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Structure of Hepatitis B Surface Antigen

CHARACTERIZATION OF THE LIPID COMPONENTS AND THEIR ASSOCIATION WITH THE VIRAL PROTEINS*

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The lipid composition of hepatitis B surface antigen (HBsAg) (subtype adw) obtained from different carriers has been determined and proven to be truly characteristic of HBsAg and not subject to individual variation. Phosphatidylcholine (~60%), cholesteryl ester (~14%), cholesterol (~15%), and triglycerides (~3%) are the main HBsAg lipid constituents. The fatty acid composition of the different HBsAg lipid components is similar to that of other normal human serum lipoprotein.

A photoactivatable hydrophobic probe, pyrenesulfonyl azide, has been used to determine what portions of the protein components of HBsAg are exposed to the HBsAg lipid matrix. Both major HBsAg protein components became randomly pyrenesulfonyl azide labeled in both the NH_2-terminal and COOH-terminal tryptic fragments, therefore suggesting they are buried within the HBsAg lipids. A model for the arrangement of proteins in HBsAg is proposed whereby regions within the NH_2-terminal and COOH-terminal parts of the two major HBsAg protein components are buried within the lipid matrix of HBsAg particles, while the antigenically important residue 122–150 region is exposed to the aqueous environment.

Hepatitis B surface antigen is a group of morphologically heterogeneous complex macromolecular structures found in the serum of patients with hepatitis B virus infection. Electron microscopy reveals three forms of HBsAg,[1] 20-nm spherical particles, tubular structures of variable lengths and a diameter of 20 nm, and 40-nm spherical "Dane" particles which are the hepatitis B virus (1–5). Of these, the 20-nm spherical particles account for the bulk of the HBsAg and are the material obtained by our purification procedures. Purified HBsAg has a molecular weight of 2–4 × 10^6 and a density of 1.20–1.21 g/ml (6, 7). Previous reports have shown HBsAg to contain protein, carbohydrate, and lipid (8, 9). Of these components, only the proteins have been well characterized (10). Indeed, a detailed description of the overall molecular composition of HBsAg has not been presented.

Much of the interest in HBsAg arises from its anticipated role in the development of a vaccine against hepatitis B virus. Currently such experimental vaccines utilize: (i) HBsAg, treated to inactivate residual hepatitis B virus (11); (ii) isolated HBsAg p-25 or gp-30 proteins (12); or (iii) synthetic peptides based on the amino acid sequence of HBsAg p-25 protein (13, 14). However, some concern has been voiced concerning the use of isolated whole HBsAg because of the possibility (although perhaps remote) of adverse autoimmune or alloimmune reactions due to the possible presence of host proteins in the purified HBsAg (actually, this vaccine has recently been approved by United States Food & Drug Administration). On the other hand, isolated HBsAg proteins, p-25 and gp-30, are poorly immunogenic (12). Nevertheless, the immunogenicity of these proteins is markedly improved when they are incorporated into lipid micelles (15). It might be expected that synthetic peptides would also be more immunogenic if presented in the form of lipid-peptide particles. Despite the apparent role of lipids in potentiating the immunogenicity and modifying the antigenicity (16) of HBsAg, no detailed information concerning the lipid composition of HBsAg has been reported. Indeed, the only information available in the literature refer to the mere identification of the major lipid classes present (8, 17). Also, no information concerning the interrelation between the protein and lipid components of HBsAg has been presented. For these reasons, we have undertaken a detailed study of the lipids of HBsAg. In addition to identifying the lipid components of HBsAg, we have used a photoactivatable probe, pyrenesulfonyl azide, to obtain data on the interaction of these lipids with the protein components of HBsAg. Pyrenesulfonyl azide partitions almost exclusively into hydrophobic lipid regions and generates a highly reactive nitrene upon exposure to UV light which covalently binds to adjacent constituents. This lipophilic probe has proven useful for studying the localization of membrane-associated proteins in vesicular stomatitis virus (18) and as a marker for the acetylcholine receptor subunits which are in contact with the membrane hydrophobic environment (19, 20). In this paper, we demonstrate that this probe is also useful for obtaining data concerning the interaction of the major protein (p-25) and glycoprotein (gp-30) components of HBsAg with its lipid matrix.

EXPERIMENTAL PROCEDURES

Materials

All electrophoresis reagents were obtained from Bio-Rad. Trypsin was obtained from Sigma. Pyrenesulfonyl azide was purchased from Molecular Probes.

Methods

Assay of HBsAg—HBsAg was assayed by counterelectrophoresis against anti-HBsAg antibodies prepared from immunized guinea pigs.

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[1] The abbreviations used are: HBsAg, hepatitis B surface antigen; PySA, pyrenesulfonyl azide; SDS, sodium dodecyl sulfate; HDL, human high density lipoprotein; TLC, thin layer chromatography; GLC, gas-liquid chromatography; PC, phosphatidylcholine.

Isolation of HBsAg—HBsAg used in these studies was obtained from the plasma of different carriers of HBsAg (adw subtype) following previously described procedures (10). The purified HBsAg samples were extensively purged with argon and stored at 3–4 °C. Storage of samples under argon proved to be important in studies involving fatty acid determination. In fact, an overall decrease in the average number of double bonds while maintaining identical chain lengths was observed in samples stored without such a precaution. This was especially noticeable for linoleic acid, the major unsaturated acyl residue of HBsAg.

