A STUDY OF STRUCTURAL AND ANTIGENIC COMPONENTS
OF HEPATITIS B SURFACE ANTIGEN

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This thesis is dedicated to my late husband, John Mathieson Kellock Mackay, whose sudden and untimely death robbed him of the opportunity of seeing this work completed.
Declaration

The following aspects of the experimental work reported in this thesis were carried out with the assistance of others:

(1) Purification and radiolabelling of HB\textsubscript{Ag} was on occasion carried out by staff of the Hepatitis Reference Laboratory.

(2) The initial observation of particle disruption following treatment with detergent and enzymes was made by myself, subsequent characterization of this material was performed by Dr. C.J. Burrell.

(3) The preparation of monospecific anti-HB\textsubscript{Ag} subtype d antiserum was performed by staff of the Hepatitis Reference Laboratory.

Composition of this thesis, and all other work described therein was performed by myself.
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Abstract

Although the spread of hepatitis B infection has largely been controlled through the use of sophisticated serological tests for the detection of hepatitis B surface antigen there is still comparatively little basic information available on the protein, lipid and carbohydrate composition of these particles, and on the arrangement of these components into a stable quaternary structure. In the present work three separate approaches have been made to extending this information.

1. Polypeptide analysis of purified $\text{HB}_s\text{Ag}$ particles by column chromatography and discontinuous or continuous polyacrylamide gel electrophoresis (PAGE) produced discrepant polypeptide profiles; in the latter system reaggregation of protein constituted a major problem, and with all techniques incomplete dissociation and/or reaggregation during handling and storage of antigen were noted. In the discontinuous PAGE system one major polypeptide of 14,000 daltons or less and one minor polypeptide of 60-70,000 daltons were consistently detected in both unlabelled and $^{125}$I labelled $\text{HB}_s\text{Ag}$. However, major limitations in the use of standard PAGE techniques for the analysis of $\text{HB}_s\text{Ag}$ polypeptides were clearly documented, which may contribute to the present confused situation in the literature relating to the number, molecular weight and immunogenicity of these components.

2. 22nm $\text{HB}_s\text{Ag}$ particles were treated with a range of denaturing agents including nonionic or anionic detergents, reducing agent, or high concentrations of urea or guanidine hydrochloride. The
particle displayed considerable resistance to disruption under strong denaturing conditions, in one instance only incomplete dissociation to a stable subunit of 25S being achieved after boiling with sodium dodecyl sulphate and reducing agent. However, prolonged incubation with high concentrations of Triton X-100 released a polypeptide of 70-80,000 daltons which retained both group specific and subtype specific activity when analysed by double antibody radioimmunoprecipitation using monospecific antisera.

3. Examination of HBSAg particles by electron microscopy following treatment with denaturing agents revealed a number of stages in the breakdown of these structures. The smallest detectable morphological structures were ring shaped units of 4-6nm diameter; a model for the assembly of these units into the complete 22nm HBSAg particle is proposed.
1.1. Summary

The following pages contain a description of the biophysical and biochemical properties of hepatitis B antigen including the evidence for its possible relationship to the hepatitis B virion. Two striking features of hepatitis B antigen, its serological and morphological stability to a variety of denaturing conditions are then summarised. The various published reports on the polypeptide composition of antigen particles are discussed in detail, together with the present state of attempts to produce antigenic and immunogenic subunits of the 22nm particle. Finally, the information on particle structure that has been obtained by electron microscopic examination of both serum samples and thin sections of liver from hepatitis patients is presented, and the various structural models that have been proposed for the 22nm particle are described.
1.2. **Historical background**

Hepatitis B antigen was discovered in the early 1960s by Blumberg and coworkers during an investigation of human serum protein polymorphisms. These polymorphisms, a complex series of genetically determined antigenic specificities on the low density serum lipoproteins, were detected by immunodiffusion using sera from multiply transfused haemophiliac patients as a source of antibody (Blumberg, Dray & Robinson, 1962). Shortly afterwards a further precipitin system was described involving an antigen found in the serum of an Australian aborigine, hence the original terminology Australia antigen (Blumberg, 1964). The occurrence of this antigen in high frequency in certain populations was first explained on a simple genetic basis, but this hypothesis was later modified by the demonstration of its association with some forms of leukemia (Blumberg, Alter & Vienich, 1965) and with institutionalised patients with Downs syndrome (Blumberg et al., 1967). A chance observation on one of the latter patients who developed this antigen during the course of investigation and subsequently manifested clinical symptoms of hepatitis, first suggested a relationship between the antigen and one of the causative agents of viral hepatitis (Blumberg et al., 1967). The temporal association between the development of hepatitis B antigen and onset of clinical hepatitis B (serum hepatitis) was rapidly confirmed by other workers (Prince, 1968; Giles et al., 1969).
At about this time examination of antigen positive sera by electron microscopy revealed the presence of spherical structures of 19-22nm diameter, and filaments of a similar width and up to 230nm in length, which resembled small virus particles (Bayer, Blumberg & Werner, 1968; Almeida et al., 1969; Almeida & Waterson, 1969). A third, less common, particle of 42nm diameter with a 27nm internal core was then described by Dane and coworkers (Dane, Cameron & Briggs, 1970); all three types of particle were clumped together by specific antibody, suggesting the presence of a common antigen on their surface. It was postulated that this latter particle, which became known as the Dane particle, might represent the true hepatitis virus and that the smaller spherical and tubular forms represented accumulations of excess viral coat material. This interpretation was strengthened by the subsequent demonstration of DNA directed DNA polymerase activity and double stranded DNA within the Dane particle (Kaplan et al., 1973; Robinson, Clayton & Greenman, 1974).

Finally, examination by electron microscopy of thin sections of liver from hepatitis patients revealed spherical intranuclear particles of 27nm diameter (Nowasowski et al., 1970; Nelson, Barker & Danovitch, 1970) which have since been shown to be immunologically indistinguishable from the core of the Dane particle (Huang & Groh, 1973; Barker et al., 1974).
1.3. **Subtypes and nomenclature**

All three particulate structures described above share a common antigenic determinant designated hepatitis B surface antigen, or \( \text{HB}_s \text{Ag} \), while the core of the Dane particle carries a different specificity, hepatitis B core antigen, \( \text{HB}_c \text{Ag} \) (Almeida, Rubenstein & Stott, 1971). \( \text{HB}_s \text{Ag} \) reactivity has been subdivided into a group specific determinant \( a \), common to all particles, and two pairs of mutually exclusive subdeterminants, \( d/y \) and \( x/w \) (Kim & Tilles, 1971; Le Bouvier, 1971; Bancroft, Mundon & Russell, 1972). Thus four primary phenotypes are found, \( adw, adr, ayw \) and \( ayr \). Since each of these phenotypes remain the same during acute or chronic infection in an individual and "breed true" when transmitted to a susceptible host it is likely that they are coded for by distinct viral genotypes (Holland, 1975).

Additional antigenic determinants include \( e, \bar{e}, h, t, \bar{a} \) and subdivisions of the group determinant \( a \) (Soulier & Courouce-Pauty, 1973). None of these specificities has yet been unequivocally shown to represent a virus coded product and as yet their relationship to the various particulate structures found in \( \text{HB}_s \text{Ag} \) positive sera is not known (Le Bouvier & Williams, 1975). Finally, Magnus & Espmark (1972) described e-antigen, an antigenic complex which appears to be specific for hepatitis B infection and is found both free in serum (Magnus, 1975) and on the surface of filaments and Dane particles (Neurath et al., 1976). Further characterisation of this antigen has not been reported.
1.4. Biophysical and biochemical properties of HBAg

1.4.1. Purification.

Since 22nm spherical particles are the most common morphological forms found in HBAg positive sera most biophysical and biochemical studies have been made on purified preparations of these particles. Purification has usually involved equilibrium and rate zonal centrifugation (Gerin et al., 1969) combined with enzyme treatment (Millman et al., 1970; Dreesman et al., 1972), column chromatography (Gerlich & May, 1973; Burrell et al., 1973), or precipitation with polyethylene glycol (De Rizzo et al., 1972) or ammonium sulphate (Booth et al., 1974). Various workers have however suggested that prolonged exposure to caesium salts during centrifugation caused a marked fall in antigen titre.

Alternative methods of purification involving attachment to concanavalin A (Neurath, Prince & Lippin, 1973a), immunoadsorption (Houwen et al., 1973; Grabow & Prozesky, 1973) or electrophoresis (Luzzio, 1975) have therefore been developed more recently.

1.4.2. Biophysical properties.

Purified 22nm particles of molecular weight $2.5 \times 10^6 - 3.7 \times 10^6$ daltons for subtype ad and $3.0 \times 10^6 - 4.6 \times 10^6$ daltons for subtype ay, calculated using the Yphantis technique (Dreesman et al., 1972; Chairez et al., 1975a; Bourbonnais et al., 1975), banded in equilibrium gradients at a density of $1.21 \text{ gm/ml}$ in caesium chloride (Gerin et al., 1969; Dreesman et al., 1972; Kim & Tilles, 1973), $1.16-1.17 \text{ gm/ml}$
in sucrose and 1.15-1.17 g/ml in potassium tartrate (Gerin et al., 1969; Dreesman et al., 1972). Initial reports of a very high sedimentation coefficient of 86S-120S (Gerin et al., 1969; Prince et al., 1971; Gerin, 1972) were probably due to measurement of either aggregates of particles or antigen-antibody complexes (Gerin et al., 1969) and more recent estimates of 32S-48S are more likely to be correct (Schober et al., 1971; Tayst et al., 1972; Kim & Tilles, 1973). In parallel with molecular weight findings, the ay subtype was found to have a significantly higher sedimentation coefficient (40S) than the ad subtype (33S) (Bourbonnais et al., 1975).

Values for the diffusion coefficient have varied from $1.94 \times 10^{-7}$ cm$^2$ sec$^{-1}$ calculated from the Stokes radius of 11nm (Bourbonnais et al., 1975) to $2.278 \times 10^{-7}$ cm$^2$ sec$^{-1}$ determined by analytical centrifugation (Kim & Tilles, 1973). Measurements of optical rotatory dispersion (O.R.D.) and circular dichroism (C.D.) spectra suggested that the particle had a very high α helical content in the region of 70-80%, much higher than that found in other, more typical, virus particles (Sukeno et al., 1972b). The 22nm particle has also been found to have a very high extinction coefficient (E$^0.1\%$) of 3.726 (Vyas et al., 1972; Dreesman et al., 1972) which has subsequently been attributed to the high percentage of tryptophan in the protein component of the particle (Rao & Vyas, 1974).

