Gel Filtration of Hepatitis B Surface Antigen: 
Increased size of the native particle

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SUMMARY

The Stokes radius of unpurified hepatitis B antigen (HBsAg) was determined by chromatography on a carefully calibrated Sepharose 4B column. A value of 14.2 nm was found by this procedure, contrasting with a published value of 11 nm for purified, pepsin-treated HBsAg. Chromatography at pH 3 appeared to reduce the Stokes radius of HBsAg to 11 nm. Evidence is presented to show that serum proteins adsorbed to HBsAg can be removed with pepsin or acid.

Hepatitis B surface antigen (HBsAg) has an average particle diam. of 20 to 22 nm, as determined by electron microscopy (Millman et al. 1970; Almeida, 1972). Recently, Bourbonnais, Guevin & Delvin (1975) have used gel filtration to show that the Stokes radius of purified, pepsin-treated HBsAg is 11 nm. A somewhat smaller radius of 9.2 nm may be calculated from the diffusion coefficient reported by Kim & Tilles (1973), who also used pepsin in their purification protocol. In these two studies, the mol. wt. of HBsAg was found to be about 2.5 x 10^6, or 3.0 x 10^6, for the ad and ay subtypes respectively. Higher values of 3.7 x 10^6 and 4.6 x 10^6 were obtained in an independent study by Chairez et al. (1975), using HBsAg purified without the use of pepsin. These particles had a diam. of 22 nm in the electron microscope; this figure is barely compatible with the higher mol. wt. estimates, since a uniform sphere of radius 11 nm and density 1.2 g/ml (Chairez et al. 1975; Gerin, Faust & Holland, 1975) has a calculated mol. wt. of 4.0 x 10^6.

In this paper, evidence is presented to show that the Stokes radius of native, unpurified HBsAg is greater than that of purified, pepsin-treated HBsAg and is also larger than the mean particle radius as determined by direct visualization. It is suggested that HBsAg may normally be associated with serum components which are digested or released in the presence of pepsin or weak acid.

An 80 x 2.5 cm chromatographic column was packed with Sepharose 4B (Pharmacia Fine Chemicals Inc., Piscataway, New Jersey), according to the manufacturer’s instructions. The column was equilibrated and run in phosphate buffered saline (PBS) at pH 7.2 (NaCl, 4.25 g/l; KH2PO4, 2.44 g/l; Na2HPO4, 8.9 g/l) at room temperature. The void volume (V0) was determined by using blue Dextran 2000 (Pharmacia). The Stokes radius of HBsAg was determined by comparing its elution behaviour with that of markers of known Stokes radius, as described below.

Bovine serum albumin, fraction V (BSA; Miles Laboratories Inc., Elkhart, Indiana) was used at 2.5 % (w/v) in PBS and was detected in column effluents by its extinction at 280 nm. Human immunoglobulin G (IgG) was represented by the antibody activity of a pool of recalcified human plasma containing antibody to HBsAg (anti-HBs). The antibody was detected in column effluents by passive haemagglutination (Vyas & Shulman, 1979) using HBsAg-coated erythrocytes (Electronucleonics Laboratories Inc., Bethesda, Maryland). In addition, immunochemically purified, 125I-labelled human anti-HBs (antibody reagent from AusRIA II, Abbott Laboratories Inc., North Chicago, Illinois) was used; it was
### Table 1. Physical characteristics and elution values for column markers chromatographed on Sepharose 4B and Sepharose CL-4B

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mol. wt.</th>
<th>Diffusion coefficient $(\text{cm}^2/\text{s} \times 10^7)$</th>
<th>Stokes radius* (nm)</th>
<th>$V_e/V_o$ Sepharose 4B</th>
<th>$V_e/V_o$ Sepharose CL-4B</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>67,000</td>
<td>5.9†</td>
<td>3.63</td>
<td>2.24</td>
<td>2.15</td>
</tr>
<tr>
<td>IgG (Human)</td>
<td>160,000</td>
<td>3.8†</td>
<td>6.2</td>
<td>2.17</td>
<td>1.99</td>
</tr>
<tr>
<td>IgM (Rabbit)</td>
<td>90,000</td>
<td>1.65‡</td>
<td>1.0</td>
<td>1.77</td>
<td>1.60</td>
</tr>
<tr>
<td>KLH (associated)</td>
<td>75,000</td>
<td>1.16§</td>
<td>1.85</td>
<td>1.46</td>
<td>1.34</td>
</tr>
<tr>
<td>φX 174</td>
<td>620,000</td>
<td>—</td>
<td>1.45§</td>
<td>1.68</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Calculated from diffusion coefficient.
† Phelps & Putnam, 1960.
‡ Calculated from S value (17 S) and mol. wt. and partial specific volume as given by Lamm & Small (1966).
§ Calculated from S value (98 S) and mol. wt. as given by Tornabene & Bartel (1962), assuming a partial specific volume of 0.727 (Wood et al. 1971).
¶ Eigner et al. (1963).