Lipid Extraction, Fractionation, and Characterization—The total lipid from HBsAg preparations was extracted by the procedure of Bligh and Dyer (21). Lipid extracts were washed with a 0.73% NaCl solution and evaporated to complete dryness. The total lipid content of HBsAg was determined gravimetrically. Dried lipid extracts were taken up to approximately 25 mg of lipid/ml in choloroform:methanol (2:1, v/v) and stored under argon at −30 °C until used for further analysis (between 1–7 days). Handling of lipid extracts was always done under argon, and all organic solvents were of high quality and glass-distilled from Burdick and Jackson Laboratories, Inc. Total, free, and esterified cholesterol and phosphorus content were determined simultaneously in the lipid extracts to avoid errors from evaporation of the chloroform:methanol solutions. Cholesterol determinations were done by a more sensitive modification of the Zlatkis *et al.* (22) and Courchaine *et al.* (23) procedures. Total lipid aliquots (6–12 μl from stock solutions) or chloroform eluates from cholesterol and cholesteryl ester TLC spots were deposited in conical centrifuge tubes, dried under a stream of argon, and dissolved in 3 ml of glacial acetic acid. Two ml of a dilute $FeCl_3$ color reagent (21 ml of concentrated H_2SO_4 plus 4 ml of a solution of 500 mg of $FeCl_3 \cdot 6H_2O$/20 ml of 85% phosphoric acid) were added and mixed, and the absorbances at 550 nm were determined after 30 min at room temperature. The calibration curve was obtained with free cholesterol (Sigma) dissolved in acetic acid (10–200 μg of cholesterol). Free cholesterol was also determined by the same procedure making use of the specific formation of a digitonide precipitate from an acetone solution of the free sterol in the presence of digitonin (Sigma). Levels of cholesteryl esters relative to the other fatty acid-containing lipids were also determined by GLC in the presence of an internal standard.

Lipid phosphorus was determined by the micromethod described by Bartlett (24) except that the Fiske and SubbaRow reagent was prepared by dissolving 6.25 mg of 1-amino-2-naphthol-4-sulfonic acid and 5 g of $Na_2S_2O_5$ up to 25 ml with distilled water. Absorbances at 830 nm were measured and interpolated into a calibration curve obtained from a KH_2PO_4 solution. This method allows for accurate quantitation of as little as 50 ng of phosphorus. Silica gel contained in phospholipid spots from TLC plates was eliminated prior to the determination of the absorbances at 830 nm by extensive centrifugation in a clinical tabletop centrifuge. Under these conditions, presence of silica gel did not interfere with the colorimetric procedure.

Neutral lipid classes were fractionated by TLC on activated (1 h, 110 °C) 0.25-mm layers of Silica Gel G (Redi-Plate, Analtech) using *n*-hexane/ethyl ether/acetic acid/methanol (60:40:1:1, by volume) as the developing solvent. After development, the plates were sprayed with a 0.05% rhodamine solution in methanol, and lipid bands were visualized under UV light. In all cases, chromatography chambers were lined with filter paper saturated with solvent for at least 1 h prior to use. Migration distances were compared with those of commerical standards (Sigma or Supelco) on the same or parallel plates.

Polar lipids were fractionated by two-dimensional TLC on activated 0.2-mm layers of linear high performance plates (10 × 10 cm; LHP-K, Whatman). Aliquots containing 100–150 μg of total lipid were developed twice (developed, dried under a stream of argon, then developed again) using chloroform/methanol/concentrated ammonia (65:25:4, by volume). Plates developed twice in the first dimension were dried for 15 min under a stream of argon and 30 min under vacuum, and then developed with chloroform/acetone/methanol/acetic acid/water (30:40:10:10:5, by volume) in the second dimension. It was crucial that all traces of NH_4OH be removed prior to chromatography in the second dimension. Lipid spots were visualized by charring with 10 N H_2SO_4 (for samples for phosphorus determinations or by spraying with Phospray (Supelco) (for identification of phosphorus-containing lipids) or with a 0.05% methanol:water (1:1, v/v) solution of 1′,7′-dichlorofluorescein (for samples for fatty acid analysis). Quantitation of the different diacyl phospholipids *versus* the corresponding alk-1-enyl (plasmalogens) derivatives was achieved through a separation-reaction-separation two-dimensional TLC procedure similar to the one described by Owens (25). This method is based on the labile character of plasmalogens to hydrolysis by acids or by $HgCl_2$ while the diacyl phospholipids are stable. The lipids are first applied on LHP-K Whatman plates and chromatographed in the first dimension as described above, and then the portion of the plate containing the fractionated lipids is sprayed with a 0.05 M solution of $HgCl_2$ and kept under a stream of argon for 30 min at room temperature (reaction step). The plates are then dried and developed in the second dimension as described above, which resulted in the concomitant separation of each phospholipid class (separated in the first dimension) and also of acid-stable (diacyl phospholipids remaining with the same R_F) and acid-labile subpopulations (as lysoforms) for each phospholipid class. Phosphorus determinations on the lipid spots allow for quantitation of the ratio of diacyl phospholipid to plasmalogen.

For preparative purposes, PC was isolated from lipid extracts by TLC on 0.25-mm layers of Silica Gel G (Redi-Plate, Analtech) using chloroform/methanol/concentrated ammonia (65:25:4, by volume) as the solvent system. The PC band was scraped off the plate and exhaustively eluted from the adsorbent as previously described (26). The positional distribution of the acyl groups in the isolated PC was determined by hydrolysis with phospholipase A_2 (EC 3.1.1.4) present in *Crotalus adamanteus* venom (Sigma) as basically described by Van Golde and Van Deenen (27). Fatty acid analysis of the initial PC and its lyso derivative (sn-1 position) was used for the calculations.

Transmethylation of total or fractionated lipid classes was performed with a sodium methoxide/methanol reagent (methanolic base, Supelco). The reagent (1 ml) was added to the dried lipid samples and kept under argon for 15–18 h at room temperature in the dark. Traces of silica gel, rhodamine, or dichlorofluorescein did not affect the transesterification or the resulting products. Fatty acid methyl esters were extracted in a water-petroleum ether system, taken to dryness under argon in small conical centrifuge tubes, and finally dissolved in 20-μl aliquots of isooctane for analysis by GLC.

GLC analysis of fatty acid methyl esters was performed on a Varian 3700 gas chromatograph equipped with a dual flame ionization detector and with 6-foot glass columns (2 mm internal diameter). Quantitation of the peak areas was achieved by automatic integration with a Hewlett-Packard 3390-A recording integrator. 10% SP-2340 cyanosilicone on 100–120 Supelcoport (Supelco) was used over a temperature range of 142–234 °C (4 min at 142 °C, linear increase from 142–234 °C at 4 °C/min, and 8 min at 234 °C) and a carrier gas flow (N_2) of 50 ml/min. Components in the chromatographed samples were identified by comparison of the retention time with known standards (Supelco and Sigma). Methyl pentadecanoate was used as an internal standard.