The isoelectric point at which particles focussed in a
The pH gradient was found to be dependent on the source, subtype and state of purification of the antigen. Unpurified antigen banded at a pH of 4.3 to 4.7 (Dreesman et al., 1972; Chairez et al., 1975) although it has been reported that spread through the entire pH range of the gradient could occur due to association of HBsAg with human serum proteins (Howard & Zuckerman, 1974). Purified HBsAg banded as major peaks at a pH of 3.9 and 4.9 for subtype ay and 3.9 or 4.5 for subtype ad, but several other minor peaks covering a pH range from 3.9 to 5.3 were also detected (Chairez et al., 1975). In one report of focusing of 125I labelled ay antigen, two discrete peaks were found at pH values of 4.7 and 4.9 (Howard & Zuckerman, 1974), but Chairez and coworkers (1975) have found that the pH of iodinated antigen (3.9-4.4 for ad; 3.9 for ay) was significantly less than that of the major non-iodinated species, and they suggested that selective labelling of particles with a more acidic pH had occurred.

1.4.3. Biochemical properties.

The original reports of the staining reactions of precipitin lines containing HBsAg indicated the presence of protein and lipid within the particle (Blumberg, 1964; Alter & Blumberg, 1966). The protein component has been estimated to constitute 70%, and the lipid component 25%, of the total particle weight (Jozwiak et al., 1971; Takahashi, 1975).

a. Protein.

The ultra-violet spectrum of antigen had a maximum at
280nm and a minimum at 250nm typical of a protein (Gerin, 1972) and also exhibited a shoulder at 285nm indicative of a high tryptophan content (Gerin, Holland & Purcell, 1972; Gerlich & May, 1973). In amino acid analyses of total protein Dreesman and coworkers (1972) demonstrated relatively large amounts of proline, leucine and serine, and lower levels of both acidic and basic amino acids than various other viruses, including polio, herpes and adenovirus 2. The high tryptophan content of 15-25% suggested by both the u-v spectrum and the high extinction coefficient has since been confirmed by direct amino acid analysis (Rao & Vyas, 1974a).

b. Carbohydrate.

The presence of carbohydrate in the 22nm particle was originally suggested by its reaction with concanavalin A (Cawley, 1972; Neurath et al., 1973a) and later confirmed by chemical analysis (Burrell et al., 1973; Chairez et al., 1973). Carbohydrate residues, which have been estimated to make up 3.6-6.5% of the total particle weight, have been found covalently attached to protein in the form of glycopeptides (Chairez et al., 1973; Gerin, Shih & Kaplan, 1975; Neurath, Hashimoto & Prince, 1975b), and to lipid in the form of non-sialic acid containing glycosphingolipids (Steirer, Huebner & Dreesman, 1974). The only individual carbohydrate residue identified to date has been sialic acid (Neurath et al., 1975b).
c. **Lipid.**

Lipid analyses have demonstrated the presence of two major lipid components, phosphatidylcholine (65%) and sphingomyelin (30%) (Kim & Bissell, 1971; Steiner et al., 1974). Minor amounts of phosphatidylethanolamine and cholesterol were also detected by Kim & Bissell (1971) but, using a more complete solvent system and more specific reagents for detection, Steiner and coworkers (1974) found minor amounts of lysophosphatidylcholine, and no phosphatidylethanolamine or cholesterol. The latter authors also detected two non-sialic acid containing glycosphingolipids which showed some similarities to the blood group lipids.

d. **Host components.**

A close association between purified HB$_s$Ag and various components of normal human serum has been observed by a number of workers. In the original report Millman and coworkers (1971) found no reaction by immunodiffusion between native purified antigen and antisera to various human serum components. However incubation with the nonionic detergent Tween 80 caused a loss of HB$_s$Ag reactivity, and an unmasking of determinants reacting with antisera to heavy and light chain IgG, C$_3$ component of complement, β lipoprotein, transferrin and albumin. Clumping of particles by anti-IgG antisera, monitored by immune electron microscopy, has been detected following treatment of HB$_s$Ag with the detergent Mucosal, or exposure to caesium chloride or glycerol (Stannard & Moodie,
1976); surface antigen activity was not destroyed by these treatments. A reaction between native purified HB$_5$Ag and antisera to lactoferrin, $\beta$ lipoprotein, $\gamma$ glycoprotein, albumin, prealbumin, apolipoprotein C, apolipoprotein D, the $\delta$ chain of IgG, thyroxine and triiodothyroxine has been detected by reverse passive haemagglutination (RHIA) (Vierucci, London & Blumberg, 1974), and by immunoadsorption to Sepharose bound antibodies (Neurath et al., 1974; 1975a).

In the latter experiments HB$_5$Ag treated with Tween 80 plus diethyl ether, Tween 80 plus tri(n-butyl) phosphate, Nonidet P40, 8M-urea, 5M-potassium iodide, or pH 2.2 retained its ability to bind to the immunoabsorbant columns suggesting that these serum components played an integral role in particle structure.

Goudeau and coworkers (1974) have however questioned the validity of the above immunoadsorption results since non-specific adsorption reactions were likely to be significant at the low protein concentrations used by Neurath and coworkers. No binding of HB$_5$Ag to insolubilised anti-normal human serum (NHS) was demonstrated in 0.5M NaCl (Goudeau et al., 1974) and no evidence was found for binding of antigen to a range of different anti-NHS components by solid phase radioimmunoassay (Schuur & Walters, 1975) or immunofluorescence (Houwen et al., 1973). $^{125}$I-labelled HB$_5$Ag showed low affinity immunoprecipitation reactions with antisera to albumin, $a_1$ lipoprotein, $\beta$ lipoprotein and IgG by double antibody radioimmunoprecipitation. The low affinity of combination with these antisera led to the
conclusion that these serum proteins were unlikely to be essential to the basic structure of the 22nm particle (Burrell, 1975).

No reaction was detected by immunodiffusion between HB Ag and rabbit antisera to adult human liver cell antigens (Burrell, 1975), but fluorescein labelled human and animal anti-HB sera were found to stain the cells lining the collecting tubes, calyces, ureter and bladder of human foetal kidney (Kistler & Sonnabend, 1974). No blocking tests were however performed in this immunofluorescence study, and a similar staining reaction was found with sera from 14 out of 100 anti-HB negative blood donors and all of 15 anti-HB negative patients with carcinoma of the kidney. A second reaction, between anti-HB and the sera of HB Ag negative pregnant women, was also detected, but no identity with HB Ag was demonstrated (Kistler & Sonnabend, 1974).

1.5. Nucleic acid and Nucleic acid polymerases

1.5.1. Ribonucleic acid.

The presence of small amounts of RNA in purified preparations of 22nm HB Ag particles has been reported in a few instances (Jozwiak et al., 1971; Kim, 1971; Sutnick et al., 1972). This RNA constituted 5% of the total particle weight and when extracted from the particle had a sedimentation coefficient of 9S (Jozwiak et al., 1971; Jozwiak & Koscielak, 1973). Preliminary analysis of base composition suggested the presence of uridine, guanosine, cytosine and adenosine phosphates in the molar ratios 1.3:1.0:1.2:1.1 (Jozwiak et al., 1971). Internal localisation of the RNA within a lipoprotein
coat was demonstrated by conversion from RNAase resistance to RNAase sensitivity following extraction with butanol or treatment with 2-mercaptoethanol (Jozwiak et al., 1971; Brzoako, Mantyjarvi & Nadalinski, 1972).

An unusual attempt to demonstrate the association of RNA with 22nm particles involved injection of $^3$H-uridine into a chimpanzee carrier of HB$_s$Ag, followed by serial blood sampling at 4 hour intervals (Jozwiak et al., 1975). Association of $^3$H-uridine with HB$_s$Ag was monitored by purification of antigen by precipitation with specific chimpanzee anti-HB$_s$ and radioactive counting. At the time of peak detection of radiolabel the nucleic acid was released from washed antigen-antibody complexes by phenol treatment and detected by absorption at 260nm. Column chromatography following hydrolysis of RNA with potassium hydroxide revealed the presence of all four ribonucleic acid bases. In vivo incorporation of $^3$H-uridine into HB$_s$Ag specific RNA was therefore postulated on the basis of these observations.

The fact that RNA was not consistently observed in association with all purified 22nm preparations has however been noted (Jozwiak et al., 1971). This observation was linked with the demonstration that many 22nm particles appeared empty in the electron microscope and hence may have represented defective structures containing either an incomplete RNA molecule, or no nucleic acid at all (Jozwiak et al., 1971; Jozwiak & Koscielak, 1973). Conversely, the demonstration of spontaneous in vitro production of filaments following incubation of 22nm spheres with heterologous RNA has led Hirschman (1976) to suggest that the association of
nucleic acid with purified 22nm particles described by other workers may have represented a nonspecific attachment of host cell RNA.

1.5.2. Deoxyribonucleic acid.

Double stranded circular DNA of mean length 0.78 ± 0.09 µm corresponding to a molecular weight of approximately 1.6 x 10^6 daltons has been detected as an internal component of the serum Dane particle (Robinson et al., 1974). Buoyant density and thermal transition studies using ^3H-DNA revealed a G + C content of 48-49%. The internal localisation of this DNA molecule was demonstrated by its cosedimentation with core particles at 110S in sucrose density gradients and by its resistance to DNAase treatment of cores (Robinson & Greenman, 1974). When released from the core by incubation with sodium dodecyl sulphate (SDS) the DNA sedimented at 15S (Kaplan et al., 1975). DNA extracted from Dane particles and characterized by restriction endonuclease cleavage and gel electrophoresis consisted of a double-stranded circle of approximately 3,600 nucleotides containing a single-stranded gap of 600-2,100 nucleotides (Summers, O'Connell & Millman, 1975). Similarly, examination of DNA by electron microscopy revealed open circular molecules with attached linear segments. These linear segments increased in number following an endogenous polymerase reaction and hence were thought to be examples of a rolling circle method of DNA replication (Overby et al., 1975).
In contrast to the results obtained with Dane particles from serum, disruption of core particles isolated from the nuclei of infected human hepatocytes released linear double stranded DNA molecules (Hirschman, Gerber & Garfinkel, 1974b). These linear molecules were seen even when nuclease inhibitors such as N-ethyl-maleimide were included during purification of the core particles. 40% of this DNA had a G + C content of 68%, and 60% of the DNA had a G + C content of 50%, the total molecular weight of the nucleic acid being estimated as $2.3 \times 10^6$ (Hirschman et al., 1974b; Hirschman, 1975). The intranuclear particle thus apparently contained 40% more DNA of high G + C content than the serum particle, although the experimental data to support the finding of linear DNA strands as large as $2.3 \times 10^6$ daltons have not been presented.

