgCl_2$ and treatment of the enzyme preparation with NP40 more than doubled the ^3H incorporation. The optimal $MgCl_2$ concentration for the reaction was over a broad concentration range above 20 mM (Fig. 2). The reaction rate was no greater when $MnCl_2$ was substituted for $MgCl_2$ over the concentration range tested. The reaction was found to be stimulated by about 25% with KCl or NH_4Cl in concentrations above 0.1 M in the reaction mixture. The enzyme activity was somewhat greater at pH 7.5 than at pH 7.0 or pH 8.0 as shown in Table 3. No sharp temperature dependence for the reaction was found and as shown in Table 3 the reaction rate was nearly the same over the temperature range from 33 to 41 C.

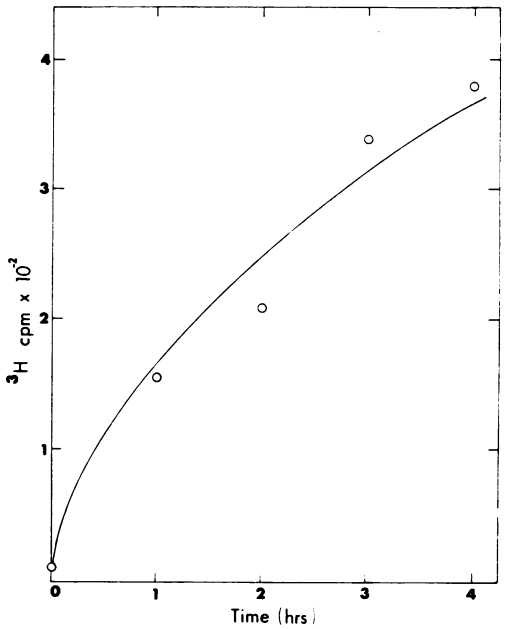

FIG. 1. *Time course of the DNA polymerase reaction. A 300-μliter amount of concentrated HBAg preparation 630198 was used in a reaction mixture with components in the proportions described in Table 1 and a volume of 1.5 ml. At the designated times of incubation at 37 C, 10 μliters of the reaction mixture was removed for counting as described in Table 1.*

TABLE 1. *DNA polymerase activity in concentrated Dane particle-rich HBAg, purified 20-nm HBAg particles and control preparations*[a]

Incubation time (min)	Enzyme preparation				
	630198b	806001	Ytr	DD	AH
0	9[b]	9	17	9	8
90	720	152	8	10	11
180	993	182	12	21	11

[a] Twenty-five-μliter samples of concentrated HBAg (630198b and 806001), highly purified 20-nm HBAg particles (Ytr and DD) and concentrated material from control plasma (AH) were made 1% with NP40 and 0.3% with mercaptoethanol. Each preparation was then added to 100 μliters of a solution containing 16 μmol of Tris-hydrochloride, pH 7.5; 4 μmol of $MgCl_2$; 12 μmol of NH_4Cl; 0.05 μmol each of dATP, dCTP, and dGTP; and 28 pmol of ^3H-TTP (45 Ci/mmol) giving a final reaction volume of 130 μliters. The reaction mixtures were incubated at 32 C and at the designated times 40 μliters from each was spotted on a disk of Whatman no. 3 paper which was washed and counted as described in Materials and Methods.

[b] Acid-precipitable ^3H in counts per minute.

TABLE 2. *Requirements for DNA polymerase reaction*

Conditions	^3H (counts/min[a])
Complete[b]	1,151
-dCTP	248
-dGTP	52
-dATP	109
-dCTP, dGTP, dATP	29
-$MgCl_2$	15
-NP40	466

[a] Acid-precipitable ^3H after 180 min of incubation at 32 C.

[b] Concentrated HBAg preparation 630198b was used for seven DNA polymerase reactions. The complete reaction mixture was as described in Table 1 and reaction components were omitted in the other reactions as indicated.

In order to determine the frequency of DNA polymerase activity in HBAg-positive and HBAg-negative plasma, concentrated preparations from eight different Dane particle-rich HBAg-positive plasmas and from seven different HBAg-negative plasmas were tested. Two such experiments are shown in Table 4. Concentrated preparations from all eight HBAg-positive plasmas contained DNA polymerase activity (Table 4, experiment 1) and none was detected in material from any of the HBAg-negative plasmas (Tables 1 and 4). The amount of enzyme activity in the 20-fold concentrated HBAg preparations was variable and did not correlate directly with the HBAg complement fixation titer in the original plasma or with HBAg subtype (Table 4). The results suggest that DNA polymerase activity occurs in a particulate fraction from HBAg-positive plasma but not in a similar fraction from normal plasma.