PySA Labeling Procedure—HBsAg at a concentration of 1.5 mg of protein/ml in 10 mM sodium phosphate buffer, pH 7.4, 0.1 mM EDTA, and 0.02% NaN_3 was placed in a 3-ml thick walled quartz cuvette containing enough dried PySA to provide a final concentration of 2 mM. All operations with PySA were performed in the dark. The mixture was stirred at room temperature until no further increase in the absorbance at 365 nm was observed (approximately 75 min). Excess PySA remaining insoluble was then removed by low speed centrifugation in a clinical centrifuge. The suspension was then exposed for 45 min to UV light (>300 nm) from a Mineral Light UVS 58 (Ultraviolet Products, Inc.) with the filter removed. A gentle stirring of the sample was maintained during the irradiation to avoid inner filter effects. The distance between the sample cuvette and the light source was approximately 3 cm. Alternatively, [^{3}H]PySA (18 Ci/mol specific activity) prepared as described elsewhere (19) was used to quantitate the extent of incorporation of PySA into HBsAg particles or its protein components. [^{3}H]Toluene was used as an internal standard in determining the efficiency of the counting. Upon irradiation, the labeled HBsAg particles were separated from nonincorporated PySA photoproducts (solubility in aqueous solutions, 10^{-5}–10^{-6} M) by passage through a Sepharose 2B column (1.5 × 45 cm) and equilibrated in the above-mentioned phosphate buffer (Fig. 3). Approximately one-third of the available PySA was found incorporated into HBsAg particles. Irradiation was also performed in the presence of a 2-fold excess (w/w) of bovine serum albumin over HBsAg protein to prevent possible PySA labeling of the HBsAg components coming from the aqueous medium rather than from within the HBsAg particle. The distribution of the [^{3}H]PySA label on the protein components of HBsAg was measured after SDS-polyacrylamide slab gel electrophoresis where either a 12–23% linear gradient or a 5–22% exponential gradient polyacrylamide gel was used. A 4% polyacrylamide stacking gel was used in either case. The gel and buffer formulations were those of O'Farrell (28). Upon visualization of the

electrophoresed gel under UV light, the gel was cut into strips and placed into scintillation vials containing 1 ml of 2% SDS. After overnight incubation and shaking of the samples at 37 °C, scintillation fluid was added, and the samples were counted.

Preparative polyacrylamide gel electrophoresis was performed using 12–22% polyacrylamide linear gradient slab gels. Upon location of the proteins under UV light, the protein strips were cut and placed in an electrophoretic apparatus as described by Allington *et al.* (29) where they were electrophoretically eluted from the gel and concentrated into a 0.15 M ammonium hydroxide solution containing 0.1% SDS. Generally, 1 h at 140 V was sufficient for a complete elution of the protein from the gel strip. SDS was then removed by the addition of 20 volumes of acetone:triethylamine:acetic acid (85:5:5, by volume) as described by Henderson *et al.* (30). Tryptic digestion of the isolated p-25 and gp-30 was performed according to previously published procedures (10). The digestion was allowed to proceed for 12 h with constant stirring at 37 °C. Following digestion, the hydrolysis mixtures were taken to dryness in a Savant Speed-Vac concentrator, and the hydrolysis products were examined by SDS-polyacrylamide gel electrophoresis.

Absorption and fluorescence emission spectra were taken in a Cary 210 (Varian) spectrophotometer and in a SLM 4000 spectrofluorimeter, respectively.

RESULTS

Purification of HBsAg—Electron micrographs obtained from HBsAg preparations purified as described under "Methods" showed a homogeneous population of particles 20 nm in diameter (Fig. 1*A*). Purified HBsAg exhibited a major protein, p-25 (M_r = 25,000), and a major glycoprotein, gp-30 (M_r = 30,000) (Fig. 1*B*), which accounted for more than 90% of the total protein content of the antigen. Amino acid sequencing data from both p-25 and gp-30 components recently demonstrated that gp-30 is the glycosylated derivative of p-25; hence, both contain the same polypeptide chain (10). Assuming identical molar extinction coefficients for both p-25 and gp-30, we have estimated an apparent p-25:gp-30 stoichiometry of approximately 2:1 by scanning at 280 nm of unstained 12–23% linear gradient polyacrylamide gels following electrophoresis of the purified antigen (Fig. 1*B*).

Lipid Composition of HBsAg—For the studies reported here, plasma from different patients known to be HBsAg carriers was utilized to obtain several HBsAg preparations. Samples 1, 3, and 5 were obtained from three different individuals. Sample 6 originated from a pool of equal amounts of HBsAg obtained from the plasma of two different carriers. Samples 2 and 4 correspond to individuals 1 and 3, respectively, except that plasma was withdrawn several weeks later. Table I shows the lipid to protein and phospholipid to cholesterol ratio for the six different preparations of HBsAg as well as the averages and standard deviations calculated from all of them. All HBsAg samples analyzed exhibited lipid to protein and phospholipid to cholesterol ratios whose individual values do not significantly differ from each other, thus having standard deviations which ranged well within experimental error. A high degree of analogy was also observed in the different samples with regard to the total fatty acid composition of lipids extracted from HBsAg preparations (Table II). The major fatty acid components were palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic (18:2) acids which accounted for more than 85% of the total fatty residues.