Hirschman (1975) has proposed that this extra DNA may represent host nucleic acid associated with the viral DNA as a result of integration of hepatitis B virus into the host cell genome during replication. This hypothesis is however inconsistent with the observation that the average G + C content of mammalian cell DNA lies between 40 and 44% (Fenner et al., 1974).

1.5.3. DNA polymerase.

DNA dependant DNA polymerase activity was first detected in association with a heavy subpopulation of Dane particles in serum. Localisation within the core particle was demonstrated by precipitation of polymerase activity by anti-HBs before detergent treatment, and by anti-HBc after treatment of Dane particles with NP40 to remove the outer coat of the particle (Kaplan et al., 1973; Robinson & Greenspan, 1974). Dependance of the enzyme on DNA as a template was
suggested by inhibition of the reaction by actinomycin D and daunomycin; however, formal confirmation of this by pretreatment with nucleases has not been possible, owing to the apparent inaccessibility of the template within the intact core (Kaplan et al., 1973). These findings all suggested that this enzyme was different from the activity originally described in pelleted HBsAg-containing sera by Hirschman and coworkers (1971) which was thought to be an RNA dependant DNA polymerase due to its sensitivity to treatment with high concentrations of RNAase.

Dane particles in serum were separated by equilibrium gradient centrifugation into two distinct populations. Particles banding at a density of 1.20 gm/ml in caesium chloride contained cores of density 1.30 gm/ml and no endogenous polymerase while heavy Dane particles, which banded at a density of 1.22 gm/ml, contained cores of 1.36 gm/ml and endogenous polymerase activity (Kaplan et al., 1976). Examination by electron microscopy of particles from these two subpopulations, after positive staining with uranyl acetate, demonstrated that heavy particles contained relatively larger amounts of nucleic acid than light particles (Gerin, Ford & Purcell, 1975; Kaplan et al., 1976). The absence of endogenous polymerase activity therefore correlated directly with core particles of lighter buoyant density and an apparently reduced DNA content.

In contrast to the results with core particles from serum, no endogenous polymerase activity was found in association with
core particles purified from human liver. The latter did however show low levels of exogenous activity using activated DNA or poly [d(A-T)n] as a template (Hirschman, Gerber & Garfinkel, 1974a). Further observations on both Dane particle and core preparations purified from chimpanzee liver have extended these findings and led to the description of two distinct DNA polymerase activities associated with the Dane particle from liver (Bradley, Fields & Maynard, 1976).

Purified, liver-derived Dane particles, identified by immune electron microscopy, banded at a density of 1.20-1.25 gm/ml in caesium chloride and contained endogenous polymerase activity. This reaction required all four deoxynucleoside 5'-triphosphates for maximal activity and synthesised a DNA-like product which was insensitive to DNAase treatment due to its internal localisation within the particle. Exogenous polymerase activity, which required addition of the template poly [d(A-T)n], banded at a density of 1.34 gm/ml characteristic of the heavy subpopulation of Dane particle cores. The exogenous reaction synthesised a product which was DNAase sensitive and, by definition, was unaffected by the removal of deoxycytosine 5'-triphosphate and deoxyguanosine 5'-triphosphate from the reaction mixture (Bradley et al., 1976). This exogenous polymerase activity therefore corresponds to that detected in core particle from liver by Hirschman and coworkers (1974a).

The properties of the exogenous polymerase enzyme make it unlikely that it alone is responsible for the synthesis of the hepatitis B virus genome (Bradley et al., 1976). It has been suggested that this enzyme activity may be a host transferase enzyme nonspecifically adsorbed to the core particle,
or that it may represent an incomplete form of the endogenous DNA polymerase activity found in core particles purified from serum and first described by Kaplan and coworkers (Bradley et al., 1976). On the other hand the endogenous polymerase activity found in liver Dane particles and serum cores shares similar properties which appear to fulfil the requirements of a bona fide viral polymerase (Bradley et al., 1976). Summers and coworkers (1975) have however suggested that the endogenous polymerase activity found in the cores of Dane particles from serum was responsible for the repair of the single-stranded gap in the double-stranded circular DNA molecule, and not for complete replication of the genome.

1.6. Stability of serological activity

Until tissue culture techniques for the growth of hepatitis B virus are developed the serological specificity shared by all three particulate structures, namely hepatitis B surface antigen (HBsAg) is the only easily measurable marker of biological activity. The effects of a wide range of denaturing conditions on the serological activity and immunogenicity of this antigen have therefore been investigated.

Surface antigen activity, detected by immunodiffusion, has been found to be stable to: storage at -10°C or -20°C for a year or longer (Millman et al., 1970); 40 cycles of freeze/thawing, or putrefaction at room temperature for 4 weeks (Kim & Bissell, 1971); pH 9-10 for 3 hours at 37°C, pH 2.7 at room
temperature or pH 4-5 at 37°C for 3 hours (Gerin et al., 1969; Kim & Bissell, 1971); heating at 56°C overnight or 80°C for 60 minutes (Millman et al., 1971; Brzosko et al., 1972). Heating at 85°C or 100°C for 60 minutes destroyed antigenic activity (Millman et al., 1971). Incubation at 37°C for up to 3 hours with the enzymes amylase, wheat germ or pancreatic lipase, phospholipase C, trypsin, pepsin, pronase, chymotrypsin, lysozyme, RNAase or DNAase had no effect on serological activity (Millman et al., 1970; 1971; Kim & Bissell, 1971; Cherchel, personal communication, 1973). Only three enzymes, subtilisin or subtilopeptidase A at 37°C for 3 hours or neuraminidase, at 37°C for 6 hours, caused any marked loss in antigen titre as measured by immunodiffusion (Kim & Bissell, 1971; Cherchel, 1973). Treatment of antigen with 1% SDS before enzyme incubation increased its sensitivity to a number of enzymes to which it was previously resistant (Kim & Bissell, 1971). Incubation with SDS alone caused an apparent fall in antigenic titre which was probably due to particle aggregation (Kim & Bissell, 1971). Fluorocarbon, diethyl ether and chloroform/methanol all had no effect on antigenicity, although the latter mixture did cause aggregation (Barker et al., 1969; Gerin et al., 1969; Kim & Bissell, 1971), whereas butanol extraction, under conditions which completely delipidated cell membranes, did bring about a loss of serological activity (Jozwiak et al., 1971).
Treatment with a range of chemical denaturing agents including 8M-urea, 5M-guanidine hydrochloride (GuHCl), 5% 2-mercaptoethanol, iodoacetamide, or guanidination with O-methylisourea had no effect on antigen titre as measured by immunodiffusion (I.D.), haemagglutination inhibition (HAI) or counter immunoelectrophoresis (CIE) (Kim & Bissell, 1971; Sukeno et al., 1972a; Vyas, Rao & Ibrahim, 1972; Rao & Vyas, 1974b). Incubation with the reducing agent dithiothreitol (DTT) caused a small loss of antigenic activity detectable by radioimmunoassay (RIA), but not by the less sensitive HAI technique (Rao & Vyas, 1974b). Reduction in the presence of 8M-urea or reduction followed by alkylation with iodoacetamide caused a complete loss of activity as measured by CIE or HAI (Sukeno et al., 1972a; Vyas et al., 1972), but only a 70-80% loss by RIA (Rao & Vyas, 1974b). Imai and coworkers (1974) have described the existence of two antigenic components on the antigen particle which differed in their sensitivity to reduction and alkylation, but it was not possible to correlate either of these components with any of the surface antigen group or subtype determinants. Reduced, alkylated antigen injected into guinea pigs induced both cell mediated immunity (Vyas et al., 1972) and low levels of humoral antibody detectable by RIA (Dreesman et al., 1973). Reoxidation of reduced non-alkylated antigen was variously reported to lead to recovery of between 6% and 80% of total antigen titre detected by RIA or CIE (Sukeno et al., 1972a; Dreesman et al., 1973; Rao & Vyas, 1974b).
The above results on the effect of reducing agents on serological activity suggested that the surface antigen determinant was a conformational antigen dependant on disulphide bonds (Vyas et al., 1972). However the finding that antigenic activity was undetectable by HAI after treatment with a combination of 8M-urea and 5M-GuHCl, or after succinylation, demonstrated that non-covalent bonds also played a role in the structure of the antigenic determinant (Rao & Vyas, 1974b). Similarly reduced, reoxidised antigen was found to have an optical density spectrum like that of reduced, alkylated antigen and typical of a denatured protein, indicating that re-folding of the protein to the native configuration was not essential for regain of serological activity (Rao & Vyas, 1974b). The latter workers therefore suggested that the loss of activity following reduction was due to masking of antibody binding sites by structural alterations in more distant areas of the polypeptide carrying the antigenic determinant.

The loss of antigenic titre following incubation with neuraminidase (Cherchel, 1973) or oxidation by periodate (Burrell et al., 1973) suggested that carbohydrate may also be involved in the antigenic determinant. In more recent work neuraminidase treatment was shown to increase the immunogenicity of purified HBsAg in rabbits, lead to a higher level of antibody production, induce a cell mediated immune response, and facilitate the clearance of antigen from the circulation (Neurath et al., 1975b).
1.7. **Morphological stability**

One of the most striking features of the 22nm HBsAg particle is its marked resistance to morphological breakdown under a wide variety of physical and chemical denaturing conditions. Disruption of particulate structure can be measured by direct visualisation in the electron microscope or by physical methods such as gradient centrifugation or column chromatography, each of which will be discussed in turn below.

Storage of sera in a frozen state for up to 30 years, heating at 100°C for 60 min or treatment with chloroform, ether or 1% SDS had no effect on the electron microscope appearance of the 22nm particle (Millman *et al.*, 1970; MacCallum, 1972), although the latter two agents were reported to cause aggregation (Gocke *et al.*, 1969; Kim & Tilles, 1973). Similarly incubation with a variety of enzymes including bromelain, amylase, neuraminidase, lipase or phospholipase C had no effect on gross particle structure (Millman *et al.*, 1970; Hirschman *et al.*, 1973). Trypsin and pronase were originally thought to have no effect on structure (Millman *et al.*, 1970) but Hirschman and coworkers (1973) reported particle destruction and granularity on electron microscope grids following such treatment. The latter workers also observed unwinding of large 40-70nm forms following incubation of partially purified antigen with chymotrypsin, but it is difficult to judge if these were originally derived from 22nm particles. Treatment
of purified HBsAg with 1% Tween 80, a non-ionic detergent, decreased the staining intensity of the small particle (Millman et al., 1971) while incubation of unpurified antigen either increased the density of the centre of the particle (Almeida et al., 1971; Hollos et al., 1973) or caused complete particle destruction (Traavik, Kjeldsberg & Siebke, 1973). Traavik and coworkers have suggested that the amount of lipid present in unpurified antigen preparations may have accounted for these differences in the degree of particle disruption by detergent treatment.

