JOURNAL OF VIROLOGY, Dec. 1980, p. 787-795
0022-538X/80/12-0787/09$02.00/0

Vol. 36, No. 3

# Structural Relationships Between the Surface Antigens of Ground Squirrel Hepatitis Virus and Human Hepatitis B Virus

WOLFRAM H. GERLICH,† MARK A. FEITELSON, PATRICIA L. MARION, AND WILLIAM S. ROBINSON*

*Division of Infectious Diseases, Department of Medicine, Stanford University, Stanford, California 94305*

Several physical, chemical, and serological properties of surface antigen particles from ground squirrel hepatitis virus (GSHsAg) and human hepatitis B virus (HBsAg) were compared. GSHsAg and HBsAg particles were purified from positive sera by gel chromatography and isopycnic centrifugation. Both antigens consisted mainly of spherical particles with an average diameter of approximately 20 nm and a buoyant density in CsCl of approximately 1.19 g/ml. Their UV absorption spectra indicated the presence of more tryptophane than tyrosine and the absence of detectable nucleic acid. GSHsAg was found to contain two major polypeptides of approximately 23,000 and 27,000 daltons, with electrophoretic migration rates distinctly faster than those of the two major polypeptides of HBsAg particles. After radiolabeling of purified antigen preparations with Bolton-Hunter reagent, the two major polypeptides of GSHsAg showed almost identical tryptic peptide maps. The tryptic peptide map of the major polypeptide from GSHsAg contained 13 of 37 spots also present in the map of the major HBsAg polypeptide, and 13 of 27 spots in the map of the major HBsAg polypeptide were also present in the map of the major GSHsAg polypeptide. This suggests considerable sequence homology between the major surface antigen polypeptides of the two viruses. However, there was only a weak serological cross-reactivity between antigens of the two viruses. Using an anti-HBs-containing serum with a relatively strong cross-reactivity, GSHsAg was found to consist of at least two antigenically different subspecies. The more strongly cross-reacting form had a slightly higher buoyant density than the other antigenic form.

When hepatitis B virus (HBV) was first characterized it was found to have several interesting differences from viruses of the recognized virus groups. Its ultrastructure and antigenic structure were considered unique (reviewed in 20). Its small circular DNA had an unusual structure, including the presence of a large single-stranded region of different length in different molecules which was repaired by a virion DNA polymerase. It caused frequent persistent infection with complete viral forms (the 42-nm Dane particle) and incomplete viral forms (22-nm spherical and filamentous particles) continuously in the blood in high concentrations, and persistent infection was associated with chronic hepatitis and hepatocellular carcinoma. Both the complete and the incomplete viral forms in blood appear to contain on their surfaces a virus-specific antigen now designated hepatitis B surface antigen (HBsAg). HBsAg is a complex antigen. The HBsAg's from all sera appear to contain a group-specific determinant *a* as well as *d* or *y* and *w* or *r* type-specific determinants (1, 15). Antigenic variation of some of these determinants and additional determinants such as *x* and *q* have also been described (4, 5). Several polypeptides isolated from purified preparations of 22-nm and filamentous HBsAg particles have been shown to contain the HBsAg specificities (7, 10, 12, 22).

Recently it has become apparent, however, that HBV is not a unique virus. Viruses with similar ultrastructural, antigenic, molecular, and biological features have been found in eastern woodchucks (25), Beechey ground squirrels in northern California (16), and domestic ducks in China (J. Summers, personal communication). These viruses provide important animal models of HBV infection in humans, and it is of interest to determine how closely related they are. Although the surface antigens of woodchuck hepatitis virus (29) and ground squirrel hepatitis virus (GSHsAg) (16) clearly cross-react in serological tests with HBsAg, it is not known how

† Permanent address: Department of Medical Microbiology, Hygiene Institute, University of Göttingen, Göttingen, Federal Republic of Germany.

similar the surface antigens of these viruses are. Here we describe physical, chemical, and serological properties of GSHsAg and compare it with HBsAg. The results clarify the degree of similarity of these two antigens and indicate that the respective viruses differ serologically more than do HBV of different HBsAg subtypes.

## MATERIALS AND METHODS

**Isolation of 20-nm HBsAg and GSHsAg particles.** HBsAg (subtype *adw*) concentrations in patient sera high in HBV-specific DNA polymerase activity (16) and GSHsAg in ground squirrel sera high in virion DNA polymerase activity (16) were determined by electroimmunodiffusion (11), using sheep antiserum (S-2/4) to HBsAg (anti-HBs). One-half to 2 ml of serum with high concentrations of HBsAg or GSHsAg was chromatographed over a Bio-Gel A-5M (Bio-Rad) column in TNE buffer (0.01 M Tris-hydrochloride [pH 7.4], 0.1 M NaCl, 5 mM EDTA) as described in the legend to Fig. 2. Fractions strongly positive for surface antigen in electroimmunodiffusion were pooled and concentrated approximately fivefold with Ficoll (Pharmacia). Each 2.5 ml of concentrated material was made up to a density of 1.30 g/ml by addition of solid CsCl and layered over 1.3 ml of a CsCl solution with a density of 1.35 g/ml in a tube for the Spinco SW40 rotor. Two-milliliter volumes of CsCl solutions with densities of 1.27, 1.24, and 1.18 g/ml were consecutively layered over the sample, followed by 0.8 ml of 0.05 M phosphate buffer, pH 7.5. The surface antigen was centrifuged to equilibrium in an SW40 rotor at 34,000 rpm for 40 h at 10°C. Antigen was again located by electroimmunodiffusion, and positive fractions were pooled and stored in CsCl at 4°C.