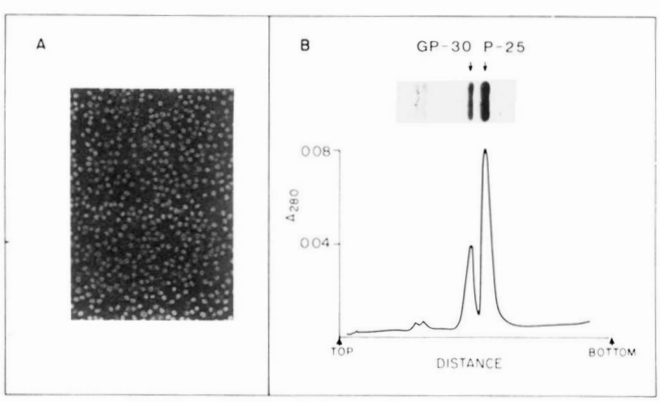

FIG. 1. **Purified HBsAg.** *A*, negative stain electron micrograph of an HBsAg sample purified as described under "Methods." A 1% solution of sodium phosphotungstate was used as the negative staining agent. *B*, SDS-polyacrylamide gel electrophoresis of purified HBsAg. The sample was dissolved in 25 μl of 62.5 mM Tris-HCl, pH 6.8, containing 10% mercaptoethanol and 2.3% SDS, and then heated in boiling water for 1 min. The solution was then made 10% in glycerol containing bromphenol blue as tracking dye and electrophoresed on a 0.75-mm thick 12–23% linear gradient polyacrylamide gel at 15 mA constant current until the dye eluted. The *lower part* of *B* shows a 280-nm scan of an unstained gel run under the conditions from above.

On the basis of these determinations, it can be concluded that lipids associated to HBsAg particles exhibit a defined compositional pattern and are not subject to individual variations. Consequently, the lipid data in subsequent tables (III and IV) are given as the averages of several separate determinations rather than on an individual basis.

The major lipid classes present within HBsAg particles (Table III) are phospholipids (~67%), free (~15%) and esterified (~14%) cholesterol, and triglycerides (~3%). The molar abundances of phospholipids and cholesteryl esters were determined by both colorimetric procedures and by GLC in the presence of an added internal standard (methyl pentadecanoate). Both methods provided values which agreed very closely. Free cholesterol was determined following two colorimetric approaches, while triglycerides relative levels were determined by GLC in the presence of an internal standard (see "Methods").

The fatty acid composition of each major lipid class is also shown in Table III. As expected from its high relative abundance, the compositional pattern observed in the fatty acids

TABLE I
Total Lipid Contents of HBsAg

	Preparation						Average ± S.D.
	1	2	3	4	5	6	
Lipid/protein[a] (weight ratio)	0.41	0.28	0.39	0.31	0.32	0.35	0.34 ± 0.05
Phospholipid/cholesterol[b] (molar ratio)	2.10	2.11	2.00	2.25	1.86	2.31	2.10 ± 0.16

[a] Total lipid and proteins were estimated gravimetrically and using the value of 3.72 as the absorbance at 280 nm for a 1 mg/ml solution of HBsAg (37), respectively.

[b] Total phosphorus and total cholesterol were determined by colorimetric procedures (see "Methods").

TABLE II
Fatty acid composition of total lipids from HBsAg (weight %)

	Preparation						Average ± S.D.
	1	2	3	4	5	6	
16:0	29.5	24.2	29.6	24.7	28.5	25.4	27.0 ± 2.3
16:1	2.0	3.2	1.9	3.5	3.5	1.6	2.6 ± 0.8
18:0	13.0	11.3	12.3	12.6	12.4	15.1	12.8 ± 1.2
18:1	17.7	16.4	20.9	23.2	25.2	19.7	20.5 ± 3.0
18:2	30.7	35.0	26.6	23.4	24.3	27.1	27.9 ± 3.9
22:1	2.5	3.0	1.9	3.6	1.6	2.7	2.6 ± 0.7
20:4	3.9	4.8	6.0	7.6	4.0	7.7	5.7 ± 1.6
22:6	0.8	2.1	0.9	1.5	0.5	0.8	1.1 ± 0.5
Average degree of unsaturation[a]	1.0	1.2	1.1	1.2	1.0	1.1	1.1 ± 0.1
Average chain length[a]	17.5	17.8	17.6	17.8	17.5	17.7	17.7 ± 0.1

[a] Average degree of unsaturation and chain length in Tables II–IV refer to the fatty acyl chains.

TABLE III

Fatty acid composition of major lipid classes (weight %)

The values indicated are the averages and standard deviations for preparations 1, 2, 3, and 5. TG, triglycerides; PL, phospholipids; CHE, cholesterol esters; CH, cholesterol.

	TG	PL	CHE	CH	Total recovery of fatty acid[a]
16:0	26.8 ± 3.8	28.5 ± 1.5	12.0 ± 1.5		25.7
16:1	6.8 ± 1.4	0.9 ± 0.2	4.7 ± 2.5		1.8
18:0	3.5 ± 1.3	15.9 ± 1.7	1.3 ± 0.4		12.9
18:1	41.0 ± 3.9	15.7 ± 2.2	22.0 ± 2.7		17.8
18:2	21.9 ± 1.3	28.0 ± 4.5	52.8 ± 3.4		31.9
22:1	ND[b]	2.3 ± 0.4	3.7 ± 1.5		2.4
20:4	ND	7.0 ± 1.7	3.5 ± 1.4		6.1
22:6	ND	1.7 ± 0.6	ND		1.3
Molar percentage[c]	3.4 ± 1.0	66.9 ± 1.8	14.2 ± 1.1	15.5 ± 1.2	
Average degree of unsaturation	0.9 ± 0.0	1.1 ± 0.1	1.5 ± 0.1		1.1
Average chain length	17.3 ± 0.1	17.7 ± 0.1	17.8 ± 0.1		17.8

[a] Recovery of fatty acids is calculated on the basis of the molar relative abundances and fatty acid composition of TG, PL, and CHE by a_i total = $\sum a_{ij} \times P_j/100$, where "$a_i$ total" represents the recovery of the different "i" fatty acids in the total lipid fraction. "a_{ij}" are the weight percentages of each "i" fatty acid in each "j" lipid class and "P_j" are the molar percentages of each "j" fatty acid containing lipid class. The values of 4.0, 79.2, and 16.8% were used as the molar percentage for TG, PL, and CHE, respectively and were obtained from the percentages given above excluding the contribution of free cholesterol.

[b] ND, not detected.

[c] Molar percentage of each lipid class based on single determination by GLC using an internal standard and colorimetric procedures (see "Methods").

present in the phospholipid fraction resembles that observed for total HBsAg lipids. Palmitic and linoleic acids (~28% each) and stearic and oleic acids (~15% each) were the major fatty residues in phospholipids, providing an average chain length of 17.7 carbons and approximately 2 double bonds per phospholipid molecule. Cholesteryl esters and triglycerides contain linoleic (~52%) and oleic (~41%) acids, respectively, as the major acyl components.