detected by means of an automatic well-type scintillation spectrometer. Rabbit immunoglobulin M (IgM) was a preparation of rabbit antibodies to sheep red blood cells with antibody activity primarily in the IgM fraction (Rabbit Haemolysin, Lot number R874010, Flow Laboratories Inc., Rockville, Maryland). The IgM was detected by direct haemagglutination of sheep erythrocytes. Each of the above serum protein markers was eluted in the same region as the corresponding human serum proteins, as detected by immunoelectrophoretic analysis of concentrated effluent pools.

Two further markers were run in order to extend the calibration of the column beyond the molecular size of HBsAg. Keyhole limpet haemocyanin (KLH) was obtained as an ammonium sulphate slurry (Calbiochem Inc., La Jolla, California). Before use, it was dialysed exhaustively against PBS at pH 7.2; in these conditions it would be expected to exist primarily in the associated form (Tornabene & Bartel, 1962). KLH was applied to the column at a concentration of approx. 1.25 μg and it was detected by its extinction at 280 nm. Bacteriophage φX 174 and its host strain of Escherichia coli were obtained from the American Type Culture Collection, Rockville, Maryland. The host was grown on Nutrient Agar (Difco Laboratories Inc., Detroit, Michigan) with 0.5% NaCl. Bacteriophage was grown and assayed by standard methods (Adams, 1959). Approx. 10⁵ p.f.u. of bacteriophage were applied to the column.

Markers, or appropriate mixtures of markers were applied to the column in a 1 ml volume. The column was run under gravity, with a pressure head of 50 cm of water, at 10 to 15 ml/h; 1.8 ml fractions were collected. The elution volume ($V_o$) was measured at the peak of concentration or activity for each marker, and the quantity $V_e/V_o$ was determined. Average values of $V_e/V_o$, obtained from at least two runs, are given in Table 1. There was no difference between elution volumes in separate experiments.

HBsAg was applied to the column as a pool of recalcified plasma obtained from voluntary blood donors positive for HBsAg by counterelectrophoresis. The antigen was detected in column effluents by radioimmunoassay (AusRIA II, Abbott Laboratories). HBsAg, purified by the method of Gerin et al. (1975) and 125I-labelled by the method of Hunter & Greenwood (1962) was obtained from Abbott Laboratories or from Dr N. Nath. Approx. 20000 cts/min of labelled antigen were applied to the column with the HBsAg positive pool. Labelled antigen was detected in column effluents by means of an automatic well-type scintillation.
Short communications

2.0

15

BSA

IgG

IgM

φX174

KLH

Stokes radius (nm)

Fig. 1. Calibration curves for Sepharose 4B (●—●) and Sepharose CL-4B (○—○). Arrows mark the \( V_e/V_o \) values for the elution peaks of: native HBsAg (N); re-chromatography of peak N after pepsin digestion (D); ultracentrifugally purified HBsAg (P₁); pepsin-Tween 80 purified HBsAg (P₂), and P₁ or P₂ after reconstitution with normal human serum (R).

spectrometer. The mean value of \( V_e/V_o \) for the unpurified HBsAg was 1.69 and that for the iodinated preparation was 1.64; both values are close to that for φX 174 and are within the range established for the markers.

The Stokes radius for each column marker was obtained from the literature, or was calculated from published physicochemical parameters (Table 1). \( V_e/V_o \) values were plotted against Stokes radii (Fig. 1) and a straight line could be closely fitted to the experimental points by the method of least squares (correlation index: 0.997). The Stokes radius of HBsAg was therefore determined by direct interpolation and was found to be 14.2 nm. This method of determination may be more reliable than that of Bourbonnais et al. (1975), who used markers of lower molecular size than HBsAg, calculating the Stokes radius indirectly, by the method of Ackers (1964) even though this may not be applicable to agarose gels.

In other studies, we chromatographed the same HBsAg preparation on polyacrylamide/agarose gel (Ultrogel AcA 22, LKB Instruments Inc., Rockville, Maryland) at pH 3.0. In these conditions, the elution volume of HBsAg could be compared with that of serum protein markers. HBsAg eluted later than IgM, and its behaviour was entirely compatible with that of a particle of Stokes radius 11 nm. When the same gel column was run at pH 7.2, HBsAg behaved as though its Stokes radius was about 14 nm (R. Y. Dodd, unpublished data).