Since the concentration of HBsAg in the human sera was, on the average, 10-fold less than concentrations of GSHsAg in the ground squirrel sera, HBsAg in serum was concentrated and partially purified by polyethylene glycol 6000 precipitation in some experiments before Bio-Gel A-5M chromatography. Briefly, 200 ml of HBsAg-positive serum was made up to 6.8% (vol/vol) with polyethylene glycol (from a 30% [wt/wt] stock) and centrifuged for 5 min at 5,000 rpm and 4°C. The resulting supernatant was made up to 12.5% with additional polyethylene glycol and centrifuged again. The 12.5% polyethylene glycol pellet was dissolved in 4 ml of TNE.

**Radiolabeling and chemical modification of HBsAg and GSHsAg.** For radioimmunoassay, purified preparations of HBsAg and GSHsAg were radiolabeled by oxidative iodination, using Iodogen (Pierce Chemical Co.; 8). Free $^{125}I$ was removed by chromatography in Sepharose 6B-Cl with 1% bovine serum albumin. Specific activities up to 10 $\mu$Ci/$\mu$g of protein were obtained. For peptide mapping, both HBsAg and GSHsAg preparations were dialyzed against 0.01 M sodium borate buffer (pH 8.5)–0.1% sodium dodecylsulfate (SDS), lyophilized, and labeled with Bolton-Hunter reagent (3) as described by Feitelson et al. (submitted for publication). Briefly, 0.2 to 0.5 $\mu$g of *p*-hydroxyphenylpropionic acid *N*-hydroxysuccinimide ester (Calbiochem) dissolved in 1 to 2 $\mu$l of dioxane was added to 50 $\mu$l of 0.5 M sodium phosphate buffer, pH 7.5, and rapidly iodinated by addition of 0.5 mCi of $^{125}I$ and 100 $\mu$g of chloramine-T. The labeled ester was immediately extracted into 100 $\mu$l of benzene, the aqueous phase was removed, and the organic phase was transferred to the lyophilized protein sample. Labeling and extraction of the ester were carried out in less than 40 s to prevent excessive hydrolysis in aqueous media. The benzene was driven off by a gentle stream of air, the vial was cooled to 0°C, and 5 $\mu$l of 0.1 M sodium borate buffer, pH 8.5, at 0°C was added to each sample. After incubation at 0°C for 1 h, the samples were stored at 4°C overnight. The next day 200 $\mu$g of *p*-hydroxyphenylpropionic acid *N*-hydroxysuccinimide ester (in dioxane) was added to each sample, and the reaction mixtures were incubated at 0°C for 3 h. The pH was then adjusted to 9 in each mixture, and the samples were reductively methylated as described previously (18), with minor modifications (M. A. Feitelson and F. O. Wettstein, submitted for publication).

Alternatively, some preparations were radiolabeled by oxidative iodination, using chloramine-T (13). All samples prepared for analysis by SDS-polyacrylamide gel electrophoresis (PAGE) were reduced in 10% 2-mercaptoethanol and 2 M urea at 100°C for 3 min and alkylated with approximately two chemical equivalents of iodoacetamide. The labeled proteins were separated from the other reaction components by chromatography on Bio-Gel P-10 in 0.05 M $NH_4HCO_3$ and 0.1% SDS, lyophilized, and redissolved in a solution with final concentrations of 2 M urea, 3% SDS, and 0.001% bromophenol blue for SDS-PAGE.

**SDS-PAGE and peptide mapping.** Discontinuous SDS-PAGE was carried out with 1-mm-thick slab gels containing 5% stacking gels (with *N,N*-methylenebisacrylamide cross-linker) and 12.5% running gels (with *N,N'*-diallyltartardiamide cross-linker). The buffer system and other conditions used were identical to those of Laemmli (14). Electrophoresis was usually carried out overnight (16 h) at a constant current of 10 mA and stopped when the dye reached the bottom of the running gel. Gels with non-radiolabeled proteins were stained with 2% Coomassie blue in 10% acetic acid and 25% isopropanol overnight, destained in the same mixture without Coomassie blue, and dried under vacuum. For radiolabeled proteins, the gels were fixed overnight in 10% acetic acid and 25% isopropanol, dried under vacuum for 2 h, and autoradiographed using an intensifying screen and Kodak XR-5 film. Individual radiolabeled bands were cut from the gel, dried under high vacuum overnight, and then rehydrated with 0.5 ml of a solution containing 250 $\mu$g of trypsin (Calbiochem) and 0.05 M $NH_4HCO_3$ at room temperature overnight. The supernatants were lyophilized, and each was redigested with 100 $\mu$g of trypsin in 20 $\mu$l of 0.01 M sodium borate buffer, pH 8.5. Two-dimensional peptide mapping was carried out on cellulose thin-layer plates (20 by 20 cm; Brinkmann) under a layer of *n*-heptane as described elsewhere (Feitelson et al., submitted for publication). Electrophoresis was carried out in the first dimension for 2.25 h at 14°C, using 8% acetic acid and 2% formic acid, pH 2, as the running buffer. After electrophoresis, thin-layer chromatography was carried out in *n*-butanol–water–ethyl acetate–acidic acid–pyridine (120:60:20:4:

1, vol/vol), using the top phase of the mixture only. Thin-layer plates were then exposed to Kodak XR-5 film, using an intensifying screen (26).

## RESULTS

**Antigenic heterogeneity of GSHsAg.** GSHsAg was detected in the sera of infected ground squirrels by its cross-reaction with antibodies against HBsAg. For its qualitative determination, a commercially available test kit for HBsAg (Ausria II, Abbott Laboratories) was used, as described earlier (16). Positive sera were assayed quantitatively by electroimmunodiffusion, using a sheep antiserum (S-2/4) directed most strongly against the group-specific *a* determinant of HBsAg. Most other antisera against HBsAg tested did not show sufficient cross-reactivity to permit quantitation of GSHsAg. In contrast to HBsAg, which produced one strong line with this antiserum (Fig. 1a, well 1), GSHsAg always produced two weaker precipitation lines of different intensities (Fig. 1a, wells 3–8). This result suggests antigenic heterogeneity in both GSHsAg and the cross-reacting antibodies. Although the relative amounts of both GSHsAg components were similar in different positive ground squirrel sera, the finding that one component formed a stronger precipitation arc closer to the origin than the other suggests that there was more cross-reacting antibody directed against the former subpopulation than against the latter. Heterogeneity in buoyant density was also demonstrated by CsCl density gradient centrifugation (Fig. 1b).