From the fatty acid composition of the phospholipids, cholesteryl esters, and triglycerides, and from the relative abundance of these components, the recovery of acyl residues throughout the analytical process can be calculated. As shown also in Table III, such a recovery accounts for most acyl residues of the experimental total fatty acid composition shown in Table II, therefore validating the techniques used for the analysis and indicating that most HBsAg lipid components were accounted for.

The phospholipid types present in the HBsAg phospholipids fraction are summarized in Table IV. The recovery of phosphate from the TLC plates was within the 90–100% range; therefore, the possibility of the existence of undetected phospholipids in the lipid extracts is unlikely. Choline phosphoglycerides accounted for approximately 90% of the total phospholipids and were almost exclusively composed of $HgCl_2$-stable diacyl derivatives (PC) since only 6% of the choline phosphoglyceride population became hydrolyzed to a lyso derivative upon treatment with $HgCl_2$ (presumably, choline plasmalogens). Conversely, ethanolamine phosphoglycerides accounted for only 2–4% of the total phospholipids and were composed of approximately equal amounts of $HgCl_2$-stable (phosphatidylethanolamine) and $HgCl_2$-labile (ethanolamine plasmalogens) forms. Minor phospholipid components were phosphatidylserine, sphingomyelin, lysophosphatidylcholine, and lysophosphatidylethanolamine. Phosphatidylinositol or cardiolipin were not detected in any preparation. Table IV also summarizes the fatty acid composition of the individual phospholipid classes. As expected from its high relative abundance, PC exhibits a fatty acid composition very similar to the one previously shown in Table III for the total population of phospholipids. Other phospholipids such as phosphatidylethanolamine or phosphatidylserine contain slightly longer fatty chains on the average than PC, although the average

TABLE IV

Fatty acid composition of phospholipid classes (weight %)

The values indicated are the average and standard deviations for preparations 1, 2, 3, and 5. PE, phosphatidylethanolamine; PS, phosphatidylserine; SPH, sphingomyelin; 1-PC, lysophatidylcholine; 1-PE, lysophosphatidylethanolamine.

	PC	PE	PS	SPH + 1-PC + 1-PE
16:0	31.6 ± 3.1	22.9 ± 0.2	16.2 ± 0.7	25.1 ± 0.9
16:1	1.1 ± 0.5	12.3 ± 2.1	3.5 ± 2.0	10.3 ± 1.4
18:0	15.7 ± 2.3	15.8 ± 0.4	26.6 ± 2.2	14.7 ± 1.1
18:1	17.4 ± 2.3	21.1 ± 2.2	17.0 ± 1.5	16.2 ± 0.7
18:2	25.8 ± 3.0	12.2 ± 0.5	8.8 ± 0.4	7.6 ± 1.2
22:1	2.4 ± 0.5	7.0 ± 0.6	16.3 ± 1.0	26.1 ± 4.9
20:4	4.4 ± 1.6	8.8 ± 2.7	11.7 ± 2.6	TR[a]
22:6	1.6 ± 0.3	ND[b]	ND	ND
Average degree of unsaturation	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	0.7 ± 0.1
Average chain length	17.6 ± 0.2	17.8 ± 0.1	18.5 ± 0.2	18.3 ± 0.3
Molar percentages[c]	87.8 ± 0.3	1.7 ± 0.8	TR	10.5 ± 1.3
Molar percentages[d]	90.8 ± 2.0	4.0 ± 1.2	1.8 ± 1.1	3.3 ± 1.8

[a] TR, traces, molar abundances lower than 0.5%.

[b] ND, not detected.

[c] Phospholipid composition based on duplicate determinations of phosphorus contents by Bartlett's procedure.

[d] Phospholipid composition based on single determinations by GLC using an internal standard.

degree of unsaturation remained fairly constant at approximately two double bonds per phospholipid molecule.

Since PC, in addition to free and esterified cholesterol, is a major lipid component of HBsAg particles, we have investigated further its chemical structure by making use of the selective cleavage of *sn*-2 positioned fatty chains by phospholipase A_2 from snake venoms. The positional distribution of acyl chains between the *sn*-1 and *sn*-2 positions of PC is depicted in Fig. 2. Main features regarding the major fatty acid components are the selective positioning of linoleic and stearic acids at *sn*-2 and *sn*-1 positions, respectively. Conversely, palmitic acid is preferentially located at the *sn*-1 position, while oleic acid is equally distributed between both *sn*-1 and *sn*-2 positions.

Studies with Pyrenesulfonyl Azide—When PySA and/or [^{3}H]PySA was incorporated into HBsAg particles and then

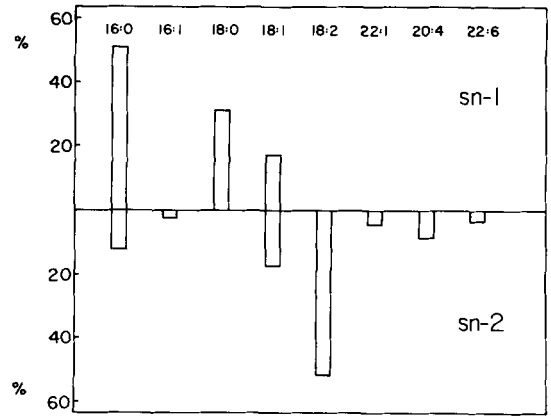

FIG. 2. **Positional distribution of acyl groups in PC from HBsAg lipids.** The *upper panel* indicates the fatty acid composition at the *sn*-1 position of PC as obtained from fatty acid analysis of the lyso-PC derivative resulting from phospholipase A_2 hydrolysis of the original PC. The *lower panel* indicates the fatty acid composition at the *sn*-2 position of PC as calculated from the difference between the fatty acid composition of the intact PC (Table IV) and that at the *sn*-1 position.