Treatment of antigen with low concentrations of DTT in the presence of 8M-urea, followed by alkylation with iodoacetamide altered particle morphology (Dreesman et al., 1973), while incubation with higher concentrations of reducing agent caused complete particle breakdown (Hirschman et al., 1973). Re-oxidation of antigen reduced in 8M-urea was reported to produce particles of 18nm diameter through reconstitution of previously disaggregated 22nm particles (Sukeno et al., 1972a), but no direct evidence of this was presented. There is one report of the production of particles of 8-10nm diameter following treatment of purified 22nm particles with low concentrations of the reducing agent 2-mercaptoethanol (Brzosko et al., 1972). These small particles had a buoyant density of 1.39gm/ml in caesium chloride and were RNAase sensitive and DNAase resistant. Higher concentrations of mercaptoethanol caused complete particle breakdown.
Succinylation (Rao & Vyas, 1974b) or reduction and alkylation of purified antigen followed by column chromatography in 1M-acetic acid (Vyas et al., 1972) or 8M-urea (Dreesman et al., 1973) did not cause any particle dissociation. Similarly incubation with 8M-urea, SDS, DTT, or iodoacetamide had no effect on the circular dichroism or optical rotatory dispersion spectra of purified antigen indicating that no gross alteration in secondary structure had occurred (Sukeno et al., 1972b). Treatment with a mixture of Nonidet P40, urea and mercaptoethanol was reported to cause an increase in the isoelectric point, thought to be due to the release of non-protein components from the particle (Howard & Zuckerman, 1974). This was said to be paralleled by a significant increase in sedimentation coefficient due to loss of lipid and an alteration in the secondary structure of the particle, but no experimental data were given.

Three procedures, all of which involve cleavage of disulphide bonds, were found to dissociate 22nm particles into smaller molecular weight components which have been isolated and characterised to varying extents. Firstly, reduction in the presence of 8M-urea followed by incubation with 0.3M-HCl and separation by sucrose gradient centrifugation and column chromatography released a small component of 4,000-10,000 daltons (Dreesman et al., 1973). Secondly, ultrasonication of antigen in 8M-urea and mercaptoethanol, followed by column chromatography revealed three peaks of molecular weight 80,000, 12,000 and 6,000 daltons, the 12,000 dalton
peak being thought to represent a dimer of the smaller peak (Rao & Vyas, 1973). Finally, heating antigen in the presence of SDS and reducing agent, a standard procedure for disruption of virus particles for polyacrylamide gel electrophoresis (Maizel, 1969) brought about dissociation into individual polypeptides.

1.8. Polypeptide composition of HB Ag

The polypeptide composition of the 22nm particle has been extensively investigated by polyacrylamide gel electrophoresis (PAGE), principally by two groups of workers using different gel systems. Dreesman and coworkers used 10% discontinuous gels (Laemmli, 1970) in the presence of urea while Gerin and coworkers favoured the continuous buffer system (Maizel, 1969) with either 5% or 10% gels containing urea. Prior to electrophoresis both groups disrupted antigen by heating for 10 min at 60°C in 1% SDS, 1% DTT and 6M-urea.

By Coomassie Brilliant Blue staining Gerin and coworkers originally detected two major polypeptides of 32,000 and 26,000 daltons and one minor polypeptide of 40,000 daltons in antigen of subtype ad purified from the serum of a chronic anicteric hepatitis patient, J.M. (Gerin et al., 1971). Later reports described three additional higher molecular weight peaks of 95,000, 75,000 and 55,000 daltons in the antigen from the same serum following separation in 10% acrylamide gels (Gerin, 1972). More recently electrophoresis in 5% acrylamide gels, said by the authors to give better separation, revealed five polypeptides of 72,000, 42,000, 36,000, 30,000
and 23,000 daltons in the same antigen, J.M. (Shih & Gerin, 1975). In the latter report the polypeptide profile of a second ad antigen, DBAA, was also presented. In this antigen only three major polypeptides of 72,000, 30,000 and 23,000 daltons were detected and the proportion of protein in each differed from that in J.M. antigen.

Electrophoresis of an ay subtype in 5% acrylamide gels gave two extra minor polypeptides of 97,000 and 54,000 daltons in addition to the five mentioned above, and there were marked subtype differences in the proportion of total protein contributed by each of the individual polypeptides (Gerin, 1972; Shih & Gerin, 1975). Periodic acid-Schiff (PAS) staining of polyacrylamide gels revealed carbohydrate in two polypeptides of 54,000 and 30,000 daltons (Shih & Gerin, 1975).

Working with eight batches of $^{125}$I-labelled antigen of subtype ad from three anicteric hepatitis patients Dreesman and coworkers originally found six polypeptides of 39,000, 32,000, 27,000, 22,000, 16,000 and 10,000 daltons plus a large peak of radiolabel at the bottom of the gel which was thought to be free $^{125}$I since it was non-precipitable by trichloracetic acid (Dreesman et al., 1972). Later reports from the same group described seven polypeptides of 120,000, 55,000, 40,000, 35,000, 27,000, 24,000 and 19,000 daltons detected by Coomassie Blue staining in an ad antigen preparation, and two additional polypeptides of 105,000 and 69,000 daltons in an ay antigen. Subtype differences in the relative distribution
of protein between the various polypeptides was observed but in both subtypes the polypeptides of 35,000, 27,000 and 24,000 daltons were identified as glycoproteins, the latter two being major constituents of the particle (Chaires et al., 1973; 1975a). Labelling of disialidated antigen with $^{14}$C sialic acid has provided independent confirmation of the presence of carbohydrate in a polypeptide of 27,000 daltons (Neurath et al., 1975b).

Major polypeptides of 28-32,000 and 24-25,000 daltons and a minor polypeptide of 34,000 daltons have been described in Coomassie Blue stained gels by other workers (Vyas et al., 1972; Gerlich & May, 1973). Variable amounts of three higher molecular weight components of 90,000, 70,000 and 50,000 daltons have also been found, but these were thought to be human serum components (Gerlich & May, 1973). Howard & Zuckerman (1974) have described two major and one minor polypeptide in an $^{125}$I-labelled av antigen, but these were of considerably higher molecular weight, namely 90,000, 82,000 and 30,000 daltons.

There have been two reports of the polypeptide composition of the various other particulate structures found in serum. Short and long filaments separated by four-step centrifugation of an av antigen contained the same polypeptides as spherical 22nm particles of the same subtype but these were present in different relative proportions when compared by the intensity of Coomassie Blue staining (Gerin, 1972). Separation of a subtype ad plasma into five morphological forms, 15-19nm
spheres, 20-22nm spheres, filaments, 23-25nm spheres, and Dane particles, followed by labelling with $^{125}$I and discontinuous gel electrophoresis revealed 7, 10, 9, 5 and 9 polypeptides respectively (Chaires et al., 1975a). Polypeptides of 69,000, 55,000 and 40-45,000 daltons were common to all five particle types but were present in different proportions in each. The other polypeptides detected ranged in molecular weight from 120,000 to 16,000 daltons.

1.9. Serologically active subunits of HB$_{Ag}$

Disruption of the 22nm particle to produce subunits which retained antigenic or immunogenic activity has been an area of both theoretical interest and possible practical relevance to the development of an hepatitis B subunit vaccine. Initial studies showed that the components of 4,000-10,000 daltons produced by reduction and acid treatment (Dreesman et al., 1973) and the 6,000 dalton unit released by reduction and ultrasonication (Rao & Vyas, 1973) both retained serological activity detectable by RIA and HAI respectively. Recently relatively large quantities of the individual polypeptides of the 22nm particle have been isolated by preparative polyacrylamide gel electrophoresis and their immunogenicity tested in guinea pigs.

Purified antigen of subtypes adw and ayw was separated by gel electrophoresis into five polypeptides of 40,000, 35,000, 27,000, 24,000 and 19,000 daltons (Dreesman et al., 1975). Antisera to individual polypeptides were produced by two injections of 0.05-0.10 µg of each polypeptide in
Freunds complete adjuvant at 14 day intervals, and humoral antibody production was measured by RIA using $^{125}$I-labelled intact adw and ayw antigens. The 24,000 dalton polypeptide from the ayw subtype and the 35,000 and 40,000 dalton polypeptides from both subtypes elicited group specific antibody responses. Guinea pigs immunized with the 19,000 and 24,000 dalton polypeptides from the adw subtype produced antibody that reacted preferentially with radiolabelled adw antigen. No antibody response was detected following injection with the 27,000 dalton polypeptide of either subtype, or with the 19,000 dalton polypeptide from ayw antigen. Cell mediated immunity to native adw and ayw antigens was detected by macrophage migration inhibition following immunisation with the 40,000 dalton polypeptide from adw antigen and to a lesser extent with the 24,000 dalton polypeptide from the same subtype (Cabral et al., 1975). This latter polypeptide also inhibited the migration of macrophages from guinea pigs inoculated with a high concentration of normal human serum indicating that it may have contained an antigenic determinant related to a human serum protein. Humoral antibodies to this polypeptide did not react with normal human serum by complement fixation (Breesman et al., 1975).

In a similar study Gerin and coworkers injected each of four polypeptides from adw antigen and six polypeptides from ayw antigen into guinea pigs and detected group and subtype specific antisera by passive haemagglutination (PHA) and RIA (Gerin, Shih & Kaplan, 1975; Shih & Gerin, 1975). Antisera
produced by the 72,000, 30,000 and 23,000 dalton polypeptides of adw antigen were characterised further by radioimmunoassay using radiolabelled intact HB\(_g\)Ag particles. Similar antibody titration curves were produced by antisera to native adw antigen and to the 72,000 and 30,000 dalton polypeptides. The titration curve of antibody to the 23,000 dalton polypeptide was unique and inhibition binding curves demonstrated that the binding efficiency of this antiserum for native adw antigen was 15 times lower than that of either of the other two anti-polypeptide sera.