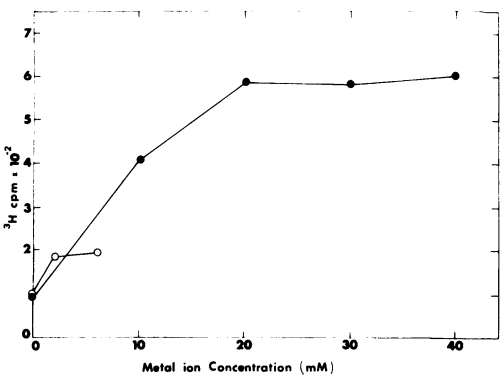

FIG. 2. *Effect of $MgCl_2$ (●) and $MnCl_2$ (○) concentrations on the DNA polymerase reaction. The reaction mixture was as described in Table 1 except that NH_4Cl was omitted and $MgCl_2$ or $MnCl_2$ concentration was as designated in the figure. Concentrated HBAg preparation 630198 was used and samples were counted as described in Table 1 after incubation for 135 min at 37 C.*

Properties of the radioactive reaction product. Some difficulty was initially encountered in efficiently extracting the radioactive reaction product from the enzyme reaction mixture. After a reaction using concentrated HBAg, ^{3}H-TTP and the other reaction components described in Table 1, SDS and mercaptoethanol were added, and the reaction mixture was extracted three times with phenol at room temperature. Only 3% of the trichloroacetic acid-precipitable ^{3}H material synthesized was found in the aqueous phase. However, when Pronase digestion was carried out at 37 C in the presence of SDS followed by phenol extraction three times at 60 C, as described in Materials and Methods, about 20% of the acid-precipitable ^{3}H material was found in the aqueous phase. Equilibrium centrifugation in a CsCl gradient

TABLE 3. *Effect of temperature and pH on the DNA polymerase reaction*[a]

pH	Temperature (C)		
	33	37	41
7.0	1,289[b]	1,505	1,577
7.5	1,749	1,808	1,905
8.0	1,155	1,137	1,246

[a] Concentrated HBAg preparation 767932 was used in DNA polymerase reactions similar to that described in Table 1 except that the Tris-hydrochloride pH and the reaction temperature were as indicated.

[b] Acid-precipitable ^{3}H (counts/min) in 25-μliter samples after 180 min of incubation.

TABLE 4. *DNA polymerase activity in different concentrated HBAg and control preparations*[a]

Expt. 1				Expt. 2		
Enzyme preparation	HBAg CF titer[b]	HBAg subtype	^{3}H (counts/min)[c]	Enzyme preparation	HBAg RIA titer[d]	^{3}H (counts/min)[c]
495528	800	ad	1,055	630198b		876
595239	800	ad	7,330	639	0	15
595839	800	ad	1,151	643	0	13
630198a	800	ad	3,961	680	0	20
672497	400	ay	1,628	684	0	15
729094	400	ay	1,087	691	0	13
767932	400	ay	6,265			
806001	200	ay	1,047			
841	0		30			

[a] The reactions were carried out as described in Table 1.

[b] Reciprocal complement fixation titer for HBAg performed on the original plasmas.

[c] Acid-precipitable ^{3}H in 40-μliter samples after 180 min of incubation at 32 C.

[d] Result of radioimmunoassay for HBAg performed on the original plasmas.

showed that all of the radioactive reaction product extracted in this way appeared in a relatively sharp band with an average density of 1.71 g per ml which was slightly higher than the density of calf thymus DNA in the same gradient (Fig. 3). This is further evidence that the radioactive product of the reaction is DNA.

When a reaction mixture after incubation for 3 h with NP40 as in Table 1 was layered over a 5 to 20% sucrose density gradient and centrifuged in an SW40 rotor for 75 min at 4 C, almost all of the acid-precipitable ^3H enzyme product sedimented in a relatively sharp band slightly slower than 114*S* ϕX 174 (31) and was estimated to have a sedimentation coefficient of about 110*S* (Fig. 4A). A small amount of ^3H material sedimented slightly slower than the main peak and can be seen as a shoulder on the trailing side of the ^3H band. Less than 8% of the acid-precipitable ^3H-product sedimented much more slowly and was found near the top of the tube. When SDS was added to a similar reaction mixture followed by incubation at 37 C for 20 min, the sedimentation of the ^3H reaction product was greatly altered (Fig. 4B). A sharp band of ^3H material sedimented more slowly than 21*S* SV40 DNA (6) and was estimated to have a sedimentation coefficient of about 15*S*. Fifteen percent of the acid-precipitable ^3H material sedimented much more slowly and appeared at the top of the tube. Heating the reaction product to 100 C in SDS followed by digestion with Pronase resulted in ^3H material sedimenting at 15*S* with a distribution similar to that shown in Fig. 4B. These experiments indicate that after the enzyme reaction in the detergent NP40, most of the acid-insoluble ^3H product is associated with a 110*S* structure and is converted to a 15*S* form by incubation with SDS.