**Size of GSHsAg.** GSHsAg-containing ground squirrel serum was chromatographed on a column of 4% agarose beads (Sepharose 4B). Total protein was detected by absorbance at 280 nm and GSHsAg was detected by electroimmunodiffusion. GSHsAg was eluted in a single 280-nm peak characteristic of serum macroglobulins (Fig. 2). In contrast, HBsAg preparations have been shown to elute with immunoglobulin M and beta-lipoprotein, but slightly before the α-macroglobulin peak (11). If the Stokes radius of human α-macroglobulin is 8.9 nm, and the diameter and composition of this molecule are the same in both species, GSHsAg would have an average diameter of 18 to 20 nm. Larger filamentous and spherical particles, detected earlier by electron microscopy (16), were apparently broken up or adsorbed to the column, because all fractions before the antigen peak were negative even by the sensitive enzyme-linked immunosorbents assay for GSHsAg (data not shown). Further, there was no detectable DNA polym-

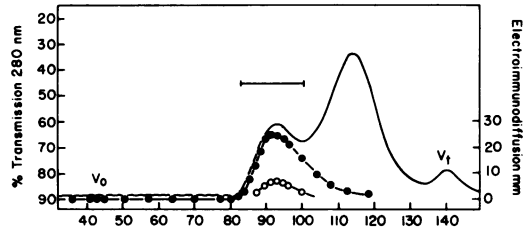


FIG. 2. *Sepharose 4B chromatography of ground squirrel serum containing GSHsAg. Serum (0.8 ml) was loaded on an 80-cm column containing 135 ml of Sepharose 4B in 0.02 M Tris-hydrochloride (pH 7.4)–0.13 M NaCl and eluted at 4 ml/h with the same buffer. The solid line is a tracing of the 280-nm absorbance of the eluate passing through a 3-mm-light path cuvette. Fractions were assayed for GSHsAg by electroimmunodiffusion as described in the legend to Fig. 1, and the lengths of the long (●) and short (○) precipitin arcs were measured. Bar shows the fractions pooled for further purification of GSHsAg.*

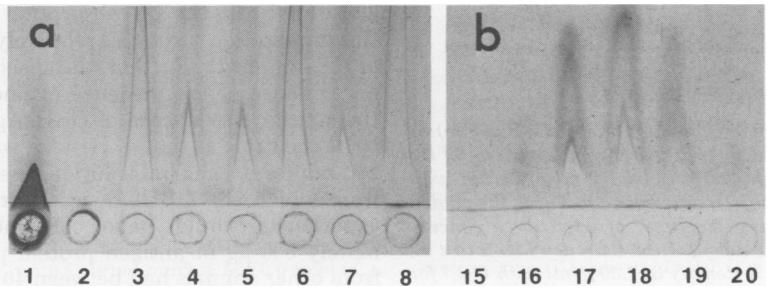


FIG. 1. *Electroimmunodiffusion of GSHsAg and HBsAg with sheep anti-HBs. Ten microliters of each HBsAg-containing sample or a 1:10 dilution of each GSHsAg-containing sample was electrophoresed at 20 mA for 8 h in a slab (25 by 75 by 1.5 mm) of 1% agarose (Bio-Rad) containing 0.034 M sodium barbital (pH 8.4) and 20% (vol/vol) antiserum. The gel was then washed, dried, and stained with 0.2% Coomassie blue in 7.5% acetic acid. (a) Well 1, human serum with 35 μg of HBsAg (adw) per ml; well 2, normal ground squirrel serum; wells 3 to 8, Ausria II-positive ground squirrel sera. (b) Wells 15 through 20, fractions 15 through 20, respectively, from the CsCl density gradient described in the legend to Fig. 3a after centrifugation to equilibrium for purification of GSHsAg.*

erase activity in any of the fractions, even though the starting material was very strongly positive.

**Density of purified GSHsAg.** GSHsAg partially purified from 0.8 ml of serum by gel chromatography was adjusted to a solution density of 1.3 g/ml with CsCl and centrifuged to equilibrium in a CsCl density gradient. Antigenic activity was detected by electroimmunodiffusion in a prominent 280-nm absorption peak at a density of 1.183 g/ml (Fig. 3a). The low base line of absorption around this peak indicated that the antigen was already highly purified after only two purification steps. HBsAg from 2 ml of serum, purified in parallel by the same procedure (Fig. 3b), was found in a 280-nm peak at a density of 1.190 g/ml. Although the starting volume of human serum was 2.5 times greater than the volume of ground squirrel serum used, the density gradient profiles showed much more GSHsAg than HBsAg. In another experiment with serum from a second positive animal, an even larger amount of GSHsAg was extracted, suggesting that the serum concentration of GSHsAg in infected ground squirrels was approximately 10 times that of HBsAg in the high-titer human sera used. Electroimmunodiffusion analysis of the three fractions making up the GSHsAg density gradient peak (fractions 17, 18, and 19, Fig. 3a) revealed two precipitation lines in the two fractions of highest density (fractions 17 and 18, Fig. 1b) and only the faint precipitation line in the antigen-containing fraction of lowest density (fraction 19, Fig. 1b). This result suggested that GSHsAg was not only antigenically heterogeneous but also heterogeneous in buoyant density. Electron microscopy of the GSHsAg peak fractions revealed both spherical and short filamentous forms with mean diameters of 18 to 20 nm. Although the morphology of these particles was similar to that of HBsAg particles, the apparent average diameter was slightly less than that reported (2, 6) for spherical HBsAg particles (20 to 22 nm).

