Examination of the Polypeptides of Hepatitis B Surface Antigen

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(Accepted 12 July 1976)

SUMMARY

When the polypeptides of hepatitis B surface antigen were examined by SDS-polyacrylamide gel electrophoresis under a variety of conditions, anomalous results were found to be due to (i) variable and at times incomplete dissociation of polypeptides after boiling with 1% SDS and reducing agent, (ii) reaggregation of solubilized material under certain electrophoretic conditions and during laboratory manipulations, and (iii) the variable presence of additional components in hepatitis B surface antigen prepared from certain individual donors. When these factors were taken into account, two major components were consistently identified by discontinuous buffer polyacrylamide gel electrophoresis, of apparent mol. wt. 6,000 to 7,000 and 12,000 to 14,000. However, in view of the demonstrated limitations of this technique in examining HBAg polypeptides, alternative methods are necessary to confirm the true mol. wt. of the unique virus-specified amino acid sequence present.

INTRODUCTION

The 22 nm virus-like particles which can be readily identified in the sera of patients with hepatitis B virus (HBV) infection contain one known virus-coded function, hepatitis B surface antigen (HBAg). The polypeptide composition of these particles has been studied by a number of workers with the aim of defining the virus gene product(s) involved in this function. Initial studies of purified 22 nm particles using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining described 2 major polypeptides of mol. wt. 24 to 26,000 and 28 to 32,000 (Gerin, Holland & Purcell, 1971; Vyas et al. 1972; Gerlich & May, 1973); additional higher mol. wt. components found by two of these authors were variable in amount and lost on further purification; they were assumed to be contaminating material. In further studies, up to 7 additional polypeptides with mol. wt. ranging from 10,000 to 120,000 have been detected both by Coomassie blue staining and location of radioactive peaks (Dreesman et al. 1972; Gerin, 1972; Howard & Zuckerman, 1974; Chairez et al. 1975); some but not all of these components contained carbohydrate detectable by PAS staining (Chairez et al. 1973; Shih & Gerin, 1975). Dreesman et al. (1975) have reported that guinea pigs immunized with many of these individual components produced antibody that reacted with HBAg. In similar work, Shih & Gerin (1975) found that all seven polypeptides examined (mol. wt. 23,000 to 97,000) elicited a humoral antibody response to HBAg. On the other hand, I. Gordon (personal communication) observed a humoral antibody response only with a polypeptide of mol. wt. 22,000, whereas all seven polypeptides examined produced delayed type hypersensitivity to purified HBAg in immunized animals.

The 42 nm Dane particle which is thought to be the hepatitis B virion (Dane, Cameron &...
Briggs, 1970) contains a circular double-stranded DNA molecule of mol. wt. approx. $1.6 \times 10^6$ (Robinson, Clayton & Greenman, 1974; Overby et al. 1975). Since the coding potential of a nucleic acid molecule of this size is limited, it is unlikely that all the polypeptide components represent unique virus gene products. At least three formal possibilities exist to explain the apparent multiplicity of polypeptides in 22 nm HBAg particles: (i) multiple proteolytic cleavages of one or several precursor polypeptides may occur during intracellular assembly, circulation in the host, or storage in the laboratory; (ii) significant and variable aggregation of a limited number of smaller polypeptides may occur during SDS-PAGE, or (iii) the preparations examined may have contained significant contaminating host-coded material. The experiments described above using individual polypeptides as immunogens are in support of either of the first two possibilities. However, the reliability of solubilization and SDS-PAGE techniques for examining HBAg polypeptides has not been examined.

In this paper, we describe certain anomalies that may occur in the examination of HBAg polypeptides by SDS-PAGE using conventional techniques.