Radioactive 15*S* material was recovered from a sucrose density gradient like that shown in Fig. 4B and tested for susceptability to DNase. Table 5 shows that 87% of the acid-precipitable 15*S* ^3H product of the reaction was converted to an acid-soluble form by the high concentration of DNase used. A similar fraction was digested by DNase at 20 μg per ml. In a parallel experiment under the same conditions 99.4% of the ^3H-DNA from chicken cells was digested to an acid-soluble form. In several experiments ^3H reaction product extracted in different ways was not completely digested by DNase as was the case in the experiment in Table 5. Reaction product prepared in this way by sedimentation after SDS treatment was also demonstrated to band in CsCl gradients at density of 1.71 g per ml as the ^3H product shown in Fig. 3. Finally,

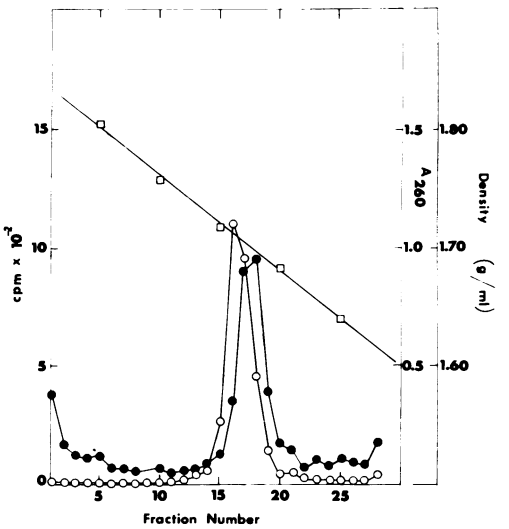

FIG. 3. *Cesium chloride equilibrium density gradient centrifugation of the DNA polymerase reaction product. A 100-μliter amount of concentrated HBAg preparation 630198 was used in a reaction mixture with components in the proportions described in Table 1 and a volume of 500 μliters. After 180 min of incubation at 35 C the radioactive reaction product was extracted with SDS and hot phenol as described in Materials and Methods. The ethanol precipitate was redissolved in 7.5 ml of 0.01 M Tris-hydrochloride, pH 7.5, 0.10 M NaCl, and 0.001 M EDTA; 50 μg of calf thymus DNA was added and then solid CsCl (10 g) was added to give a final solution density of 1.71 g/ml. Centrifugation in a Spinco 50 Ti rotor was at 44,000 rpm for 40 h at 20 C. Absorbance at 260 nm (●), solution density (□), and acid-precipitable radioactivity (○) were determined on fractions collected from the bottom of the tube.*

the acid-insoluble reaction product was not made acid soluble by digestion with 0.5 N NaOH and ^3H material treated this way banded at a density of 1.71 g per ml in a CsCl density gradient.

Further characterization of the DNA polymerase-containing structures from plasma. Although DNA polymerase activity appeared to be regularly present in HBAg-positive plasma (Table 4) and could be further localized to a crude, washed particulate fraction containing HBAg reacting particles, the enzyme was not associated with highly purified 20-nm HBAg particles (Table 1). In order to further identify the DNA polymerase-containing structure in HBAg-positive plasma, concentrated HBAg preparations were further fractionated by sedimentation in sucrose density gradients. Figure 5A shows the results of an experiment in which a concentrated HBAg preparation was layered over a preformed 15 to 65% sucrose density