irradiated for 45 min under the conditions described under "Methods," about 80% of the photoactivatable probe became photolyzed to reactive nitrenes. Since complete photolysis of PySA would take approximately 45 min more, we decided to use the above conditions of 80% photolysis to avoid possible damage of the protein components of HBsAg by prolonged ultraviolet exposure. Under these conditions, the PySA-labeled antigen preparation completely retained its antigenic activity as judged by counterelectrophoresis against anti-HBsAg. After removal of the nonsolubilized hydrophobic probe from the irradiated mixture, the soluble nonincorporated [^{3}H]PySA photolysis products (the solubility limit of PySA or PySA photoproducts in aqueous solutions is in the 10^{-5}–10^{-6} M range) were separated from [^{3}H]PySA-labeled HBsAg particles by chromatography on Sepharose 2B (Fig. 3). The first peak centered around fraction number 37 and contained approximately one-third of the total radioactivity applied to the column. The absorbance spectrum of a pool from fractions 25 to 47 is shown in Fig. 4; the 280-nm region of the spectrum coincides almost exactly with that of purified HBsAg alone. The specific activity of the [^{3}H]PySA-labeled HBsAg was approximately 0.085 μmol of PySA/mg of protein (about 10^6 cpm/mg of protein). On the other hand, the absorption spectrum of pooled fractions 48–62 (Fig. 4) was identical with the one observed for the PySA photoproducts obtained by irradiation in the absence of HBsAg particles. When the irradiation was performed in the presence of water-soluble scavengers (bovine serum albumin, 2-fold excess by weight over HBsAg protein), the extent of PySA incorporation into HBsAg remained unchanged. Furthermore, the ratio of absorbances at 280 nm to 364 nm (as indicative of protein/PySA ratio) upon irradiation in the presence of the albumin was identical with that obtained in its absence.

Polyacrylamide gel electrophoresis in the presence of SDS and reducing agents such as mercaptoethanol is the only method by which we have been able to separate and, subsequently, isolate p-25 and gp-30 (31). The distribution of [^{3}H]PySA photoproducts among HBsAg components was determined after SDS-gel electrophoresis. The electrophoretic pattern of HBsAg protein components (protein stain) is not changed by photolabeling, which indicates that no apparent loss of polypeptide material occurred as a result of cross-linking or any other kind of polymerization or hydrolysis reaction. Both major protein components, p-25 and gp-30, were labeled (Fig. 5) as demonstrated by the incorporation of radioactivity and by the fluorescence of both p-25 and gp-30 bands. Radioactivity measurements and scanning at 354 nm of unstained gels gave a ratio of p-25 to gp-30 bands of approximately 2:1 which coincides with the p-25/gp-30 stoichiometry obtained by 280-nm absorbance scans (Fig. 1*B*). These results indicated that both protein and glycoprotein were labeled by PySA to the same extent and suggested their buried character within the lipid matrix. The specific activities of both protein components were approximately 0.011 μmol of PySA/mg of protein. Nevertheless, most of the radioactivity incorporated into HBsAg particles is associated with nonprotein components. About 87% of the incorporated radioactivity was found to be associated with a fluorescent band that did not stain with Coomassie blue and migrated close to the dye front in SDS gels. This is likely to be associated with the lipid components of HBsAg as previously demonstrated with the acetylcholine receptor system (19).

PySA-labeled p-25 and gp-30 were electrophoresed out of the gels into a buffered solution containing SDS. The fluorescence emission spectra of both purified labeled components is

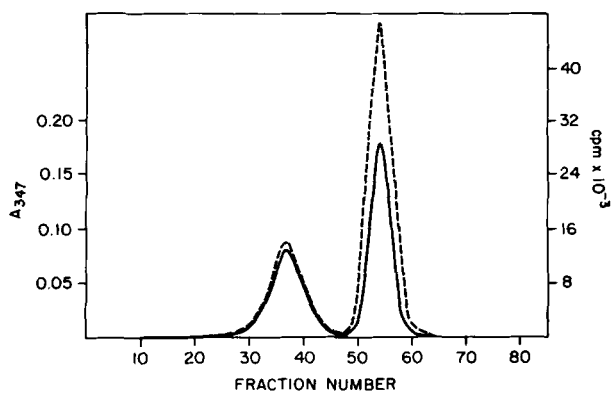

FIG. 3. **Removal of nonassociated [^{3}H]PySA photoproducts from [^{3}H]PySA-labeled HBsAg particles after irradiation.** Gel filtration chromatography in Sepharose 2B column (see "Methods") was performed. Fractions of 1.2 ml were collected. ——, represents the continuous monitoring of absorbance at 280 nm; - - -, indicates the radioactivity of 100-μl aliquots of each fraction.

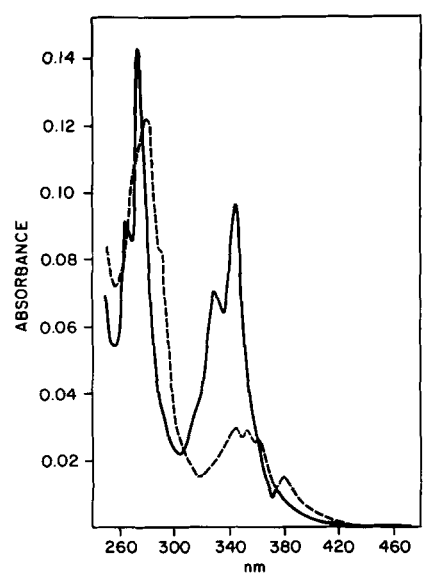

FIG. 4. **Absorption spectra of pooled fractions from Sepharose 2B column.** ——, represents the spectrum of pooled fractions 48–62; - - -, represents the spectra of pooled fractions 25–47.

shown in Fig. 6. Upon excitation at the excitation maximum (346 nm), labeled protein and glycoprotein exhibited emission maxima at 385, 404, and 424 nm which are in agreement with the results obtained by Sator *et al.* (19). When compared to the emission spectrum of the intact labeled HBsAg particle, these emission maxima were shifted toward longer wavelengths indicating a more polar environment of the fluorophore in the presence of SDS than in the native HBsAg particle. Also, an increased 385 to 404 nm ratio, characteristic of protein-associated PySA, was observed in the SDS-purified samples.