**METHODS**

**Purification and radiolabelling of HBAg.** HBAg positive plasma was collected from healthy blood donors and stored at $-20$ °C until used. Samples of 15 to 30 ml were purified as previously described by gel chromatography and equilibrium gradient sedimentation (Burrell, 1975; Burrell et al. 1976); with the exception of gel chromatography, which was carried out at room temperature for 16 h, all purification steps were done at 4 °C. Purified antigen preparations were stored in phosphate buffered saline (PBS) containing 0.01% sodium azide and approx. 25% (w/v) sucrose and 14% (w/v) CsCl, at 4 °C in sealed glass test tubes; preparations stored longer than 4 weeks were generally not examined. Radiolabelling and subsequent further purification of radiolabelled HBAg by gel filtration and rate-zonal gradient sedimentation were carried out as previously described.

**Sucrose gradient sedimentation.** Samples for sucrose gradient analysis (100 µl) were layered over pre-formed 5 to 20% sucrose gradients in PBS containing 0.001% SDS, and centrifuged for 2.5 h at 42000 rev/min at 4 °C in a Spinco SW 50L rotor. Samples (250 µl) for radioactive counting were collected by puncturing the bottom of centrifuge tubes; all centrifuge tubes were examined routinely for the presence of pelleted radioactive material. Sedimentation coefficients were calculated by comparison with 18S and 28S mouse liver RNA internal markers (a gift from Dr K. Jones, Dept. of Genetics, University of Edinburgh).

**Disruption of material prior to PAGE.** Samples were disrupted either by incubating at 37 °C overnight or boiling for 2 min with 1% (w/v) SDS and 65 mM-dithiothreitol (DTT) in 0.1 M-Na phosphate, pH 7.2, prior to continuous buffer PAGE, or 0.0625 M-tris, pH 6.8, prior to discontinuous buffer PAGE (disc-PAGE). In some experiments the concentration of DTT was increased to 1 M, or 8 M-urea was included during disruption; where specified, free sulphhydryl groups were alkylated after disruption by treatment with 100 mM-iodoacetamide for 60 min at 4 °C in the dark, followed by overnight dialysis against either 0.1 M-Na phosphate or 0.0625 M-tris, each containing 0.1% SDS.

**Polyacrylamide gel electrophoresis.** PAGE was carried out in 85 x 7 mm cylindrical gels at acrylamide concentrations of 7.5 to 15% (w/v), using a constant, N,N-methylenebisacrylamide: acrylamide ratio of 1:40. Continuous buffer gels (Maizel, 1969) containing 0.1% SDS in 0.1 M-Na phosphate buffer, pH 7.2, were polymerized overnight at room temperature by the addition of 0.04% TEMED and 0.1% ammonium persulphate (final concentration).
Disrupted antigen samples containing bromophenol blue tracking dye and 5% (v/v) glycerol or 10% (w/v) sucrose in a total volume of 100 µl were electrophoresed for 5 h at a constant voltage of 40 V, giving a current of 10 mA/gel. Electrophoresis buffer consisted of 0.1% SDS in 0.1 M-Na phosphate, pH 7.2.

Discontinuous buffer gels (Laemmli, 1970) consisted of a resolving gel of high acrylamide concentration polymerized in 0.375 M-tris/HCl buffer, pH 8.8, and 0.1% SDS, overlaid with a low concentration (3%) stacking gel containing 0.1% SDS in 0.125 M-tris/HCl, pH 6.8. Samples were electrophoresed for 4 h at a constant current of 3 mA/gel, using electrophoresis buffer containing 0.1% SDS in 0.025 M-tris/HCl and 0.192 M-glycine, pH 8.3.

Unlabelled polypeptides were located by staining with 0.25% Coomassie brilliant blue in acetic acid:methanol:water (5:45:50) for 60 min, followed by destaining for 24 h in several changes of acetic acid:methanol:water. Polypeptides were detected by scanning at 580 nm in a Gilford gel scanner, and mol. wt. determined by comparison with standard proteins electrophoresed in parallel.