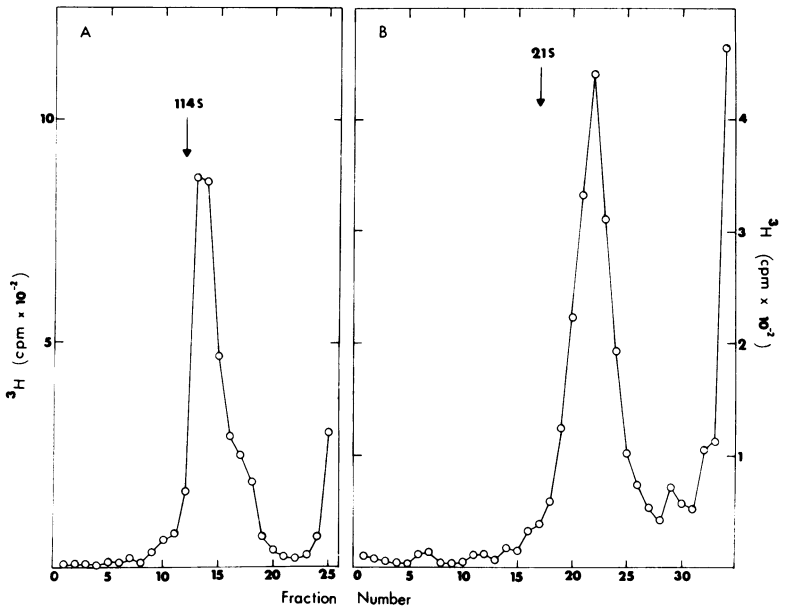

FIG. 4. *Sedimentation of the HBAg DNA polymerase ^{3}H-product in sucrose density gradients. A DNA polymerase reaction was carried out by using a concentrated HBAg preparation made up to 1% NP40 and 0.05% mercaptoethanol. ^{3}H-TTP and other reaction components were in the proportions described in Table 1. After incubation for 3 h at 35 C, Sample A from the reaction mixture was layered over a 5 to 20% sucrose density gradient containing 0.01 M Tris-hydrochloride, pH 7.4, 0.10 M NaCl, and 0.001 M EDTA and centrifuged in a Spinco SW40 rotor at 40,000 rpm for 75 min at 4 C. ^{3}H-φX174 was sedimented in an identical gradient in the same rotor. Sample B from the reaction mixture was made up to 1% SDS and incubated for 20 min at 37 C. It was then layered over a 5 to 20% sucrose density gradient and centrifuged in a Spinco SW65 rotor at 50,000 rpm for 3 h at 4 C. ^{3}H-DNA from SV40 virus was sedimented in an identical gradient in the same rotor. Acid-precipitable ^{3}H (O) was determined on each fraction collected from the bottom of the tubes. The relative positions of 114S φX174 and 21S SV40 DNA are designated in Fig. A and B, respectively.*

TABLE 5. *DNase treatment of the DNA polymerase product*[a]

^{3}H-DNA	+DNase	-DNase
767932	75[b]	564
Chicken	177	28,294

[a] Concentrated HBAg preparation 767932 was used in a reaction as described in Table 1 and the reaction product was incubated with SDS and sedimented in a sucrose density gradient as shown in Fig. 4B. The radioactive material recovered from the middle of the gradient was adjusted to 10 mM $MgCl_2$ and a sample was then incubated with DNase (250 μg per ml) and another sample without DNase at 37 C for 1 h. Similarly, ^{3}H-DNA from chicken cells was incubated with and without DNase.

[b] Acid-precipitable ^{3}H in counts per minute.

gradient and centrifuged at 50,000 rpm for 3 h in a Spinco SW65 rotor. Each fraction collected from the bottom of the gradient was then assayed for DNA polymerase activity and for HBAg and core antigens by radioimmunoassays. Although there was a peak of HBAg near the middle of the tube, a significant amount of antigen was distributed throughout the upper half of the gradient. The core antigen formed a sharper band at a slightly higher sucrose density than the HBAg peak. DNA polymerase activity likewise was found in a relatively narrow band at a still higher sucrose density than the core antigen peak but completely overlapped by both the core antigen and HBAg. Examination of the peak fraction of DNA polymerase activity (fraction 8) by electron microscopy revealed the presence of mostly 42-nm Dane particles and a few long 20-nm diameter filaments.

Figure 5B shows a parallel sucrose gradient fractionation of a concentrated HBAg preparation after incubation with NP40 and mercaptoethanol for 3 h at 37 C. Again each gradient fraction was assayed for DNA polymerase activity, HBAg, and core antigen. NP40 and mercaptoethanol treatment resulted in a shift in the position of the HBAg peak toward the top of the gradient and little or no antigen was detected above the background in the bottom half of the tube where the core antigen and DNA polymer-

ase activity were found. The position of the core antigen in the gradient was unchanged from that in Fig. 5A. The DNA polymerase activity also remained in the same relative position as in Fig. 5A but it clearly formed a wider band after NP40 and mercaptoethanol treatment. Examination of the peak fraction of DNA polymerase activity (fraction 7) by electron microscopy revealed that typical 28-nm Dane cores were the only recognizable particulate structure. No Dane particles, filaments or 20-nm forms, were seen.