Further confirmatory studies were all performed using Sepharose CL-4B (Pharmacia), a crosslinked agarose gel with properties similar to those of Sepharose 4B, at pH 7.2. The column was poured and calibrated as described above, but the bacteriophage was omitted. A straight line could be fitted to the experimental points with a correlation index of 0.995 (Fig. 1). On this column, the Stokes radius of unpurified HBsAg was 13.7 nm, confirming our previous observations. Mixtures of native and radiolabelled antigen were chromatographed and the HBsAg-containing peak was pooled, concentrated by pressure dialysis and digested with pepsin, using the conditions described by Bourbonnais et al. (1975).

Pepsin digestion resulted in a small, but reproducible shift in the Stokes radius of the
Short communications

HBsAg to 12.2 nm (D, Fig. 1); in addition, about 30% of the radioactivity was released as low mol. wt. fragments. These effects were not seen upon re-chromatography of control samples of the same fraction which were untreated, or exposed to acid in the absence of pepsin.

Purified HBsAg, provided by Dr J. Gerin, or 125I-labelled, purified HBsAg preparations, obtained from Dr N. Nath, were chromatographed in the absence of any added serum. Approx. 60% of the applied label and a significant proportion of the unlabelled antigen were retained on the column. The remaining isotopic and antigenic activity emerged at an elution volume compatible with a Stokes radius of 5.8 nm for ultracentrifugally purified HBsAg (Gerin et al. 1975; P1, Fig. 1) and 8 nm for an antigen purified by a pepsin-Tween 80 method (Nath et al. 1976; P2, Fig. 1).

Because purified HBsAg is bound to the gel medium, these values do not necessarily reflect the true Stokes radius of these HBsAg preparations. However, when these same preparations were chromatographed in the presence of normal human serum, 100% of the activity was recovered in a single peak with an elution volume equivalent to a Stokes radius of 14.2 nm (R, Fig. 1). The effect was exactly the same when the serum was heated to 56 °C for 30 min before the addition of purified antigen.

Analytical gel filtration clearly shows that HBsAg has an effective, or Stokes, radius of about 14 nm in the presence of serum. This value is significantly larger than the value of 10 to 11 nm which would be expected on the basis of visual observation and physicochemical studies of purified HBsAg (Bourbonnais et al. 1975). We suggest that this size increment is due to the adsorption of some serum component, or components, by the HBsAg. The adsorbed material is released at pH 3 and is susceptible to the digestive action of pepsin. The removal of this material by the purification procedures of Nath et al. (1975) or Gerin et al. (1975) results in a profound change in the gel filtration behaviour of HBsAg. Reconstitution of such preparations with normal human serum results in the recovery of the 14.2 nm Stokes radius which is characteristic of the native antigen. It is possible that this could be due to reassembly of a subunit structure, as suggested by Cossart (1972), but electron microscopic studies of purified HBsAg (Kim & Tilles, 1973; Chairez et al. 1975; Gerin et al. 1975), do not support this concept. Recent work by Hirschman (1976) has shown that exogenous RNA promotes the self-assembly of purified HBsAg into larger, more elongated forms, an observation strikingly analogous to those reported here.

We do not suggest that our results account for observations relating to the presence of host components in purified HBsAg (Neurath, Prince & Lippin, 1974; Burrell, 1975); presumably a more intimate relationship between HBsAg and host determinants was detected in those studies. Our findings, however, indicate two factors which may affect the ease of purification of HBsAg. Firstly, there is an affinity between HBsAg and unidentified serum factors and secondly, the Stokes radius of native HBsAg is such that the antigen cannot be separated from IgM by methods which depend upon molecular size.

We are unable to suggest the identity of the HBsAg-binding component, or components, present in human serum. Neither antibody to HBsAg nor complement components are involved, since the effect is seen with heated, normal serum which has no detectable antibodies to HBsAg. Even though the size relationships which we have described are clear, it is not possible to estimate the mol. wt. of the binding components, since particles of widely differing shapes may have the same Stokes radius.

It is clear that HBsAg must circulate in the body in an associated form which is rather different from that which has been described for pure HBsAg. This association between HBsAg and serum components may be responsible for the rather benign relationship
between the antigen and the immune response of the carrier. Adsorption of host components could block, or modulate the expression of HBsAg determinants or promote their recognition as a self antigen.

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American National Red Cross
Blood Research Laboratory
9312 Old Georgetown Road
Bethesda, Maryland 20014, U.S.A.

REFERENCES


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