Gels containing 125I-labelled material were cut into 1.5 mm slices and the iodinated peaks located by counting each slice in an LKB Wallac Gamma Counter. For mol. wt. estimations, 10 µg each of bovine serum albumin (BSA), ovalbumin (OA) and lysozyme (L) were added to the antigen sample prior to disruption and electrophoresed in the same gel. After slicing and counting, the marker proteins were detected by staining with Coomassie brilliant blue.

Reagents. SDS, specially pure grade, was obtained from B.D.H., Poole, Dorset, acrylamide from Eastman Kodak Company, Rochester, N.Y., and BSA (Fraction V, Bovine Plasma) from Armour Pharmaceutical Company, Eastbourne. Neuraminidase (from Clostridium perfringens, type VI), lysozyme and ovalbumin were from Sigma Chemical Company, Surrey, and mixed glycosidases (from Turbo cornutus) from Miles Laboratories, Slough. All other chemicals were best grade available from B.D.H.

RESULTS

Solubilization of radiolabelled HBsAg with detergent and reducing agent

Solubilization of 125I-HBsAg by treatment with 1% SDS and 65 mM-DTT was examined in sucrose velocity gradients as a preliminary step to PAGE analysis of individual polypeptides. Incubation at room temperature for 15 min released 30 to 50% of the labelled material and altered the sedimentation coefficient of the particle from 36S to 25S. With increased time or temperature of incubation, relatively less 25S component was produced, together with increasing amounts of radiolabel at the top of the gradient (Fig. 1). In most cases heating at 100 °C for 2 min was sufficient to dissociate the particle completely into low mol. wt. components, as confirmed by disc-PAGE and column chromatography (see below). However, with HBsAg purified from one plasma source the 25S component remained after treatment with SDS and reducing agent even at this temperature.

Irrespective of the source from which it was prepared the 25S component, once produced, was resistant to complete dissociation under a variety of denaturing conditions. Boiling with 8 M-urea alone, 8 M-urea and 1% SDS and 65 mM-DTT, 1% SDS and 1 M-DTT at pH 5.6 or 7.2, or incubating overnight at 37 °C with 1% SDS and 0.5 M-DTT caused some further breakdown in particulate structure. Labelled material now sedimented around 5 to 10S but was still too large to enter a discontinuous buffer 10% acrylamide gel, and therefore it was not possible to determine its polypeptide composition.

These findings indicated that treatment at 100 °C for 2 min with 1% SDS and 65 mM-DTT would in most situations disrupt radiolabelled HBsAg into low mol. wt. material, but that
the 25S component, once formed, could not be adequately dissociated by further extensive disruptive procedures.

**PAGE of HBsAg polypeptides detected by Coomassie blue staining**

Preparations of HBsAg of both subtypes (ad and ay) at different stages of purification were heated to 100 °C for 2 min with 1% SDS and 65 mM-DTT and examined by PAGE using the discontinuous buffer system. Analysis of HBsAg preparations after 2 cycles of equilibrium gradient sedimentation revealed nine or more polypeptides covering the mol. wt. range from 130,000 to 140,000 (Fig. 2a). Material at this stage of purity gave a faint precipitin line by gel diffusion with antibody to whole human serum. After further purification by rate zonal sedimentation the relative intensity of peaks 1, 2, 4, 5 and 6 was significantly decreased. These polypeptides probably represented contaminating human serum components differing from HBsAg in sedimentation coefficient and they are therefore not indicated on Fig. 2b and c. In 10% gels two major polypeptides, 3 and 9, of mol wt. 66 to 70,000 and 12 to 14,000, and two minor polypeptides, 7 and 8, of mol wt. 17,000 and 23,000 were detected (Fig. 2b). Increasing the acrylamide concentration to 12.5% decreased the height of peak 9 and gave a clearer resolution of peaks 7 and 8 which also increased in apparent mol. wt., now appearing as broad peaks at 26 to 30,000 and 20 to 24,000 respectively.
Polypeptides of HB$_{Ag}$