Figure 5C shows a parallel experiment in which a DNA polymerase reaction with concentrated HBAg and ^{3}H-TTP as described in Table 1 was carried out for 3 h in the presence of NP40 and mercaptoethanol and then the reaction mixture was fractionated in a sucrose density gradient as in Fig. 5A. The HBAg and core antigen were in the same relative positions as in Fig. 5B. Almost all of the acid-precipitable ^{3}H-DNA formed in the reaction before sucrose gradient sedimentation was found to be in the same position in the gradient as the DNA polymerase activity in the experiment in Fig. 5B. Only about 5% of the acid-precipitable ^{3}H-DNA remained at the top of the tube. Again only 28-nm Dane cores were observed by electron microscopy in the peak sucrose gradient fraction of acid-precipitable ^{3}H (fraction 8).

These experiments indicate that the DNA polymerase activity is in a particulate structure with a buoyant density within the density range of Dane particles and Dane cores but with an average density slightly higher than the average density of cores. Since after NP40 treatment of the concentrated HBAg preparation the only particulate structure detected by electron microscopy within the sucrose gradient band of enzyme activity was 28-nm Dane cores, it seems very likely that the activity was associated with a subpopulation of cores. It is also apparent that the DNA product of the reaction remains associated with the enzyme containing particulate structure and very little newly synthesized DNA is released from this structure. The experiment in Fig. 4A shows that this structure sediments around 110S.

The ability of the DNA polymerase containing structure alone to carry out active DNA

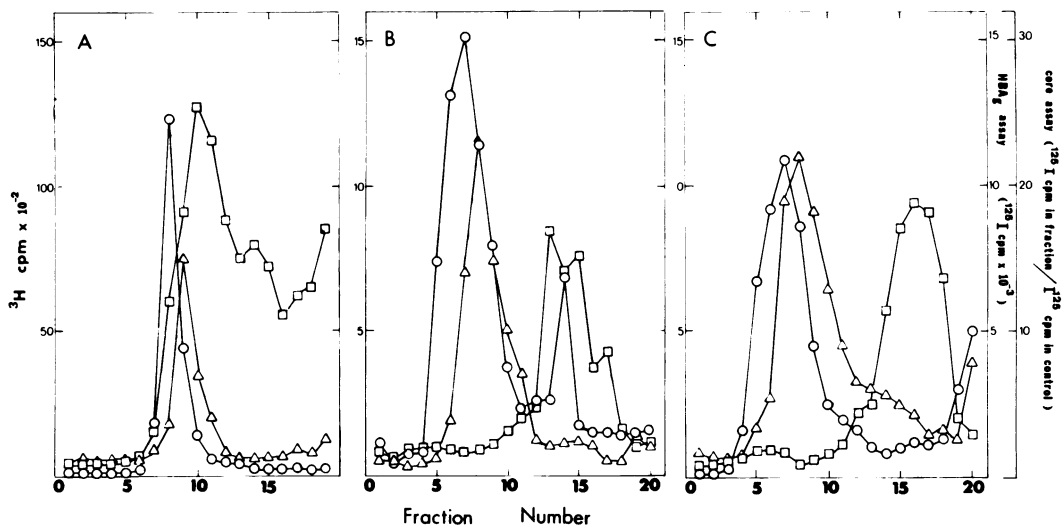

FIG. 5. *DNA polymerase activity in sucrose density gradients after centrifugation of concentrated HBAg. After sonic treatment of concentrated HBAg preparation 630198b to disrupt aggregates, a 150-μliter sample was diluted to 0.5 ml with buffer containing 0.01 M Tris-hydrochloride, pH 7.4, and 0.10 M NaCl and layered on a 15 to 65% sucrose gradient containing 0.01 M Tris-hydrochloride, pH 7.4, and 0.10 M NaCl (A); a second 150-μliter sample was incubated for 3 h at 35 C with 1% NP40 and 0.05% mercaptoethanol, diluted to 0.5 ml, and layered over a second 15 to 65% sucrose gradient (B); and a third 150-μliter sample was used in a 3-h, 0.5-ml enzyme reaction with ^{3}H-TTP, as described in Table 1, and then layered over a third 15 to 65% sucrose gradient (C). The three gradients were then centrifuged in a Spinco SW65 rotor at 50,000 rpm for 3 h at 4 C and fractions were collected from the bottom of each tube. Ten μliters from each fraction was used for radioimmunoassay of HBAg (□) and 25 μliters was used for the core assay (△). Acid-precipitable ^{3}H (○) after a 3-h DNA polymerase reaction with ^{3}H-TTP as described in Table 1 was determined by using 150-μliter samples from each fraction of sucrose gradients (A) and (B). Acid-precipitable ^{3}H (○) was determined directly for 150-μliter samples from the fraction of sucrose gradient (C).*