Tryptic hydrolysis of either purified protein component of HBsAg, p-25 and gp-30, does not give rise to soluble peptides even after prolonged digestion (about 18 h) at 37 °C with trypsin at an enzyme:substrate ratio of 1:25 (10). Nonetheless, both p-25 and gp-30 are cleaved at lysine 122, each giving two peptides. p-25 is cleaved into p-25-1 and p-25-2 which represent the NH_2-terminal 122 amino acids and the carboxyl-terminal 104 residues, respectively. Cleavage of gp-30 provides gp-30-1 and gp-30-2, representing the same polypeptide portions as in cleavage of p-25, except that their resolution by SDS-polyacrylamide gel electrophoresis is less satisfactory due to the presence of the carbohydrate moiety on the gp-30-2 fragment (10). Fig. 7 shows the results obtained from the tryptic hydrolysis of PySA-labeled p-25 and gp-30. Once again, the polypeptide pattern upon digestion with trypsin is not modified by PySA labeling and coincides exactly with previously reported ones for unmodified p-25 and gp-30 (10). PySA fluorescence labeling was observed in both the NH_2-terminal and COOH-terminal tryptic fragments from either protein or glycoprotein.

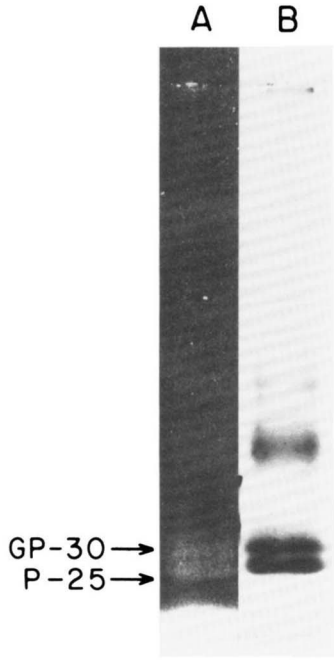

FIG. 5. **SDS-polyacrylamide gel electrophoresis of PySA-labeled HBsAg.** The samples were treated, and the electrophoresis was performed as described for Fig. 1, except that a 5–23% exponential gradient polyacrylamide gel was used. *Lane A* shows the unstained gel visualized under long wavelength UV light. *Lane B* shows the same gel as lane A, except that it is now stained with Coomassie blue.

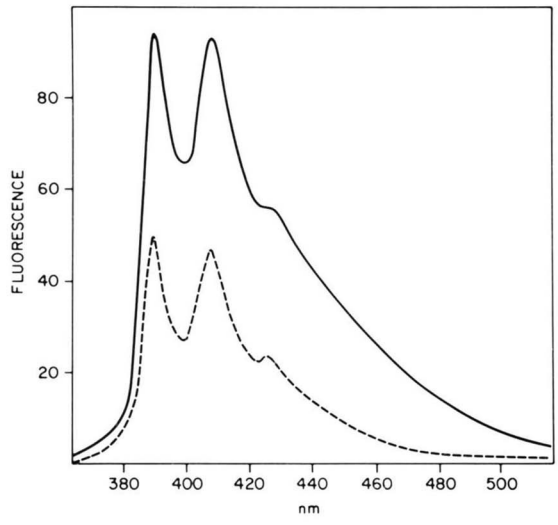

FIG. 6. **Fluorescence emission spectra of PySA-labeled p-25 and gp-30.** Both protein and glycoprotein were isolated as described under "Methods." Excitation was at 346 nm.

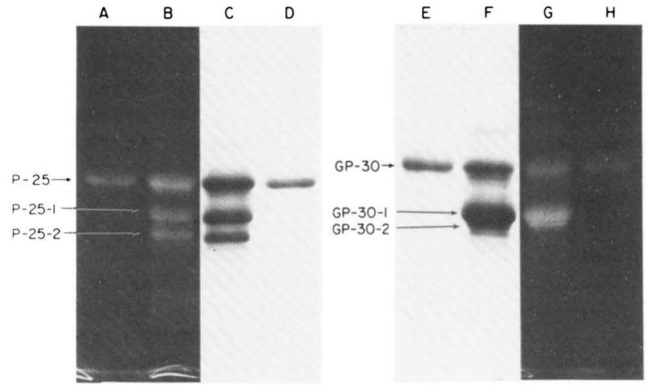

FIG. 7. **SDS-polyacrylamide gel electrophoresis of tryptic fragments of purified p-25 and gp-30.** The samples were treated, and the electrophoresis was performed as described for Fig. 1 on a 12–23% linear gradient gel. Lanes *A*, *B*, *G*, and *H* corresponded to gel visualized under long wavelength UV light. Lanes *C*–*F* correspond to the same gel stained with Coomassie blue. *A*, undigested p-25; *B*, trypsin-digested p-25; *C*, trypsin-digested p-25; *D*, undigested p-25; *E*, undigested gp-30; *F*, trypsin-digested gp-30; *G*, trypsin-digested gp-30; *H*, undigested gp-30.

DISCUSSION

After extensive purification, HBsAg derived from human plasma has been shown to contain two proteins, p-25, the major protein, and gp-30, the glycosylated form of p-25. p-25 and gp-30 have been shown to bear distinctive HBsAg antigenic activities as discrete amino acid sequences (32). These components account for more than 90% of the protein of HBsAg but only about 70% of the total mass of HBsAg. The remaining components of HBsAg, namely the lipids and carbohydrates, have not been characterized, nor has their role with regard to the structure or antigenic activity of HBsAg been elucidated.

We describe in this paper that the lipid composition of exhaustively purified HBsAg, subtype adw, does not vary between different preparations from individual HBsAg carriers or between preparations from the same individual whose plasma was obtained at different times. Therefore, we can conclude that the described features of HBsAg lipids are truly characteristic of HBsAg. HBsAg lipids account for approximately 25% of the HBsAg weight and are exclusively composed of phospholipids, cholesterol, cholesteryl esters, and triglycerides. No detectable amounts of any other lipid components were observed in any preparation. In this regard, HBsAg has been previously reported to contain glycolipids (8) which could presumably represent antigenic determinants, as described for other immunogenic systems (33). However, all

of our preparations were examined for glycolipids, but none were detected.