Fig. 2. Densitometer scan of polyacrylamide gels of Coomassie blue stained polypeptides of hepatitis B antigen at different stages of purification. (a) Column chromatography and 2 cycles of CsCl/sucrose equilibrium gradient sedimentation, 10 % discontinuous gel. (b) Column chromatography, 2 cycles of equilibrium gradient sedimentation followed by rate-zonal sedimentation in a sucrose density gradient, 10 % discontinuous gel. (c) Identical material to (b) separated on a 12.5 % discontinuous gel.
(Fig. 2c). Similar results were obtained using continuous buffer PAGE with the exception that the band of mol. wt. 12 to 14000 was not detected at any acrylamide concentration.

It was apparent that gel concentration, buffer conditions, the extent of solubilization of HB,Ag prior to PAGE, and the degree of purity of the preparations, were all likely to be affecting the number and apparent mol. wt. of the polypeptides detected. Accordingly, these variables were examined in more detail, using $^{125}$I-HB,Ag purified further by gel filtration in Sepharose 6B and rate-zonal gradient sedimentation, as previously described.

**PAGE of radiolabelled HB,Ag**

When $^{125}$I-HB,Ag was extracted with chloroform:methanol (2:1, v/v), less than 1% of the radioactivity partitioned in the organic phase, suggesting that chloroform-soluble lipid had not been radiolabelled to any great extent, and that the major labelled component was protein.

All $^{125}$I-HB,Ag preparations were reduced and alkylated as described in Methods. Using the continuous buffer system and 10% polyacrylamide gels, most of the radioactivity remained at the gel origin; minor peaks of 96000, 79000, 60000, 52000, 45000, 31000 and 26000 were detected corresponding to those described by other workers, but these contributed an insignificant proportion of the total radioactivity. Disruption of labelled antigen at pH 5.6 to eliminate protein aggregation caused by disulphide interchange reactions (Tanford, 1968), reduced the amount of non-migrating radiolabel but had no effect on the number or apparent mol. wt. of the polypeptides seen. Addition of 8 M-urea prior to disruption and inclusion of 4 M-urea in resolving gels had no effect on the polypeptide profile obtained.

Using a discontinuous buffer system and 10% acrylamide gels there was no accumulation of label at the gel origin and only 2 polypeptides of mol. wt. 60 to 65000 and 12 to 14000 were observed (Fig. 3a). The possibility that material larger than 12000 to 14000 mol. wt. might remain in the stacking state in these gels if its mobility exceeded that of the trailing glycinate ion at pH 8.3 (mobility = 0.5 units; Ornstein, 1964) was eliminated since similar profiles were obtained using tris/glycine buffer at pH values up to 9.5 (mobility = -15 units). Incubation for 72 h at 37°C with 0.05% neuraminidase and 0.1% mixed glycosidases also had no effect on the radioactivity profile indicating that charged sialic acid residues and carbohydrate were unlikely to be affecting electrophoretic mobility of labelled antigen in this system.

In an attempt to resolve material migrating with the lysozyme marker, the acrylamide concentration was increased to 12.5% or 15%. The rapidly migrating material was resolved as a single sharp peak of 12000 daltons but an increasing proportion of radioactivity remained at the gel origin and as background along the length of the gel; no new peaks were resolved (Fig. 3b and c). Inclusion of 4 M-urea had no effect on the polypeptide profile obtained. In all continuous and discontinuous gel runs internal marker proteins migrated according to the logarithm of their mol. wt.