1.10. Structure of hepatitis B antigen particles

1.10.1. Particles derived from serum.

Electron microscopy has demonstrated that typical HB\(_g\)Ag carrier sera contained between \(10^3\) and \(10^9\) particles per ml. (Almeida, 1972). In a study of 36 antigen positive blood donor sera the percentage of small spherical particles was never less than 70\% whereas filamentous forms were absent in 5 sera, and in the remaining 31 accounted for between 1\% and 16\% of the total particle count. Dane particles occurred with much lower frequency and contributed 2-13\% of the total particles in 5 of these sera but were undetectable in the other 31 (Woolf et al., 1975).

Rate zonal gradient centrifugation separated the small spherical particles into two subpopulations of 15-19nm and 20-22nm diameter (Chairez et al., 1975): filaments had the same diameter as the larger of these subpopulations and a variable length from 50 to 400nm (Muscatello et al., 1973).
Spherical and tubular particles penetrated by negative stain to reveal a central electron-dense hole or channel of 5-10nm have been described (Bayer et al., 1968; Muscatello et al., 1973; Traavik et al., 1973); in two instances the proportion of such particles was increased following centrifugation in cesium chloride (Hirschman et al., 1973; Ackerman et al., 1974). Subunits of 3-4nm diameter have been observed on the surface of the small particle and striations with a 3nm periodicity have also been observed in the filaments (Bayer et al., 1968; Muscatello et al., 1973; Hirschman et al., 1973). Particle size and substructure has however been found to be dependent on the composition and ionic strength of the suspending buffer (Hirschman et al., 1973) and on the type of negative stain used (Muscatello et al., 1973).

The Dane particle of 42nm diameter appears to be made up of an outer membrane 6nm wide separated by a space of 4nm from the 27nm diameter inner core; the core itself contains an outer limiting layer 2nm thick (Dane et al., 1970). Tadpole-like Dane particles with "tails" resembling filaments have also been described (Dane et al., 1970; Jokelainen et al., 1970; Zalan et al., 1971). The use of rotational techniques to reinforce hexagonal and pentagonal symmetry in micrographs demonstrated icosahedral symmetry in the core particle, and at higher magnification surface structures suggestive of capsomere arrangement were observed (Zalan et al., 1971). Staining with potassium permanganate indicated the presence of lipid in the outer surface of the core as well as in the coat of the Dane particle (Jokelainen et al.,
Core structures were not found free in serum but could be released from Dane particles by treatment with non-ionic detergent (Almeida et al., 1971; Kaplan et al., 1973; Lipman, Hierholzer & Schleuderberg, 1973). Released cores isolated by cesium chloride centrifugation were present as aggregated masses surrounded by an aureola containing regularly spaced projections. These projections were 14nm long with knob-like tips, giving the core an overall diameter of 55nm, and appeared to originate from discrete sites on the surface of the core particle (Lipman et al., 1973).

1.10.2. Particles derived from liver.

Examination by electron microscopy of thin sections or tissue homogenates from livers of patients with hepatitis B infection has supported the view that the liver is one site of synthesis and maturation of the various particulate structures associated with this disease.

Particles of 18-26nm diameter have been observed in the nuclei of liver cells after thin sectioning (Nowasowski et al., 1970; Nelson et al., 1970; Huang, 1971; Gerber & Hadziyannis, 1974). These were thought to be identical to the 25-27nm diameter particles found in liver homogenates after negative staining, the size discrepancy being due to the difference in preparative techniques (Almeida et al., 1970). Many of these particles appeared hollow, but some showed a distinct electron dense central region surrounded by an outer layer made up of subunits of 2.5nm diameter (Nowasowski et al., 1970; Huang, 1971). Hexagonal or pentagonal particle symmetry was observed with 6 morphological subunits along each edge, suggesting a minimum of 162 subunits in
the complete core structure (Huang, 1971; Skikne & Talbot, 1974; Yamada & Kosaka, 1975). The particles were generally grouped as irregular shaped aggregates within the nucleus, usually separated by a clear zone from the surrounding nuclear or nucleolar chromatin, but occasional chain-like arrays were also seen (Nowasowski et al., 1970; Huang et al., 1972). The serological identity of these nuclear particles with cores of Dane particles from serum was first demonstrated by immunofluorescence (Brzosko et al., 1973; Edgington, 1974) and confirmed by immunoelectron microscopy (Huang & Groh, 1973).

Within the liver cell cytoplasm occasional spherical particles of similar size and morphology to nuclear particles were seen either randomly distributed or in small aggregates (Huang, 1971). Filamentous structures 25-29 nm wide and up to 1 µm in length were observed within phagosomes and in the distended smooth endoplasmic reticulum (Huang, 1971; Huang et al., 1974; Gerber & Hadziyannis, 1974; Gudat et al., 1975). Circular or ellipsoid structures, occasionally containing an eccentric electron dense dot, the so-called "owls-eye" appearance (Stein, Fainaru & Stein, 1972), have been interpreted as representing cross sections of this filamentous material (Gerber et al., 1974a; Huang et al., 1974). Larger, 35-40 nm membrane bound particles, often in the form of tadpole-like structures were found in close association with the filamentous material (Huang, 1971; Huang et al., 1974; Gudat et al., 1975), but no particles were observed budding from the endoplasmic reticulum (Gerber et al., 1974a; 1974b). The filamentous and circular structures found in the cytoplasm, and the surrounding endoplasmic reticulum, were all found to react with anti-HB_Ag by immunofluorescence and immunoelectron microscopy (Gerber et al., 1974a). Occasional weak cytoplasmic staining observed with anti-HB_Ag appeared to be
related to the ultrastructural finding of Dane particle-like structures in some cells (Huang, 1975).

Examination of human liver homogenates after negative staining revealed the presence of enveloped and non-enveloped particles and filamentous structures. The enveloped particles, which were 42-44nm in diameter, were morphologically indistinguishable from Dane particles in serum (Huang et al., 1974). Non-enveloped particles displayed icosahedral symmetry with spike-like outer projections 7-10nm long ending in knob shaped structures (Huang et al., 1974; Hirschman et al., 1974a). These particles were found to be unstable after storage at -20°C and spontaneously dissociated into individual capsomeres (Hirschman et al., 1974a). The filamentous structures, which were 18-24nm wide and occasionally had a central electron-dense channel running along the long axis, were frequently seen in long intertwined bundles (Huang et al., 1974). The typical 22nm spherical particle predominating in serum was not recognisable in negatively stained liver homogenates from the same individual (Huang et al., 1974). In contrast, negative staining of a liver homogenate from an experimentally infected chimpanzee revealed both typical 22nm spherical particles and 27nm core particles (Barker et al., 1974). Antisera raised in guinea pigs against a purified chimpanzee core antigen preparation reacted specifically with the detergent-released core components of Dane particles from serum, confirming that cores from these two sources were antigenically indistinguishable (Barker et al., 1974).

1.10.3. Structural models.

A number of structural models have been proposed for the spherical 22nm particle and the associated filaments, primarily on the basis of protein-protein interactions.
Subunits of 3-4nm diameter have been observed on the surface of the 22nm particle by a number of workers, but in most instances no ordered arrangement could be detected (Bayer et al., 1968; Almeida et al., 1971; Muscatello et al., 1973). One group have described larger subunits of 6nm diameter, each with a central hole, and proposed that the particle contained 32 of these subunits arranged in overall cubic symmetry (Brzosko et al., 1970).

Optical diffraction studies on tubular particles have demonstrated the presence of a helical lattice of pitch 15-20° and approximately 16 subunits per thread (Muscatello et al., 1973; Hull, 1976). Spiral unwinding of both spherical and tubular structures seen under certain ionic conditions has led to the suggestion that the spherical particles were formed by partial unwinding of one end of a tubule followed by budding off of a sphere (Hirschman et al., 1973; Muscatello et al., 1973; Traavik et al., 1973). Similarly ring forms of the 22nm particle containing a central hole of 5-10nm diameter have been frequently described (Muscatello et al., 1973; Hirschman et al., 1973; Ackerman et al., 1974). By analogy with the structure of tobacco mosaic virus Hirschman and coworkers (1973) have suggested that these ring forms represented discs of protein and that the spherical 22nm particles were "lock washers" in which the protein strands had coiled over to fill the empty space.

Detection of two fundamental polypeptides of 27,000 and 23,000 daltons by polyacrylamide gel electrophoresis has led to the suggestion that the 22nm particle was made up of 80-100 of these polypeptide units. In this instance the principal bonding forces holding the particle together were thought to be of a hydrophobic nature due to
the high tryptophan content of the protein (Gerlich & May, 1973).
Finally, there is one report which suggested that the 22nm particle
was made up of an outer shell 5-6nm thick which was sensitive to
reducing agent, and an inner core of 8-10nm which was destroyed by
RNAase treatment (Brzosko et al., 1972).
1.11 Conclusions and scope of thesis

It is apparent from the work described above that detailed information on the biochemical constituents of hepatitis B surface antigen is scant, and, in the case of the polypeptide composition, apparently conflicting. Moreover, little progress has been made in describing the structural configuration of the 22nm HBsAg particle. The following work attempts to examine certain of these areas in some detail. Firstly, the effects of a variety of chemical denaturing agents on the morphological stability of the 22nm particle, and attempts at the production of serologically active subunits, have been monitored by gradient centrifugation and double antibody radioimmunoprecipitation. Secondly, the polypeptide composition of purified 22nm particles has been investigated using both continuous and discontinuous polyacrylamide gel electrophoresis and column chromatography techniques. Finally, breakdown of the 22nm particle into smaller morphological units has been examined by direct visualisation in the electron microscope in an attempt to describe a structural model of this particle.
MATERIALS AND METHODS
2.1. Source of HB\textsubscript{Ag}

22nm HB\textsubscript{Ag} particles were purified from plasma from individual healthy blood donors. All antigen positive plasma samples were stored at -20°C, and irradiated with u-v light for 25 min immediately before use (Results Section 3.1).