synthesis dependent on all four deoxribonucleoside triphosphates, suggests that a nucleic acid molecule which can serve as a template or primer for the reaction is part of the enzyme complex. To test this possibility, a concentrated HBAg preparation was treated with NP40 and then digested with high concentrations of RNase or DNase before testing its enzyme activity. Table 6 shows that neither nuclease decreased at all the ability of the enzyme preparation to incorporate ^{3}H-TTP into new DNA. RNase under the same conditions completely digested a sample of ^{3}H-RNA to an acid-soluble form. Similarly, DNase in the presence of the same concentrated HBAg preparation completely digested a sample of ^{3}H-DNA indicating that the DNase was completely active in the presence of NP40 and the concentrated HBAg preparation. These results suggest that either the DNA polymerase reaction requires neither RNA nor DNA or that the template molecule is in a protected state within the enzyme-containing particle and is not available as substrate for RNase or DNase.

TABLE 6. *Effect of DNase and RNase pretreatment on the DNA polymerase activity*[a]

Sample	Treatment	^{3}H (counts/min)
1 630198b	None	6,270
2 630198b	DNase	6,283
3 630198b	RNase	8,075
4 RSV(RAV-1) ^{3}H-RNA	None	43,363
5 RSV(RAV-1) ^{3}H-RNA	RNase	58
6 Chicken ^{3}H-DNA	None	54,353
7 Chicken ^{3}H-DNA	DNase	489

[a] Three 70-μliter samples of concentrated HBAg preparation 630198b were each made up to a final volume of 200 μliters containing 2% NP40 and 20 mM $MgCl_2$. Sample no. 1 contained no nuclease, sample no. 2 contained DNase at 0.5 mg/ml, and sample no. 3 contained RNase at 0.5 mg/ml. All three were incubated at 35 C for 60 min and then layered on discontinuous sucrose gradients consisting of 4 ml of 20% sucrose and 1 ml of 65% sucrose. After centrifugation in a Spinco SW50.1 rotor at 50,000 rpm for 2 h at 4 C, 250-μliter fractions were collected from each tube. Each fraction was assayed for DNA polymerase activity by using reaction components in the proportions described in Table 1. The sum of acid-precipitable ^{3}H of the fractions in each gradient with enzyme activity is listed in the ^{3}H counts per minute column. Acid-precipitable ^{3}H-RNA was determined after incubation of equal samples of RSV(RAV-1) ^{3}H-RNA in NP40 and $MgCl_2$ for 3 h at 35 C without RNase (no. 4) and with RNase 0.5 mg/ml (no. 5). Acid-precipitable ^{3}H-DNA was determined after incubation of equal samples of chicken ^{3}H-DNA in solution with NP40, $MgCl_2$ and concentrated HBAg preparation 630198b for 3 h at 35 C without DNase (no. 6) and with DNase 0.5 mg/ml (no. 7).

Several synthetic and natural DNA's and RNA's were tested for their ability to stimulate the DNA polymerase reaction. Table 7 shows that none of them stimulated the reaction carried out by either of two concentrated HBAg preparations. Poly dAT inhibited the reaction by an unknown mechanism. None of the nucleic acid preparations when added to a control preparation without HBAg effected the background level of incorporation. The RSV(RAV-1)-catalyzed DNA polymerase reaction, on the other hand, was greatly stimulated by several of the additions. "Nicked" or "activated" calf thymus DNA and poly dAT were the most active. Native calf thymus DNA, heated calf thymus DNA, heated and unheated serum DNA, and poly rA-oligo dT also stimulated the incoporation of ^{3}H-TTP over the level observed with no additions. These results suggest that either the DNA polymerase in the concentrated HBAg preparations will not use any of the nucleic acid molecules tested as a template or primer, or the enzyme is protected within the enzyme-containing particle so that none of the nucleic acid molecules added can reach the enzyme.

Finally, the ^{3}H-DNA product of the DNA polymerase reaction was tested for susceptibil-

TABLE 7. *Effect of added DNA or RNA on the DNA polymerase activity*[a]

Additions to the reaction	Enzyme preparation			
	630198b	595239	841	RSV (RAV-1)
None	1,246[b]	2,759	157	2,377
Calf thymus DNA	1,267	2,391	100	14,490
Heated calf thymus DNA	1,167	2,623	111	17,030
"Activated" calf thymus DNA	1,718	3,024	134	80,700
Lupus serum DNA	1,008	2,448	118	16,672
Heated lupus serum DNA	1,029	2,571	176	14,526
Poly rA-oligo dT	1,020	2,564	76	6,945
Poly dAT	227	330	71	35,695
Sendai virus RNA	958	2,416	152	2,959

[a] Nine reaction mixtures were made up as described in Table 1 with each enzyme preparation and 20 μg of the nucleic acid preparations listed in the table were included in the reactions indicated.