It was concluded that, in the continuous buffer system, re-aggregation of most of the labelled material was occurring at the origin of 10% gels; in discontinuous systems, such aggregation was not marked and two labelled components of mol. wt. 60 to 65000 and 12 to 14000 could be resolved. These components correlated closely with the 2 major polypeptides reproducibly detected by Coomassie blue staining. Discrepancies between continuous and disc-PAGE mol. wt. estimates of a structural protein of *Bacillus subtilis* phage φ 29 (Camacho et al. 1975), and between disc-PAGE profiles of foot-and-mouth disease virus polypeptides using SDS from different commercial sources (Swaney, van de Woude &
Fig. 3. Distribution of radioactivity after disc-PAGE of 131I-labelled HB,Ag polypeptides using different acrylamide concentrations: (a) 10% acrylamide; (b) 12.5% acrylamide; (c) 15% acrylamide.
Bachrach, 1974) have been reported. In each case altering the conditions of PAGE led to inversion of the relative mobilities of two polypeptides but neither study reported the reaggregation of polypeptides in the continuous PAGE system, which was a marked feature in the present work.

In view of the effect of different electrophoretic conditions on polypeptide profiles independent analyses of HBsAg polypeptides were made using gel chromatography.

Gel chromatography of HBsAg polypeptides

Samples of 125I-labelled HBsAg were disrupted as for PAGE, and chromatographed in a 1.6 x 15 cm column of Sepharose 6B using either 0.1 M-Na phosphate buffer, pH 7.2, or 0.025 M-tris and 0.192 M-glycine, pH 8.3, both containing 0.1 % SDS, as eluant. Internal protein markers (BSA, ovalbumin and lysozyme) denatured under identical conditions, were included in each sample and detected by extinction at 280 nm; these markers chromatographed according to the logarithms of their mol. wt. In all experiments, a consistent elution profile of radioactivity was seen (Fig. 4); an insignificant proportion of radiolabel was recovered in the void volume, and peaks II and III corresponded to mol. wt. of 60 to 65000 and 15 to 20000 by comparison with internal protein standards. Although this type of experiment did not give a clear separation of radiolabelled components, the general correlation between gel chromatography and disc-PAGE results suggested that major artefacts in apparent mol. wt. were unlikely to be present in the disc-PAGE results shown in Fig. 3.
Polypeptides of HBsAg

When the rapidly migrating material isolated by disc-PAGE (12,000 to 14,000 daltons) was eluted from gel slices and chromatographed on Sepharose 6B, a single uniform peak of 15,000 to 20,000 was obtained. However, disc-PAGE of peak II or peak III after isolation by column chromatography revealed a large number of discrete radiolabelled components of apparent mol. wt. 1,200 to 10,000. Heating to 100 °C prior to PAGE had no effect on the radioactivity profile seen. These additional components were not present when identical samples of radiolabelled antigen were examined directly by disc-PAGE. This finding provided further evidence that significant reaggregation of HBsAg polypeptides could occur during laboratory manipulations.

Analysis of material released from 25S component

\(^{125}\)I-HB\(_s\)Ag preparations were treated with 1 % SDS and 65 mM-DTT at room temperature for 15 min, alkylated, and spun on sucrose velocity gradients. Radiolabelled material released during formation of the 25S component was analysed by continuous buffer PAGE in 10 % gels.

Immediate examination of the released material revealed a single component of mol. wt. 83,000 to 87,000, but after boiling for 2 min with SDS and DTT before electrophoresis this large component dissociated to give a single polypeptide of mol. wt. 14,000. Mild denaturation of HBsAg therefore led to the initial release of a homogeneous aggregate of a smaller polypeptide. Such aggregates may be related to the polypeptides of mol. wt. 82,000 and 90,000 described by Howard & Zuckerman (1974).

Proteins released during formation of the 25S component differed from the total \(^{125}\)I-labelled protein in that it did not accumulate at the origin of a continuous gel and could be dissociated into a single polypeptide of mol. wt. 14,000. These results suggests that the major 14,000 mol. wt. polypeptide was not an artefact of the disc gel system but could also be detected on continuous gels under conditions where reaggregation at the gel origin had not occurred.

DISCUSSION

The results described above clearly demonstrate certain difficulties in the analysis of the polypeptides of HBsAg using SDS-PAGE.