2.2. Radiolabelling of purified HB\textsubscript{Ag}

2.2.1. Chloramine T labelling of intact HB\textsubscript{Ag}

Samples of the antigen peak from the 2nd caesium chloride (CsCl)/sucrose gradient (Section 3.1.) were labelled with $^{125}\text{I}$ according to the method of Hunter & Greenwood (1962). 20 µl of HB\textsubscript{Ag} in phosphate buffered saline (PBS), pH 7.2, containing approximately 10 µg of protein as calculated from the extinction coefficient $E_{\text{O.1%}} = 3.726$ (Vyas et al., 1972; Dreesman et al., 1972), was added to 1mCi of $^{125}\text{I}$, followed by 20 µl of chloramine T (2.5mg/ml). After 60 sec at room temperature the reaction was terminated by adding 20 µl of sodium metabisulphite ($\text{Na}_2\text{S}_2\text{O}_5$: 6mg/ml) and 0.5 ml of PBS containing 0.5% bovine serum albumin (BSA). Iodinated HB\textsubscript{Ag} was separated from free $^{125}\text{I}$ by chromatography on a 9 x 22mm column of Sepharose 6B equilibrated in PBS containing 0.5% BSA and 0.02% sodium azide (RIP buffer). Fractions containing the $^{125}\text{I}$-HB\textsubscript{Ag} peak, detected by double antibody radiocoomunoprecipitation (DA-RIP), were pooled and centrifuged on a 5-20% sucrose velocity gradient in RIP buffer (42,000 rev/min for 2h at 4°C in Spinco SW.50L rotor). A labelling efficiency of 5-25% was routinely obtained.
yielding between 9 and 13 μCi $^{125}$I per μg HB$_g$Ag.

2.2.2. $^{125}$I labelling of HB$_g$Ag after disruption.

HB$_g$Ag was purified by 2 cycles of equilibrium gradient centrifugation, followed by a 5-20% sucrose velocity gradient (42,000 rev/min for 2h at 4°C in SW.50L). The peak antigen fraction detected by inhibition DA-RIP assay was disrupted and labelled with $^{125}$I by chloramine T oxidation according to the method of Stanley & Haslam (1971). 30 μl of HB$_g$Ag containing 10 μg of protein was boiled for 2 min in 1% sodium dodecyl sulphate (SDS) and 0.065M-dithiothreitol (DTT) in 0.0625M-tris/HCl, pH 6.8, containing 0.1% SDS. After alkylation with 0.075M-iodoacetamide at 4°C for 60 min the sample was dialysed for 18h against tris/HCl buffer. 40 μl of the disrupted antigen was then labelled with 100 μCi of $^{125}$I by chloramine T oxidation as described above. 20 μl of sodium metabisulphite and 200 μl of tris/HCl buffer was added to terminate the reaction and the mixture was dialysed for 48h against several changes of 500 vol of tris/HCl buffer.

2.2.3. Radiolabelling with iodinated 3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester ($^{125}$I-ester).

Labelling of HB$_g$Ag with $^{125}$I-ester was carried out according to the method of Bolton & Hunter (1973). 20 μl of purified antigen containing 8 μg of protein was shaken with 1.3 mCi of $^{125}$I-ester in 10 μl of 0.1M-borate buffer, pH 8.5, at 4°C for 30 min. 0.4ml of 0.2M-glycine in 0.1M-borate was added, and,
after a further 5 min at 4°C the mixture was separated on a column of Sepharose 6B in RIP buffer, as previously described.

2.3. Double antibody radioimmunoprecipitation (DA-RIP)

The optimal conditions for the precipitation of rabbit IgG, to be used as the first antibody, were determined in two steps. To define the optimal ratio of rabbit IgG to donkey anti-rabbit IgG (DARS), complexes of 125I-HBsAg (50 c.p.m.) and rabbit anti-HBs (diluted 1/400) were incubated at 4°C overnight with a constant volume of neat DARS and serial dilutions of carrier non-immune rabbit serum (NIRS) from neat to 1/64. As the ratio of DARS to rabbit IgG increased the precipitation level rose to a plateau. The optimal ratio of second to first antibody was chosen on this plateau region so that small volume fluctuations did not affect the stability of the system.

Secondly, the ratio of first to second antibody was kept constant while the DARS and carrier NIRS were diluted out in parallel in a similar reaction; this determined the limiting dilutions of both reagents, before precipitation levels began to decline. For each new batch of 125I labelled HBsAg rabbit anti-HBs dilution curves were determined. Antibody dilutions from 1/100 to 1/25,600 in carrier NIRS 1/160 were titrated with a constant amount of 125I labelled HBsAg and the appropriate dilution of DARS. For maximal sensitivity of HBsAg detection the antibody dilution which gave a precipitation level just down from the plateau region of the curve was chosen.

The radioimmunoprecipitation system used for the detection
of both unlabelled and $^{125}$I labelled HB$_3$Ag was essentially similar to that of Hollinger and coworkers (1971). Generally 50 µl of $^{125}$I-HB$_3$Ag containing approximately 50 c.p.s. of $^{125}$I and 1ng of HB$_3$Ag protein was incubated with 50 µl of first antibody at $4^\circ$C for 18h in the presence of carrier NIRS. 100 µl of the appropriate dilution of second antibody was then added and the reaction allowed to reach equilibrium at $4^\circ$C for 18h. The reaction mixture was then made up to 1.5ml with RIP buffer containing 0.1mg/ml soluble starch and spun at 2,000 rev/min for 30 min at $4^\circ$C in a Mistral 6L. The supernatant fluid was decanted from the pelleted antigen-antibody complexes and, after counting in an LKB Wallac Gamma Counter, the percentage of $^{125}$I labelled HB$_3$Ag precipitated was calculated from the following formula:

$$\% \text{ of } ^{125}\text{I-HB}_3\text{Ag precipitated} = \frac{\text{c.p.s. } ^{125}\text{I in pellet}}{\text{c.p.s. } ^{125}\text{I in pellet} + \text{c.p.s. } ^{125}\text{I in supernatant}} \times 100\%$$

Unlabelled HB$_3$Ag was detected by inhibition of the precipitation of $^{125}$I labelled HB$_3$Ag. 5-10 µl samples of purified antigen were diluted to 100 µl with RIP buffer and incubated with 50 µl of first antibody at $37^\circ$C for 3h or $4^\circ$C for 18h prior to addition of $^{125}$I labelled HB$_3$Ag.

2.4. Gradient centrifugation

2.4.1. Equilibrium centrifugation.

100 µl samples for buoyant density analysis were layered over 4.8ml of 28% (w/v) CsCl in PBS, pH 7.2, and centrifuged to equilibrium at 40,000 rev/min for 18h at $4^\circ$C in a Spinco
Density determinations were made by weighing 100 µl samples of the gradient fractions in a tared pipette.

2.4.2. **Velocity gradient centrifugation.**

100 µl HBsAg samples were centrifuged on 5-20% (w/v) linear sucrose gradients in the appropriate buffer (4,200 rev/min at 4°C for 2h in SW.50L). After centrifugation 200-250 µl samples were collected by needle puncture of the bottom of the tube, and drop counting (Martin & Ames, 1961). Tubes in which radiolabelled HBsAg had been centrifuged were routinely counted for the presence of any pelleted material. Sedimentation coefficients were calculated by comparison with purified 18S and 28S mouse liver ribosomal RNA spun in the same gradient and detected by absorbance at 260nm.

2.5. **Isoelectric focusing.**

The isoelectric point of 125I-HBsAg was determined by focusing in short sucrose gradients in a standard polyacrylamide gel apparatus, according to the method of Korant & Lonberg-Holm (1974). 3ml continuous 10-40% (w/v) sucrose gradients containing 0.01% Triton X-100 and 1% ampholine carrier ampholytes pH range 3.5-10, in 0.01M-Na phosphate buffer were formed in 7 x 85mm glass tubes sealed at the bottom with dialysis tubing. Before gradient formation 200 µl of 40% sucrose in 1% (w/v) H2SO4 was introduced into the bottom of the tube, directly in contact with the dialysis tubing. Samples of 125I-HBsAg for analysis were dissolved in 15%
sucrose and introduced into the tubes during gradient formation. The anode solution in the lower reservoir consisted of 1% (v/v) H$_2$SO$_4$, in 40% sucrose, pH 2.0, and the upper reservoir contained 2% (v/v) mono-ethanolamine, pH 12.0. The electrophoresis apparatus was cooled by enclosing it in a polystyrene container with the lower buffer chamber surrounded by ice. Voltage was set at 240V to give an initial current of 4mA per gradient, which declined to 0.5mA after 1h. After a further 3h at 300V the tubes were removed and the gradients fractionated by puncture of the dialysis membrane. The $^{125}$I peaks were located by gamma counting, and the pH determined after dilution of the samples with 0.5ml distilled water.

2.6. Disruption of 22nm HB$_g$Ag particles

50 µl samples of purified HB$_g$Ag containing 20-30 µg protein in PBS, pH 7.2, or 20 µl samples of $^{125}$I labelled antigen containing 5 x 10$^2$ to 5 x 10$^3$ c.p.s. in RIP buffer were disrupted under the following conditions.

2.6.1. Nonionic detergents: 1% to 5% (v/v) Tween 80 or Triton X-100 at 37°C for 3-24h in PBS, pH 7.2. In some instances 0.065M-DTT in 0.5M-tris/HCl, pH 8.2, was added to 1% Triton X-100 solutions and $^{125}$I-HB$_g$Ag incubated at 37°C for 2h or 65°C for 10 min. In certain experiments the concentration of Triton X-100 was kept constant at 1% and varying amounts of BSA added to the reaction mixture to alter the detergent to protein ratio from 0.01:1.0 to 6.0:1.0
on a weight to weight basis.

2.6.2. **Sodium dodecyl sulphate**: 1% (w/v) SDS in 0.0625M tris/HCl, pH 6.8, at 37°C for 16h or 100°C for 2 min.

2.6.3. **Enzymes**: 0.1% trypsin, bromelain or phospholipase C at 37°C for 2h in PBS, pH 7.2; 0.05% neuraminidase in 0.05M-Na acetate buffer, pH 5.0, or 0.1% mixed glycosidases in 0.1M-Na phosphate, pH 6.3, at 37°C for 72h.

2.6.4. **Detergent and enzymes**: Sequential treatment involved incubation of ^125_I-HB Ag with 1% SDS or 1% Triton X-100 at 37°C for 3h followed by isolation of detergent treated antigen by velocity gradient centrifugation, and digestion of this material with 0.1% trypsin at 37°C for 3h in PBS, pH 7.2. For combined treatment ^125_I-HB Ag was incubated with a mixture of 1% detergent and 0.1% trypsin at 37°C for 4h.

2.6.5. **Sodium dodecyl sulphate and reducing agent**: 1% SDS and 0.065M-DTT in 0.1M-Na phosphate, pH 7.2, or 0.0625M-tris/HCl, pH 6.8, at room temperature for 15 min or 30 min, 37°C for 30 min or 100°C for 2 min.