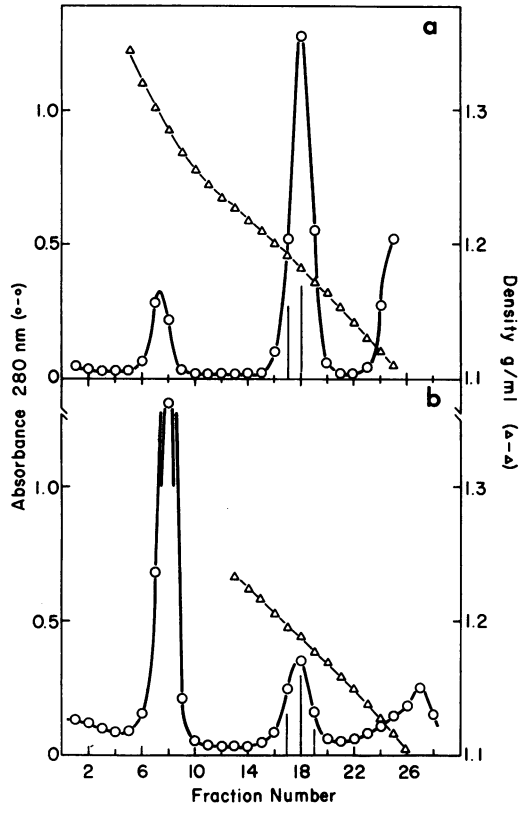


FIG. 3. *Isopycnic centrifugation of GSHsAg (a) or HBsAg (b) in CsCl. After gel chromatography of 0.8 ml of serum with GSHsAg and 2 ml of serum with HBsAg as described in the legend to Fig. 2, the pooled antigen-containing fractions of each were concentrated by osmotic dehydration with dry Ficoll to 4 ml and adjusted to a density of 1.30 g/ml with CsCl for centrifugation in a CsCl density gradient as described in the text. After centrifugation in a Spinco SW40 rotor for 40 h at 34,000 rpm and 10°C, fractions were collected from the bottom of the tube. Absorbance at 280 nm (○) in a cuvette with a 1-cm light path and solution density (△) by refractometry were determined for each fraction. Bars represent the lengths of the precipitation arcs in the electroimmunodiffusion assay for GSHsAg or HBsAg.*

**UV absorption spectrum of purified GSHsAg.** The UV absorption spectrum of GSHsAg, like that of HBsAg (11), had peaks at 280 and 290.5 nm as well as a shoulder at 275 nm (Fig. 4), which were indicative of a high tryptophane/tyrosine ratio. Since most proteins have a low tryptophane/tyrosine ratio, and the absorption spectrum of tyrosine obscures the 290.5-nm peak, the UV absorption spectrum of this preparation suggests a relatively high degree of HBsAg purity. Further, the spectrum showed no evidence of the presence of nucleic acid. If the specific absorbance of GSHsAg is assumed to be the same as that of HBsAg, which is 4.2 at 280 nm for a 1-mg/ml solution, then the serum from which the GSHsAg was purified in this experiment would have contained approximately 300 μg of antigen protein per ml. Sera from other animals had between 40 and 300% of that concentration when tested in electroimmunodiffusion.

**Serological cross-reactivity of antibodies against HBsAg and GSHsAg in radioimmunoassay.** Antibodies directed against HBsAg or GSHsAg determinants were detected in sera by solid-phase radioimmunoassay (21). Briefly, dilutions of test sera were added to U-

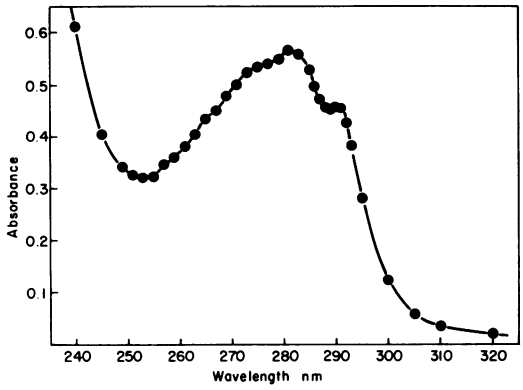


FIG. 4. *UV absorption spectrum of purified GSHsAg. Fraction 19 from the CsCl density gradient shown in Fig. 3a was measured in a Zeiss PM2 spectrophotometer at 0.1-mm slit width.*

shaped wells of microtiter plates coated with GSHsAg or HBsAg, and antibody binding was detected by subsequent binding of $^{125}$I-labeled antigen of the same type used to coat the wells. Mouse and ground squirrel antisera against GSHsAg and guinea pig and sheep antisera against HBsAg were titrated by this assay, using purified GSHsAg or HBsAg as the reagent (Fig. 5). All antisera from four mice immunized with GSHsAg bound labeled GSHsAg to saturation (75% of the radioactivity added) at low dilutions, and 50% was bound when antiserum dilutions between 1:2,000 and 1:10,000 were used. With HBsAg as reagent, two of the four antisera were negative (<0.3% binding), and the other two sera bound 0.5 and 2% of the added $^{125}$I-labeled HBsAg at a dilution of 1:10 (Fig. 5a and b). Using HBsAg, antibody could be detected in the two anti-HBs-containing sera diluted up to 1:10,000. The sheep antiserum (S-2/4) bound up to 48% of GSHsAg when used undiluted and 28% at a 1:10 dilution (Fig. 5c), and the guinea pig antiserum bound only 5.6% of GSHsAg at a 1:10 dilution (Fig. 5d). It should be noted that the sheep antiserum S-2/4 was directed mainly against the *a* determinant of HBsAg because it was prepared by boosting with an HBsAg subtype different from that used for the primary inoculation. Six monoclonal antibodies to HBsAg (a gift of Liz Jones) were also tested with GSHsAg. Only one bound 0.5% of the GSHsAg at dilutions up to 1:100, whereas the other five antibodies showed no detectable binding in this sensitive assay.