[b] Acid-precipitable ^{3}H (counts/min) in 40-μliter samples of each reaction mixture were determined after 180 min of incubation at 35 C.

ity to DNase before extraction from the reaction mixture. Table 8 shows that a high concentration of DNase failed to convert any of the acid-precipitable ^{3}H-DNA product of reactions with two different concentrated HBAg preparations to an acid-soluble form. In a similar experiment with RSV(RAV-1), more than 95% of the ^{3}H-DNA product was rendered acid soluble by DNase under the same conditions. These results indicate that not only does the DNA reaction product remain associated with the 110S enzyme-containing particle (Fig. 4 and 5) but the product is not available as substrate for DNase before extraction from the particle.

Effect of nucleic acid polymerase inhibitors on the HBAg enzyme reaction. Rifampin, daunomycin, and actinomycin D were each added at 2, 10, and 50 μg per ml to DNA polymerase reactions with a concentrated HBAg preparation. Figure 6 shows that rifampin had little effect on the incorporation of ^{3}H-TTP into DNA, daunomycin at all three concentrations inhibited the reaction by around 50%, and actinomycin D inhibited the reaction by more than 75%.

DISCUSSION

Our experiments indicate that DNA polymerase activity is present in preparations of HBAg concentrated from human plasma by simple high-speed centrifugation and not in similarly concentrated material from HBAg-negative plasma. There were no exceptions among eight HBAg-positive plasmas, selected for a higher than average Dane particle concentration, and seven control plasmas. Lower levels of enzyme activity could be detected in unconcentrated HBAg-positive plasma. Hirschman, et al. (13) found similar enzyme activity in material pelleted by centrifugation from three HBAg-positive sera and not in two controls.

TABLE 8. *DNase treatment after the DNA polymerase reaction*[a]

Enzyme preparation	Treatment	^{3}H (counts/min)
630198a	None	5,534
630198a	DNase	5,197
595239	None	9,642
595239	DNase	9,056
RSV(RAV-1)	None	24,695
RSV(RAV-1)	DNase	1,171

[a] Enzyme reactions were carried out by using 50 μliters of each enzyme preparation and other reactants in the proportions described in Table 1. After 180 min at 35 C the reaction mixtures were divided into two 100-μliter samples, DNase at 500 μg/ml was added to one sample and both were incubated for 60 min. Acid-precipitable ^{3}H remaining in each sample was then determined using Whatman no. 3 paper disks.

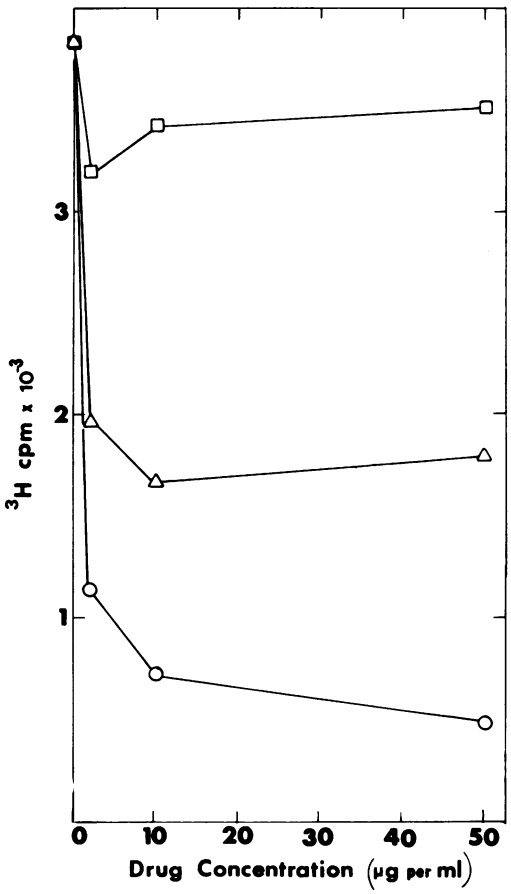

FIG. 6. *Effect of rifampin (□), daunomycin (Δ), and actinomycin D (O) on the DNA polymerase reaction. DNA polymerase reactions using concentrated HBAg preparation 595239 were carried out as described in Table 1 and each drug was added to a reaction mixture in the concentration indicated in the figure. Acid-precipitable ^{3}H was determined on 40-μliter samples of each reaction mixture after 180 min of incubation at 35 C.*