Firstly, standard dissociating conditions (heating in the presence of 1 % SDS and 65 mM-DTT) resulted in stepwise particle solubilization and the production of a 25S antigen component. In some cases disruption was incomplete after 2 min at 100 °C, and in all instances the 25S component, once produced, was remarkably resistant to further disruption.

Secondly, disrupted \(^{125}\)I-HB\(_s\)Ag polypeptides underwent considerable reaggregation at the origin of 10 % acrylamide gels using the continuous buffer system, but to a much smaller extent with the discontinuous buffer system. The reasons for this are not apparent; however, we consider it possible that similar reaggregation may be partly responsible for the production of high mol. wt. components in HBsAg reported by other workers using continuous buffer PAGE.

Thirdly, the proportion of radiolabelled material or the intensity of the stained protein band of 12,000 to 14,000 mol. wt. decreased with increasing acrylamide gel concentration. It was not clear whether this material was contributing to discrete peaks of apparently higher mol. wt., or to the general background level of material along the gel. In addition, the apparent mol. wt. of the two diffuse components of 17,000 to 24,000 and 23,000 to 30,000, when present, varied with the concentration of acrylamide used. These effects were not
seen with the sharp band of 66 to 70,000 mol. wt. detected by Coomassie blue or the 60 to 65,000 radiolabelled peak.

Finally, the two diffuse components of 17,000 to 24,000 and 23,000 to 30,000 probably correspond to the two major polypeptides described by other workers (Gerin et al. 1971; Vyas et al. 1972; Gerlich & May, 1973); in our hands their presence was variable. In batches of purified antigen in which these two polypeptides were detectable by Coomassie blue staining, they could also be seen as minor peaks after disc gel analysis of 125I-labelled samples of the same antigen. When present these components always occurred together in approx. equal amounts; their presence or absence was unrelated to degree of purity, subtype or length of storage of the purified antigen, but appeared to be related to the source of the material. Their presence could be explained by proteolytic cleavage of a larger polypeptide, occurring with material from some sources but not from others. Variable proteolytic degradation of human erythrocyte membrane proteins due to the action of leucocyte proteinases has been recognized (Fairbanks, Steck & Wallach, 1971); it is reasonable to expect that the proteins of HBsAg prepared from donations of blood may be susceptible to similar proteolytic action.

SDS-PAGE has proved a powerful analytical tool for polypeptide analysis of water-soluble proteins and virus particles (Shapiro, Viñuela & Maizel, 1967; Weber & Osborn, 1969; Laemmli, 1970). However, such techniques have proved inadequate for the complete dissociation of certain water insoluble proteins including bovine cell membrane (Maddy & Dunn, 1973; Green et al. 1974; Frank & Rodbard, 1975) and brain myelin proteins (Katzman, 1971; Morell, Wiggins & Gray, 1975). We have demonstrated above various patterns of incomplete dissociation and reaggregation of HBsAg polypeptides after treatment with SDS and DTT. Reports that different HBsAg polypeptides contain common immunogenic sites (Dreesman et al. 1975; Shih & Gerin, 1975; I. Gordon, personal communication) should be reconsidered with these cautions in mind.

This paper examines in detail the reliability of solubilization and SDS-PAGE techniques for the analysis of HBsAg polypeptides. Two major polypeptides of mol. wt. 60,000 to 70,000 and 12,000 to 14,000 were consistently detected in purified HBsAg. However, in view of the described anomalous behaviour of this material, it cannot be concluded that each of these represent distinct fully unfolded protein monomers. Alternative methods, such as analysis of tryptic peptides, are needed to define the true size of the unique amino acid sequence present in these particles.

We wish to thank Professor B. P. Marmion for his encouragement and advice during this work. This project was financed by a grant from the Scottish Home and Health Department. Patricia Mackay was supported by a Scholarship from the Faculty of Science, University of Edinburgh.

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(Received 22 March 1976)