Except where otherwise stated all samples of disrupted HB Ag were separated by velocity gradient centrifugation as previously described. A 1/100 dilution of the initial concentration of disrupting agent was incorporated into the gradients before centrifugation.
2.7. Polyacrylamide gel electrophoresis (PAGE)

2.7.1. Non-dissociating conditions.

Purified, and partially purified samples of HB$_5$Ag were dissolved in 0.0625M tris/HCl, pH 6.8, and separated on 4% polyacrylamide gels in a discontinuous buffer system. Conditions for electrophoresis, staining and destaining were as described below, with the exception that SDS was omitted from the system.

2.7.2. Dissociating conditions.

Samples of HB$_5$Ag were disrupted either by incubating at 37°C overnight or boiling for 2 min with 1% SDS and 0.065M-DTT, in 0.1M-Na phosphate, pH 7.2, prior to continuous buffer PAGE, or in 0.0625M-tris/HCl, pH 6.8 prior to discontinuous buffer PAGE (disc-PAGE). In some experiments the concentration of DTT was increased to 1M, or 8M-urea was included during disruption; where specified, free sulphhydryl groups were alkylated after disruption by treatment with 0.075M-iodoacetamide for 60 min at 4°C in the dark, followed by overnight dialysis against either 0.1M-Na phosphate or 0.0625M-tris/HCl, each containing 0.1% SDS. PAGE was carried out in 85 x 7mm cylindrical gels at acrylamide concentrations of 7.5%-15% (w/v), using a constant N,N,N',N'-methylenebisacylamide:acrylamide ratio of 1:40.

Continuous buffer gels (Maizel, 1969) containing 0.1% SDS in 0.1M-Na phosphate, pH 7.2, were polymerised overnight at room temperature by the addition of 0.01% N,N,N',N'-Tetramethylethylenediamine (TEMED) and 0.1% ammonium persulphate.
(final concentrations). 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 5h at a constant voltage of 40V, giving a current of 10mA per gel. Electrophoresis buffer consisted of 0.1% SDS in 0.1M-Na phosphate, pH 7.2.

Discontinuous buffer gels (Laemmli, 1970) consisted of a resolving gel of high acrylamide concentration polymerised in 0.375M-tris/HCl, pH 8.8, and 0.1% SDS, overlaid with a low concentration (3%) stacking gel containing 0.1% SDS in 0.125M-tris/HCl, pH 6.8. Samples were electrophoresed for 4h at a constant current of 3mA per gel, using electrophoresis buffer containing 0.1% SDS in 0.025M-tris/HCl and 0.192M-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 2h in several changes of acetic acid:methanol:water. Polypeptides were detected by scanning at 580nm in a Gilford gel scanner, and molecular weights determined by comparison with standard proteins electrophoresed in parallel.

Gels containing 125I-labelled material were cut into 1.5mm slices and the iodinated peaks located by counting each slice in a gamma counter. For molecular weight estimations, 10 μg each of bovine serum albumin (BSA), ovalbumin (OA) and lysozyme (LY) 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 the gel slices with Coomassie Brilliant Blue.
2.8. **Column chromatography**

1.6 x 15 cm and 1.6 x 30 cm columns were packed under gravity with 30 ml and 60 ml respectively of Sepharose 6B or Sephadex G-200, and allowed to equilibrate in the appropriate buffer for 2 days before use.

2.8.1. **Chromatography using sodium dodecyl sulphate.**

$^{125}$I-HB$_5$Ag disrupted and alkylated as for PAGE was mixed with 1 mg each of BSA, ovalbumin and lysozyme disrupted in an identical manner, and applied to the column in a total volume of 300 µl. In certain cases 50 µl of 2 mg/ml Blue dextran was added immediately before chromatography to indicate the void volume of the column. Columns were eluted with either 0.1 M Na phosphate, pH 7.2 or 0.025 M tris/HCl and 0.192 M glycine, pH 3.3, both containing 0.1% SDS, at a flow rate of 1.5 ml/hour. Marker proteins were located by continuous monitoring of the eluant at 280 nm in LKB Uvicord, and 0.35 ml fractions were collected for location of radioactive peaks by gamma counting.

2.8.2. **Chromatography using guanidine hydrochloride (GuHCl).**

Column chromatography in GuHCl was essentially as described by Green & Bolognesi (1974). Samples of $^{125}$I-HB$_5$Ag were disrupted by incubating with 8 M GuHCl, 0.02 M DTT, 0.01 M EDTA in 0.05 M tris/HCl, pH 8.5, at 37°C for 72 h, 80°C for 10 min or 100°C for 2 min, and mixed with 2 mg of each of the marker proteins (BSA, OV and LY) treated identically. The pH of the sample was reduced to 5-6 by addition of 1 drop of glacial acetic acid and applied to the column in a total volume of 0.6 ml.
The eluting buffer consisted of 6M-GuHCl and 0.01M-DTT in 0.02M-Na phosphate pH 6.5 or 0.05M-Na acetate, pH 5.0. In some experiments 1% Triton X-100 was added to the sample during disruption, and 0.1% Triton to the eluting buffer. The column was eluted by upward displacement at a flow rate of 2ml/hour, and fractions collected every 30 min.

2.9. Renaturation of separated polypeptides

2.9.1. Following PAGE.

Following PAGE of ^125^I-HBsAg and location of peaks by gamma counting the separated polypeptides were eluted and renatured as described by Kennedy (1974). Gel slices were crushed by forcing through a plastic syringe into 1ml of 0.1M-tris, pH 7.5, containing 0.1% SDS. After incubation for 16h at 37°C with occasional mixing, the large gel fragments were precipitated by centrifugation at 2,500g for 15 min, and re-incubated for 4h with 0.5ml of fresh buffer. Gel fragments were re-pelleted, the supernatant fluid added to that from the first centrifugation, and the mixture further clarified by centrifugation at 12,000g for 30 min and filtration through a 0.45 μm cellulose acetate membrane. Triton X-100 was added to the filtrate to a final concentration of 0.05% and the mixture dialysed for 5 days at room temperature against 7 changes of 1000ml of 0.05M-tris/HCl containing 0.1M-NaCl and 0.05% Triton X-100, pH 7.4. Dialysed samples were concentrated x10 with polyethylene glycol (PEG; molecular weight 20,000) and assayed for serological activity by DA-RIP.
2.9.2. Following GuHCl chromatography.

Disrupted $^{125}\text{I-}H_B\text{Ag}$ was separated by column chromatography in 6M-GuHCl and fractions constituting a peak of $^{125}\text{I}$ label were pooled. These pooled fractions were dialysed for 2 days against 2 changes of 500mL of $10^{-3}\text{M-EDTA, }7.5 \times 10^{-4}\text{M-DTT}$ and 0.1% Triton X-100 in 0.02M-Na phosphate, pH 6.5, followed by 2 changes of $5 \times 10^{-4}\text{M-DTT}$ and 0.01% Triton X-100 in 0.02M-Na phosphate. After dialysis samples were concentrated by PEG and assayed by DA-RIP.

2.10. Electron microscopy

Samples of purified $H_B\text{Ag}$ were examined either after disruption in suspension as described earlier, or were disrupted "in situ" following adsorption to collodion/carbon coated copper grids. The latter technique, which was a modification of the method described by Milne & Luisoni (1975) for immuno-electron microscopy, overcame the problem of aggregation and precipitation of $H_B\text{Ag}$ following treatment with certain denaturing agents. A 30 $\mu$L drop of $H_B\text{Ag}$ in PBS, pH 7.2, was placed on a Teflon coated glass microscope slide and the grid was inverted and allowed to float on the surface of the drop. After 5 min at room temperature the grid was removed, excess moisture drawn off with filter paper, and the grid was then inverted onto a drop of a suitable denaturing agent. The slide was then placed in a moist chamber and incubated at room temperature or 37°C for the required length of time. After incubation the grid was washed by immersion in distilled water for 30 sec., and excess moisture
drawn off with filter paper. The grid was then inverted over a drop of 2% (w/v) uranyl acetate, pH 4.5, for 10 min at room temperature in a moist chamber, removed, and allowed to dry on filter paper for at least 30 min before viewing.

Antigen disrupted in suspension was adsorbed directly onto grids and stained with uranyl acetate as described above.

Grids were viewed in an Hitachi HU12A electron microscope at an accelerating voltage of 75kV.
2.11. **Materials**

\(^{125}\)I (carrier free) was obtained from the Radiochemical Centre, Amersham, and \(^{125}\)I-3-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester was a gift from Dr A.E. Bolton, M.R.C. Radioimmunoassay Team, Forrest Road, Edinburgh. Rabbit anti-HBs and rabbit anti-whole human serum, used as first antibodies in the DA-RIP system, were from Hoechst Pharmaceuticals, Hounslow, Middlesex and donkey anti-rabbit IgG (DARS), used as second antibody, was from Wellcome Reagents Ltd., Kent, England. 18S and 28S mouse liver ribosomal RNA was a gift from Dr K.W. Jones, Department of Genetics, University of Edinburgh. Ampholine carrier ampholytes, pH range 3.5-10 were obtained from LKB Instruments Ltd., South Croydon, Surrey, and Blue dextran, Sepharose 6B and Sephadex G-200 from Pharmacia (G.B.) Ltd., London. SDS, specially pure grade and Triton X-100 were obtained from British Drug Houses, Poole, Dorset, and Tween 80 from Sigma Chemical Company, Surrey. Trypsin (ex. bovine pancreas, 2x crystallised, salt-free) and bromelain (ex. pineapple stem juice) were obtained from Koch-Light Laboratories, Colnbrook, England; mixed glycosidases (ex. *Turbo cornutus*) from Miles Laboratories, Slough, England; neuraminidase (type VI ex. *Cl. perfringens*) and phospholipase C (type III ex. *B. cereus*) from Sigma Chemical Co. Guanidine hydrochloride (grade I), lysozyme and ovalbumin were from Sigma; acrylamide from Eastman Kodak Company, Rochester, New York; BSA (Fraction V, Bovine Plasma) from Armour Pharmaceutical Company, Eastbourne; PEG (mol. wt. 20,000) from Union Carbide Ltd., London and anti-HBs coated latex, "Antigex", from Pfizer Inc, New York. All other chemicals were best grade available from B.D.H.
RESULTS
3.1. Purification of 22nm HB\textsubscript{Ag} particles