Our results strongly suggest that the DNA polymerase activity is associated with a subpopulation of 28-nm cores released spontaneously or by NP40 detergent treatment from 42-nm Dane particles. This is suggested by the results in Fig. 5B showing that the enzyme activity in NP40-treated preparations bands in a density gradient within the density range of core antigen but at a buoyant density slightly higher than the average for core antigen. The only particulate structures found by electron

microscopy in this region of the gradient were typical 28-nm cores. Why the cores that are capable of actively synthesizing DNA are more dense than the average is unknown but such cores could contain nucleic acid in contrast to empty cores which would be less dense and lack the capacity to incorporate ^{3}H-TTP into DNA. It is clear that the most common HBAg-containing structures such as the 20-nm particles do not contain DNA polymerase activity.

The evidence that the enzyme-containing particles or cores may contain nucleic acid is indirect. First, the ability of cores to synthesize DNA without addition of a DNA or RNA template and the requirement of all four deoxyribonucleoside triphosphates for incorporation of ^{3}H-TTP suggest that a DNA or RNA template within the core probably directs the synthesis of DNA. Although DNA polymerases have been shown to catalyze unprimed reactions, such reactions have not been found to require four deoxyribonucleoside triphosphates (29). The DNA and RNA polymerases found in known animal viruses use viral nucleic acids as templates for DNA or RNA synthesis (15, 32). Second, the inhibition of the reaction by actinomycin D and daunomycin suggests that the reaction is dependent on a DNA template. These compounds inhibit DNA-dependent DNA or RNA synthesis exclusively by specific binding to DNA templates containing deoxyguanidine residues (12, 25). There is greater binding to double-stranded DNA than to single stranded, and no binding to RNA. The levels of inhibition observed in this study are consistent with those reported for other DNA-dependent DNA polymerase reactions (25).

The experiments here also suggest that the DNA polymerase and the postulated DNA template are probably within the core which is impermeable to large molecules such as protein and nucleic acid molecules but permeable to small molecules, and the DNA reaction product is synthesized within the core and very little, if any, is released as free DNA. Evidence that the reaction product remains associated with the enzyme-containing particle or core after the reaction is seen when comparing Fig. 5B and C where ^{3}H enzyme product in NP40-treated concentrated HBAg preparations follows the enzyme activity in density gradients. The NP40-treated particle or core has a sedimentation coefficient (110*S*) very near that of ϕX174 (114*S*) (Fig. 4A). The observations that (i) ^{3}H-DNA product before disruption of the cores is not digested by DNase (Table 8); (ii) the ability of core particles to synthesize DNA is unaffected by either DNase or RNase pretreatment (Table 6); and (iii) the reaction carried out by cores is not stimulated by any of the nucleic acid molecules tested (Table 7) suggest that the DNA reaction product, the postulated DNA template, and the enzyme are all in protected positions within the core and are not available for interaction with large DNase or nucleic acid molecules. Smaller molecules such as $MgCl_2$, deoxyribonucleoside triphosphates, actinomycin D, and daunomycin, on the other hand, must freely enter the core.

We have little information about the mechanism of the enzyme reaction or the structure of the DNA product. After disruption of the core by SDS alone, SDS followed by phenol extraction or 0.5 M NaOH, the ^{3}H-DNA product had a buoyant density of 1.71 in CsCl, suggesting that it had very little, if any, protein associated with it. The reason for the resistance of a small fraction (13%) of the ^{3}H-DNA product to DNase digestion (Table 5) is not clear but the presence of a small amount of associated protein protecting the DNA has not been excluded. ^{3}H-DNA released from cores by SDS treatment at 37 C had a sedimentation coefficient around 15*S* (Fig. 4B). Heating to 100 C for 10 min in a reaction mixture containing SDS did not alter the sedimentation behavior of the 15*S* ^{3}H-DNA, indicating that a significant irreversible conformationl change did not result from heating under these conditions. Further work is being done to determine the structure of DNA polymerase product.

Our evidence that the 28-nm core structures from the 42-nm Dane particles contain DNA polymerase activity and endogenous template in addition to the morphological structure of the particle (7) are consistant with the hypothesis that the Dane particle is a virus. It is thus quite possible that the Dane particle is the human hepatitis B virion and the core structure is the virion nucleocapsid. If the DNA polymerase described here is a virion enzyme and if the reaction is DNA dependent, the enzyme would be unique because no similar virion enzymes have been described.

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