When compared to normal human serum lipoproteins, HBsAg was found to have some similarities with HDL which, for instance, has approximately the same density and contains a protein (Apo A-I) whose molecular weight is similar to that of HBsAg protein components (34). The major lipid components of HDL are also glycerides (~10%), cholesteryl esters (~20%), free cholesterol (~5%), and phospholipids (~41%) (35). Total cholesterol content of HDL is approximately that of HBsAg, even though the relative proportion of cholesterol to cholesteryl esters is approximately 1:1 for HBsAg and 1:3.5–4 for HDL. HBsAg also contains more phospholipids and less triglycerides than HDL. However, the fatty acid composition of the major lipid classes of HBsAg coincides almost exactly with that described for HDL_{2+3}, even though most serum lipoproteins seem to exhibit this feature as a common property regardless of their other differences (35). Nevertheless, because of the similarity between HDL and HBsAg lipids we examined our purified HBsAg preparations for HDL contamination. Automatic Edman degradation of HBsAg (data not shown) resulted in the liberation of phenylthiohydantoin-Met. Methionine has been demonstrated to be the NH_2-terminal residue of both p-25 and gp-30 (10). There was no detectable phenylthiohydantoin-Asp, which would be the expected derivative of the NH_2-terminal residue of Apo A-I (34), which is the major protein constituent of HDL, or any other amino acid, indicating that if HDL or any other contaminant was present, they were in very minor amounts and below the level of detection. Additionally, the absence of Apo A-II, the second most abundant apoprotein of HDL (34), was shown by SDS-gel electrophoresis. Apo A-II, M_r = ~8500, would have moved ahead of p-25, and no traces of protein were observed within that molecular weight range. The Edman degradation procedure would not have been useful with Apo A-II since it has the NH_2 terminus blocked by pyrrolidonecarboxylic acid (34). Additionally, counterelectrophoresis against anti-HDL revealed an HDL content in several HBsAg preparations which was lower than 2–3% and would not account for significant amounts of lipid in HBsAg preparations.

Choline phosphoglyceride is the major lipid component of HBsAg, and it is composed almost exclusively of $HgCl_2$-stable diacyl derivatives (PC) which contain palmitic (~31%), stearic (~16%), oleic (~18%), and linoleic (~26%) acids as the major fatty acid components. From the positional distribution of the acyl chains obtained by hydrolysis with phospholipase A_2, it seems likely that the main molecular species of PC is 1-palmitoyl, 2-linoleoyl-*sn*-glycero-3-phosphorylcholine, which would account for approximately 50% of the PC population plus other minor species composed of combinations of stearic or oleic acids in the *sn*-1 position with either palmitic or oleic acids in the *sn*-2 position.

In order to provide some initial information on the structural organization of the lipid-protein complex in HBsAg we have used the photoactivatable hydrophobic probe PySA which, once incorporated and irradiated under adequate conditions, should become attached to those portions of the HBsAg protein components in contact with the lipid hydrophobic matrix. PySA has been successfully utilized as a marker for the acetylcholine receptor subunits which are in contact with the membrane hydrophobic matrix (19, 20) and also to identify membrane-associated proteins in vesicular stomatitis virus (18). When the photoactivatable PySA is irradiated following incorporation within HBsAg particles, both p-25 and gp-30 components became labeled by this probe, indicating that they are at least partially buried within the lipid environment. The distribution of the label between p-25 and gp-30 can be estimated by either radioactivity (using [^{3}H]PySA), absorbance scans on unstained gels at 354 nm, or fluorescence measurements on electrophoretically eluted p-25 and gp-30 fractions. By all of these, the stoichiometry of PySA labeling is very similar to the stoichiometry of p-25 to gp-30 obtained from 280-nm scans of unstained gels, suggesting a similar exposure to the lipid matrix for both protein components. Indeed, when the labeled p-25 and gp-30 were digested with trypsin and the fragments separated on SDS-polyacrylamide gels, both the COOH-terminal and the NH_2-terminal tryptic fragments of each were found to be labeled. These results are not unexpected since proteins p-25 and gp-30 have identical amino acid sequences (10) and might be expected to interact with the lipids in the same or similar manner. Also these results are consistent with the fact that there are extensive hydrophobic amino acid sequences in both of the tryptic fragments produced (18). Protection against PySA labeling of HBsAg components by the presence of an excess of bovine serum albumin failed. Indeed, the extent and the pattern of labeling as judged by the absorbance ratio 280:346 nm remained unchanged. Additionally, the PySA photoproducts which were associated with bovine serum albumin exhibited the characteristic absorption spectra of free PySA photoproducts which differ from the spectrum of protein-bound PySA photoproducts. This suggests that those PySA photoproducts which are generated in the aqueous solution fail to react with protein, the reaction apparently being quenched by water. On the other hand we may conclude that the PySA labeling, which we observed in the case of HBsAg, occurred with those PySA photoproducts generated within the hydrophobic environment of the HBsAg particle.

The labeling pattern of HBsAg protein components described above is not the result of any observable modification of the protein arrangement due to the incorporation of PySA or the irradiation procedure. In fact, PySA-labeled HBsAg exhibit no measurable decreased antigenic activity when compared with unlabeled HBsAg samples which would have presumably been lost if protein rearrangement had taken place.

Intact HBsAg particles also exhibit a water-exposed region at residues 122–150 of both p-25 and gp-30. This region appears to be an anchoring site for carbohydrates, and also it has been demonstrated that it contains amino acid sequences characteristic for adw and ayw subtypes.[2] These results and the above-described PySA labeling pattern are compatible with a model for HBsAg whereby regions within both the COOH-terminal and NH_2-terminal portions of p-25 and gp-30 are buried within the lipid matrix, while the antigenically important 122–150 region is sticking out of the HBsAg particle, thus exposed to the aqueous surroundings. The identification of those specific amino acids which comprise the regions in contact with lipid cannot be determined from these studies. However, the use of additional methods to cleave the protein into smaller fragments may allow such specific assignments. Such experiments are currently in progress.

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