**SDS-PAGE analysis of GSHsAg- and HBsAg-associated proteins.** Purified preparations of HBsAg and GSHsAg each containing 50 μg of protein were reduced, alkylated, and analyzed by SDS-PAGE, and the protein bands were revealed by Coomassie blue staining. The HBsAg preparation yielded two major polypeptides with apparent sizes of 25,000 and 29,000 daltons (Fig. 6a, lane 2), similar to the findings in previous studies (7, 10, 12, 17, 22). Two major polypeptides were also found in the GSHsAg preparation (Fig. 6a, lane 1), and although their apparent sizes were similar, they consistently migrated faster during electrophoresis (apparent sizes of 23,000 and 27,000 daltons) than the corresponding major HBsAg polypeptides. Surface antigen preparations radiolabeled by chloramine-T (Fig. 6b) or Bolton-Hunter reagent (data not shown) revealed the same two major components by autoradiography.

Several minor components migrating more slowly in SDS-PAGE than the major polypeptides were also found in surface antigen preparations from both viruses when Coomassie blue staining or either method of radiolabeling was used to detect polypeptides. Minor polypeptide components have been repeatedly found in HBsAg preparations previously (7, 10, 12, 17, 22).

Table 1 summarizes the findings from several SDS-PAGE experiments in which polypeptides were detected by Coomassie blue staining or by autoradiography of preparations radioiodinated by the iodogen procedure. None of the minor Coomassie blue staining polypeptides with apparent molecular weights greater than 30,000 in GSHsAg preparations comigrated with any of those from HBsAg. In contrast, the major and

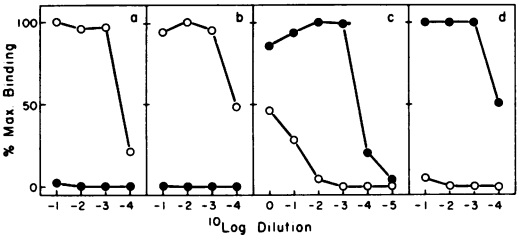


FIG. 5. *Cross-reactivity of antisera against GSHsAg and HBsAg in solid-phase radioimmunoassay. A 50-μl portion of mouse antisera against purified GSHsAg (a and b), sheep antiserum against HBsAg determinant a (c), or guinea pig antiserum against HBsAg subtype adw (d), each diluted in 1% bovine serum albumin (BSA), was incubated in microtiter wells precoated with 10 ng of purified HBsAg (●) or GSHsAg (○) for 15 h at 4°C. After two washings with BSA, 40,000 cpm of $^{125}$I-labeled HBsAg (2.5 μCi/μg) or 8,000 cpm of $^{125}$I-labeled GSHsAg (0.5 μCi/μg) in 50 μl of BSA were incubated for 24 h at 4°C. After three washings the wells were counted. Approximately 75% of either radiolabeled antigen was the maximum amount bound. The background with normal human serum instead of antiserum was between 0.2 and 0.4% (data not shown).*

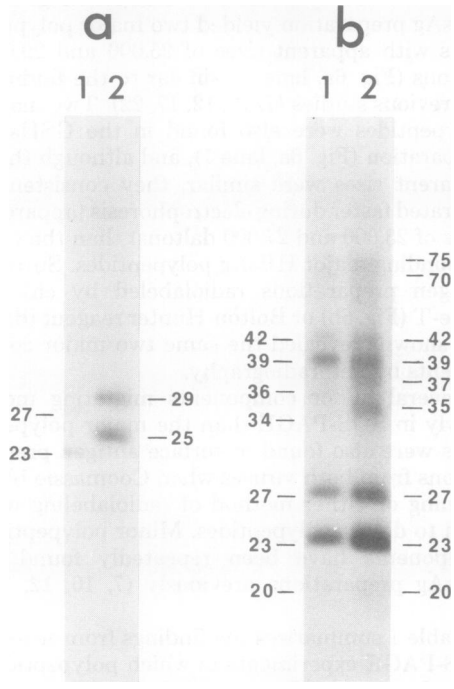


FIG. 6. *Polypeptides of GSHsAg and HBsAg particle preparations. Purified preparations of GSHsAg (a, lane 1) and HBsAg (a, lane 2) were analyzed by SDS-PAGE and Coomassie blue staining. Purified GSHsAg preparations from ground squirrels 83 (b, lane 1) and 147 (b, lane 2) were radioiodinated by the iodogen method and analyzed by SDS-PAGE and autoradiography.*

TABLE 1. *Apparent molecular weights ($\times 10^{-3}$) of polypeptides separated by SDS-PAGE of purified GSHsAg and HBsAg preparations*

| GSHsAg | | | HBsAg (Coomassie blue) |
|---|---|---|---|
| Animal no. 83 | | Animal no. 147 ($^{125}$I) | |
| Coomassie blue | $^{125}$I | | |
| 75 | | 75 | 72 |
| 70 | | 70 | |
| | | | 63 |
| | | | 56 |
| | | | 54 |
| | | | 49 |
| 43 | 42 | 42 | |
| 39 | 39 | 39 | |
| | 36 | 37 | |
| 34 | 34 | 35 | |
| | | | 31 |
| | | | 29[a] |
| 27[a] | 27[a] | 27[a] | |
| | | | 25[a] |
| 23[a] | 23[a] | 23[a] | |
| | 20 | 20 | |

[a] Major components.

most of the minor $^{125}$I-labeled polypeptides of GSHsAg preparations from the sera of two different ground squirrels (no. 83 and 147) radiolabeled by the iodogen procedure appeared to be identical (Table 1). There was, however, variability in the intensity and position of two of the minor components migrating in the 34,000- to 37,000-molecular-weight range (Table 1, Fig. 6b) in the two GSHsAg preparations. Radiolabeling of both HBsAg and GSHsAg by the chloramine-T, iodogen, and Bolton-Hunter procedures revealed several autoradiographic bands in the 14,000- to 20,000-molecular-weight range which were not detected on gels stained with Coomassie blue (Fig. 6, Table 1).

**Tryptic peptide mapping of the major polypeptides of GSHsAg and HBsAg.** The relationship between the two major polypeptides of GSHsAg was examined by tryptic $^{125}$I-labeled peptide mapping of each after labeling antigen preparations with Bolton-Hunter reagent and recovering the appropriate $^{125}$I-labeled polypeptide from a polyacrylamide gel after SDS-PAGE. Figure 7a shows the map of $^{125}$I-labeled peptides detected by autoradiography for the 23,000-dalton polypeptide (P-23) of GSHsAg. The peptide map of the 27,000-dalton polypeptide (P-27) of GSHsAg was very similar but not identical. Figure 7b shows a composite diagram of the peptide maps of the two GSHsAg polypeptides. The spot at position 1 was detected in the map of P-27 and not in that of P-23. The spot at position 2 was very heavily labeled in P-23 and barely detectable in P-27. Thus, the two major polypeptides of GSHsAg are closely related in their primary sequences. Figure 7c shows the $^{125}$I-labeled peptide map of the 25,000-dalton polypeptide (P-25) derived from HBsAg, and a diagram of this map is shown in Fig. 7d. The tryptic $^{125}$I-labeled peptides in the maps of the major polypeptides of the two viruses were compared in autoradiograms of the two mapped separately and by digesting and mapping a mixture of the two on a single thin-layer plate. Thirteen $^{125}$I-labeled peptides in the map of GSHsAg P-23 were found to be identical to spots in the map of HBsAg P-25, and the remainder of the spots were different in the two maps. Specifically, the shaded spots (13 of 37 spots) in the diagram of the peptide map of GSHsAg P-23 (Fig. 7b) are identical to spots present in the map of HBsAg P-25. The unshaded spots are unique to GSHsAg P-23. The shaded spots (13 of 27 spots) in the diagram of the peptide map of HBsAg P-25 (Fig. 7d) are those also found in the peptide map of GSHsAg P-23. The unshaded spots were unique to HBsAg P-25. It can be concluded that, although the major polypeptides of GSHsAg and HBsAg

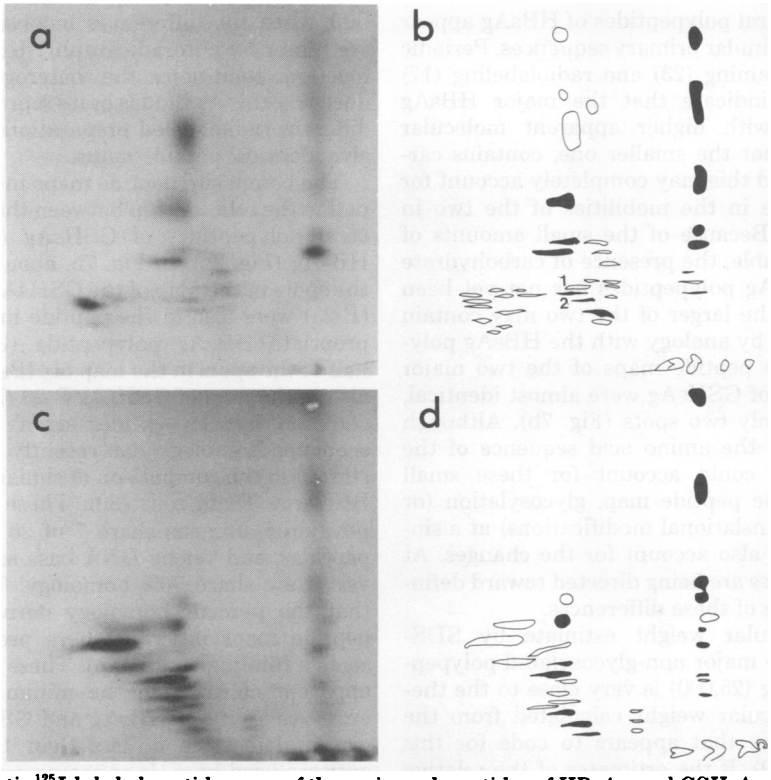


FIG. 7. *Tryptic $^{125}$I-labeled peptide maps of the major polypeptides of HBsAg and GSHsAg radioiodinated by the Bolton-Hunter method. (a) $^{125}$I-labeled peptide map of the 23,000-dalton polypeptide (P-23) of GSHsAg. (b) Diagram of the composite map of the same polypeptide and the 27,000-dalton polypeptide of GSHsAg. The darkened spots are identical to spots in the map of the 25,000-dalton polypeptide (P-25) of HBsAg. (c) $^{125}$I-labeled peptide map of HBsAg P-25. (d) Diagram of the map of the same polypeptide, with the darkened spots corresponding to those identical to spots in the map of GSHsAg P-23.*

possess different mobilities on SDS-PAGE, they share a considerable amount of structural homology.

## DISCUSSION

By physical, chemical, and serological techniques the similarities and differences between the spherical surface antigen particles from HBV and ground squirrel hepatitis virus were investigated. The most numerous particles bearing the surface antigens of both viruses had similar sizes and morphologies, the same buoyant density, unusually high tryptophane/tyrosine ratios, serological cross-reactivity, and a pair of major structural polypeptides with considerable homology in primary structure. These common features suggest that the molecular architecture of intact GSHsAg particles is probably similar to that of HBsAg particles. With one antiserum (S-2/4) from a sheep immunized with HBsAg, sufficient cross-reactivity was found to detect and quantitate GSHsAg in electroimmunodiffusion and by enzyme immunoassay. Surprisingly, this antiserum distinguished between two antigenic forms in GSHsAg, although no such heterogeneity has been described for HBsAg. Since this antiserum was raised against HBsAg, it is probable that HBsAg itself has a similar heterogeneity, which may be obscured by the presence of many common determinants on partially heterogeneous particles. The uneven density distribution of the stronger cross-reactive components suggests that its specificity is related to glycosylation or masking by lipid. That there are common determinants on HBsAg and GSHsAg suggests a similar configuration of the polypeptide chains at the surface of the particles. It will be of interest to determine whether chemical reduction of GSHsAg reduces its antigenicity as much as has been reported for HBsAg (27).

It is of interest that GSHsAg particles contain two major structural polypeptides similar but not identical in size to the major components of HBsAg, providing further evidence for a similarity in structure of the surface antigen particles of the two viruses. Based upon amino acid composition (22, 28), limited sequencing data (19), and similar antigenic properties (12, 22), the two

major structural polypeptides of HBsAg appear to have very similar primary sequences. Periodic acid-Schiff staining (23) and radiolabeling (17) experiments indicate that the major HBsAg polypeptide with higher apparent molecular weight, and not the smaller one, contains carbohydrate and this may completely account for the difference in the mobilities of the two in SDS-PAGE. Because of the small amounts of antigen available, the presence of carbohydrate in the GSHsAg polypeptides has not yet been studied, but the larger of the two may contain carbohydrate by analogy with the HBsAg polypeptides. The peptide maps of the two major polypeptides of GSHsAg were almost identical, differing in only two spots (Fig. 7b). Although differences in the amino acid sequence of the two proteins could account for these small changes in the peptide map, glycosylation (or other post-translational modifications) at a single site could also account for the changes. At present, studies are being directed toward defining the nature of these differences.

The molecular weight estimate by SDS-PAGE for the major non-glycosylated polypeptide of HBsAg (25,000) is very close to the theoretical molecular weight calculated from the DNA sequence that appears to code for this polypeptide (9). If the estimates of the relative sizes of the major polypeptides of GSHsAg and HBsAg are accurate, an approximately 10% smaller DNA coding sequence would be required to specify the 23,000-dalton GSHsAg polypeptide than to specify the 25,000-dalton non-glycosylated component of HBsAg.

For peptide mapping, the major polypeptides from surface antigen particles of both viruses were radiolabeled in vitro by the Bolton-Hunter procedure, which has been shown to label proteins more uniformly than oxidative iodination (Feitelson et al., submitted for publication). When interpreting structural relationships from peptide maps of proteins labeled with Bolton-Hunter reagent and digested with trypsin, it is important to note that each spot does not necessarily correspond to a single tryptic peptide. Depending upon the number of lysine residues with free epsilon amino groups in a given peptide and the extent to which these groups are acylated by the Bolton-Hunter reagent, this reaction introduces some chemical heterogeneity into a population of otherwise identical protein molecules. The relatively large number of spots, therefore, is a reflection of this chemical heterogeneity introduced by the acylation procedure. This is in contrast to the relatively few spots generated from direct iodination with chloramine-T or iodogen where this chemical heterogeneity is not present. This is especially important when the differences involve spots which are minor by autoradiography. Under identical reaction conditions the heterogeneity introduced by this method is quite reproducible since different radiolabeled preparations of a protein give identical peptide maps.

The composite peptide maps in Fig. 7b and d define the relationship between the major structural polypeptides of GSHsAg (Fig. 7a) and HBsAg (Fig. 7c). In Fig. 7b, about one-third of the spots in the map of the GSHsAg polypeptide (P-23) were also in the peptide map of the appropriate HBsAg polypeptide (P-25). Nearly half of the spots in the map of HBsAg P-25 were also in the map of GSHsAg P-23 (Fig. 7d). That common tryptic peptides signify considerable sequence homology has recently been demonstrated in the comparison of simian virus 40 and BK virus T antigens (30). These different papovavirus antigens share 7 of 20 or 21 tryptic peptides, and yet by DNA base sequence analysis, they share 70% homology. This suggests that the percent homology derived from the peptide maps described here probably represents a minimum estimate. These results are in apparent contrast to the minimal serological cross-reactivity of HBsAg and GSHsAg, which was undetectable or less than $10^{-4}$ with the antisera used here. It appears possible that the selective pressure of the host immune response results in greater variability in the part of the protein manifesting surface antigenic reactivity than in the overall sequence. The core antigens from ground squirrel hepatitis virus and HBV have more serological cross-reactivity than the surface antigens (W. Gerlich et al., unpublished data). This observation confirms for this group of viruses the greater variability of virion surface than internal antigens found for many enveloped viruses. As the molecular biology of the ground squirrel virus is further elucidated and compared with that of HBV, the extent to which this virus-host system can be used as a model for HBV infection in humans will become more apparent.

## ACKNOWLEDGMENTS

This study was supported by Public Health Service research grant AI 13526 from the National Institutes of Health. M.A.F. was supported by postdoctoral fellowship PF-1792 from the American Cancer Society. W.H.G. received grant Ge 345/5 from the Deutsche Forschungsgemeinschaft.

## LITERATURE CITED

1. **Bancroft, W. H., F. K. Mundo, and P. K. Russel.** 1977. Detection of additional determinants of hepatitis B antigen. J. Immunol. **109**:842–848.
2. **Bayer, M. E., B. S. Blumberg, and B. Werner.** 1968. Particles associated with Australia antigen in the sera of patients with leukemia, Down's syndrome and hepatitis. Nature (London) **218**:1057–1059.

3. **Bolton, A. E., and W. M. Hunter.** 1973. The labelling of proteins to high specific radioactivities by conjugation to a $^{125}$I-containing acylating agent. Biochem. J. **133**: 529–539.
4. **Courouce, A. M., P. V. Holland, J. Y. Muller, and J. P. Soulier.** 1976. HB$_s$ antigen subtypes. Proceedings of the international workshop on HB$_s$ antigen subtypes. Bibl. Haematol. (Basel) **42**:1–158.
5. **Courouce-Pauty, A. M., and J. P. Soulier.** 1974. Further data on HB$_s$ antigen subtypes—geographical distribution. Vox Sang. **27**:533–549.
6. **Dane, D. S., C. H. Cameron, and M. Briggs.** 1970. Virus-like particles in serum of patients with Australia antigen associated hepatitis. Lancet **ii**:695–698.
7. **Dressman, G. R., R. Chairez, M. Suarez, F. B. Hollinger, R. J. Courtney, and J. L. Melnick.** 1975. Production of antibody to individual polypeptides derived from purified hepatitis B antigen. J. Virol. **16**:508–515.
8. **Fraker, P. J., and J. C. Speck.** 1978. Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenyl-glycoluril. Biochem. Biophys. Res. Commun. **80**:849–857.
9. **Galibert, F., E. Mardart, F. Fitoussi, P. Tiollais, and P. Charnay.** 1979. Nucleotide sequence of hepatitis B virus genome (subtype ayw) cloned in E. coli. Nature (London) **281**:646–650.
10. **Gerin, J. L.** 1974. Structure of hepatitis B antigen (HB Ag), p. 215–224. *In* W. S. Robinson and C. F. Fox (ed.), Mechanisms of virus disease. W. A. Benjamin, Menlo Park, Calif.
11. **Gerlich, W., and R. Thomssen.** 1975. Standardized detection of hepatitis B surface antigen: determination of its serum concentration in weight units per volume. Dev. Biol. Stand. **30**:78–86.
12. **Gold, J. W. M., J. W. Shih, R. H. Purcell, and J. L. Gerin.** 1976. Characterization of antibodies to the structural polypeptides of HB$_s$Ag: evidence for subtype-specific determinants. J. Immunol. **117**:1404–1406.
13. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. Preparation of $^{131}$I-labelled human growth hormone of high specific radioactivity. Biochem. J. **89**:114–123.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T$_4$. Nature (London) **227**:680–685.
15. **LeBouvier, G. L.** 1971. The heterogeneity of Australia antigen. J. Infect. Dis. **123**:671–675.
16. **Marion, P. L., L. Oshiro, D. C. Regnery, G. H. Scullard, and W. S. Robinson.** 1980. A virus in Beechey ground squirrels which is related to hepatitis B virus of man. Proc. Natl. Acad. Sci. U.S.A. **77**:2941–2945.
17. **Marion, P. L., F. H. Salazar, J. J. Alexander, and W. S. Robinson.** 1979. Polypeptides of hepatitis B surface antigen produced by a hepatoma cell line. J. Virol. **32**: 796–802.
18. **Means, G. E., and R. E. Freeney.** 1968. Reductive alkylation of amino groups in proteins. Biochemistry **7**: 2192–2201.
19. **Peterson, D. L., I. M. Roberts, and G. N. Vyas.** 1977. Partial amino acid sequence of two major component polypeptides of hepatitis B surface antigen. Proc. Natl. Acad. Sci. U.S.A. **74**:1530–1534.
20. **Robinson, W. S.** 1977. The genome of hepatitis B virus. Annu. Rev. Microbiol. **31**:357–377.
21. **Schober, A., R. M. Biswar, and R. Thomssen.** 1975. Direct solid phase radioimmunoassay (dsp-RIA) for detection of antibody to hepatitis B surface (HB$_s$) antigen. Dev. Biol. Stand. **30**:93–102.
22. **Shih, J. W., and J. L. Gerin.** 1975. Immunochemistry of hepatitis B surface antigen (HB$_s$Ag): preparation and characterization of antibodies to the constituent polypeptides. J. Immunol. **115**:634–639.
23. **Shih, J. W., and J. L. Gerin.** 1976. Proteins of hepatitis B surface antigen. J. Virol. **21**:347–357.
24. **Shih, J. W., and J. L. Gerin.** 1977. Proteins of hepatitis B surface antigen: amino acid composition of the major polypeptides. J. Virol. **21**:1219–1222.
25. **Summers, J., J. M. Smolec, and R. Snyder.** 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proc. Natl. Acad. Sci. U.S.A. **75**:4533–4537.
26. **Swanstrom, R., and P. R. Shank.** 1978. X-ray intensifying screens greatly enhance the detection by autoradiography of the radioactive isotopes $^{32}$P and $^{125}$I. Anal. Biochem. **86**:184–192.
27. **Vyas, G. N., K. R. Rao, and A. B. Ibrahim.** 1972. Australia antigen (hepatitis B antigen): a conformational antigen dependent on disulfide bonds. Science **178**:1300–1301.
28. **Vyas, G. N., E. W. Williams, G. G. B. Klaus, and H. E. Bond.** 1972. Hepatitis-associated Australia antigen: protein, peptides and amino acid composition of purified antigen with tissue in determining sensitivity of the hemagglutination test. J. Immunol. **108**:1114–1118.
29. **Werner, B. G., J. M. Smolec, R. Snyder, and J. Summers.** 1979. Serologic relationship of woodchuck hepatitis virus and human hepatitis B virus. J. Virol. **32**: 314–322.
30. **Yang, R. C. A., A. Young, and R. Wu.** 1980. BK virus DNA sequence coding for the t and T antigens and evaluation of methods for determining sequence homology. J. Virol. **34**:416–430.