Purified 22nm particles were prepared from HB\textsubscript{Ag} positive plasma with an antigen titre of $> 1$ in 16 by counter current immunoelectrophoresis (CIE). In a typical purification 15-20ml of serum was irradiated for 25 min with a dosage of 750 $\mu$w/cm$^2$ of u-v light, diluted with an equal volume of PBS, and chromatographed on a 90 x 3.2cm column of Sepharose 6B. Fractions containing HB\textsubscript{Ag}, which eluted immediately after the void volume of the column, were identified by latex agglutination (Fig. 1). Strongly positive fractions were pooled, concentrated 10 fold by dialysis against polyethylene glycol and centrifuged to equilibrium in a discontinuous 0-50\% (w/v) sucrose gradient made up in 14.3\% (w/v) caesium chloride (40,000 rev/min for 18h, Spinco SW.50L rotor). The antigen peak located by latex agglutination was then rebanded under identical conditions and the protein peaks from this second equilibrium gradient were located by measurement of optical density (O.D.) at 280nm. The position of the peak of HB\textsubscript{Ag} serological activity, which coincided with the O.D. peak, varied from 1.20 to 1.22g/ml in CsCl/sucrose depending on the source from which the antigen was purified (Fig. 2). At this stage of purity a faint precipitation line could be obtained by immunodiffusion against rabbit anti-whole human serum. Similarly, acrylamide gel analysis of the peak antigen fraction on 4\% acrylamide gels under non-dissociating conditions revealed a major high molecular weight band near the gel origin corresponding to 22nm HB\textsubscript{Ag} particles, plus
Fig. 1. Optical density profile following column chromatography of serum containing HB$_s$Ag on Sepharose 6B at a flow rate of 24 ml/hour. 12 ml fractions were collected and HB$_s$Ag detected by latex agglutination. Vo: void volume.
Optical density profile at 280 nm after second CsCl/sucrose equilibrium gradient centrifugation (---). The antigen titre by latex agglutination is superimposed (○—○). Fraction 11 was used for subsequent radioactive labelling and further purification. Pooled fractions 10-13 were used for evaluation of detergent treatment of HBsAg by gradient centrifugation and electron microscopy.
minor bands of lower molecular weight presumably representing small amounts of contaminating human serum components.

Except where otherwise stated material at this stage of purification was used for evaluation of the effect of detergent treatment on structure of the 22nm HB\textsubscript{s}Ag particle.

The peak antigen fraction from the second equilibrium gradient, generally having a titre of 1 in 160 to 1 in 320 by latex agglutination was either radiolabelled with \(^{125}\text{I}\) (Section 2.2), or dialysed overnight against PBS and isolated on a 5-20\% (w/v) linear sucrose gradient. Peak antigen fractions detected by DA-RIP (Fig. 3) showed no reaction with anti-whole human serum by immunodiffusion and no evidence of contaminating human serum proteins by non-dissociating PAGE. Examination of this material by electron microscopy revealed an homogeneous population of spherical particles of 18-24nm diameter (Fig. 4).

3.1.1. Evaluation of radiolabelled HB\textsubscript{s}Ag.

Except where otherwise stated all these results refer to \(^{125}\text{I}-\text{HB}_s\text{Ag}\) labelled by chloramine T oxidation.

a. Integrity of antigenic sites and degree of purity.

\text{HB}_s\text{Ag} radiolabelled with \(^{125}\text{I}\) and purified further, as described in Materials and Methods, was reacted with an excess of rabbit anti-\text{HB}_s in a DA-RIP assay. More than 95\% of the labelled material was precipitated under these conditions. Since rabbit anti-whole human serum activity had been detected in certain batches of rabbit anti-\text{HB}_s, it was necessary to determine whether simultaneous precipitation of any contaminating radiolabelled human serum components in the \(^{125}\text{I}-\text{HB}_s\text{Ag}\) preparation
Fig. 3. Velocity gradient centrifugation of fraction 11 from second CaCl2/sucrose equilibrium gradient. Antigen was detected by inhibition of precipitation of 125I-labelled HBsAg in a DA-RIP.
Fig. 4. Electron micrograph of negatively stained preparation of purified HB Ag particles; fraction 11 from sucrose velocity gradient, fig. 3.
Final mag. 75,000x.
was contributing to this high level of precipitation.

Antibody dilution curves of rabbit anti-HBs in the presence of either RIP buffer or a 1 in 6 dilution of normal human serum were however identical, indicating the absence of any free containing material in the 125I-HBsAg pool (Fig. 5a). In contrast, reaction of this 125I labelled HBsAg against dilutions of rabbit anti-whole human serum as first antibody in a DA-RIP assay revealed the presence of human serum antigenic determinants on the surface of the particle (Fig. 5b). These determinants were stable to low pH, nonionic detergent and ether treatment of 125I-HBsAg and may represent either cross-reacting groups or human serum proteins tightly bound to the 22nm particle (Burrell, 1975).

b. Chemical nature of 125I-HBsAg labelled by chloramine T oxidation.

When 125I-HBsAg 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.

c. Biophysical properties.

Buoyant density

Equilibrium gradient centrifugation of freshly labelled 125I-HBsAg revealed a single homogeneous peak at a density of 1.22g/ml in caesium chloride, compared to 1.20g/ml for purified unlabelled antigen in a similar gradient. Iodination therefore caused an increase in buoyant density of 0.02g/ml which increased further on storage in RIP buffer to 1.24 to 1.25g/ml, possibly
Fig. 5. Standard antiserum dilution curves of $^{125}\text{I}$-labelled HB$_3$Ag. (a) rabbit anti-HB$_3$ in RIP buffer (•—•) or 1 in 6 dilution of normal human serum (○—○). (b) rabbit anti-whole human serum in RIP buffer (•—•).
due to adsorption of BSA to the particles. A buoyant density of 1.20 gm/ml for unlabelled HB Ag corresponds closely with the value of 1.21 gm/ml reported by other workers (Gerin et al., 1969; Hillman et al., 1970; Dreessen et al., 1972).

**Sedimentation coefficient**

The sedimentation coefficient of $^{125}I$-HB Ag was determined by velocity gradient centrifugation using 18S and 28S mouse liver ribosomal RNA as internal markers. The sedimentation coefficient varied depending on the source from which the antigen was purified, but was constant with antigen from any one source, and invariably fell within the limits 32S-36S. This corresponded to previously reported values of 32S-40S (Tayot et al., 1972; Kim & Tilles, 1973), but in this work no correlation between antigen subtype and sedimentation coefficient was observed, in contrast to the findings of Bourbonnais and coworkers (1975).

**Isoelectric point**

$^{125}I$ labelled HB Ag, subtype ad, focussed as a major peak at a pI of 4.8 and a minor peak at 4.1. These values agreed with those reported by Chairez and coworkers (1975b) for the major population of purified unlabelled antigen of this subtype, but were significantly higher than the reported values for the major population of antigen after $^{125}I$ labelling.
3.2. Effects of detergent treatment on 22nm HB₈ Ag particles

3.2.1. Nonionic detergents.

a. Sedimentation properties of ¹²⁵I-HB₈ Ag

Incubation of ¹²⁵I-HB₈ Ag with Tween 80 at concentrations up to 5% at 37°C for 24h had no effect on the sedimentation coefficient of antigen and did not release any low molecular weight material from the particle. In contrast the effect of incubation with Triton X-100 was found to be dependent on the ratio of detergent to total protein in the reaction mixture. Samples of ¹²⁵I-HB₈ Ag were incubated at 37°C for 3h with 1% Triton X-100 in PBS, pH 7.2, at detergent to protein ratios from 0.01:1.0 to 6.0:1.0 (Section 2.6.1.).

At a ratio of 0.05:1.0 and below the sedimentation coefficient of the bulk of ¹²⁵I labelled antigen was increased to >60S, with the release of 1% of the total radiolabel as low molecular weight material at the top of the gradient (Fig. 6a).

Increasing the relative amount of detergent to 0.25:1.0 caused the ¹²⁵I-HB₈ Ag to band as a major homogeneous peak of 42S, and a minor, more heterogeneous, peak of 12-18S (Fig. 6b). Finally, at a ratio of 0.5:1.0 and above the major radiolabelled peak sedimented at 36S, in the same position as untreated antigen, with the solubilization of 28% of the total ¹²⁵I label (Fig. 6c).

Increasing the time of incubation to 72h, or inclusion of 0.065M-DTT in the reaction mixture, followed by incubation at 37°C for 24h in PBS, pH 7.2, or at 65°C for 10 min in 0.6M tris/HCl, pH 8.2, did not increase the amount of radiolabel solubilized from the particles. The 36S ¹²⁵I-HB₈ Ag isolated after
Fig. 6. Radioactivity profiles (-----) after centrifuging 
$^{125}$I-HB$_b$Ag through 5-20% linear sucrose gradients at 42,000
rev/min for 2h in a Spinco SW.50.L rotor at 4°C. Samples
were incubated with 1% Triton X-100 at 37°C for 3h at
detergent to protein ratios of (a) 0.05:1.0, (b) 0.25:1.0,
(c) 0.5:1.0. Serological activity (-----) was detected
by DA-RIP using rabbit anti-HB$_b$ as first antibody. Gradients
were made up in PBS, pH 7.2, containing 0.01% Triton X-100.
detergent treatment at a ratio of > 0.5:1.0 banded in CsCl equilibrium gradients at the same density as untreated $^{125}\text{I-HB}_2\text{Ag}$ particles.

b. Effect on the serological activity of $^{125}\text{I-HB}_2\text{Ag}$

Following the separation of Triton treated $^{125}\text{I-HB}_2\text{Ag}$ by velocity gradient centrifugation, the gradient fractions were diluted in RIP buffer to contain approximately equal amounts of radioactivity and 50 µl samples analysed by DA-RIP, as described in Section 2.3. All the particulate radiolabelled material retained immunoprecipitability by anti-HB$_2$ comparable to that of untreated antigen (Fig. 6a, b & c). The low molecular weight material released from the particle was partially precipitable by anti-HB$_2$, the level of precipitation decreasing as the relative detergent to protein ratio increased (Table 1). The small heterogeneous/peak released at a detergent to protein ratio of 0.25:1.0 did not show any precipitation with anti-HB$_2$.

The polypeptide composition of the serologically active material released from the particle at a detergent to protein ratio of 0.5:1.0 was analysed by continuous and discontinuous PAGE. The two final gradient fractions (Fig. 6c) were precipitated with an excess of rabbit anti-HB$_2$ in a DA-RIP reaction, pelleted and washed twice with PBS, pH 7.2. The pelleted $^{125}$I labelled material was then solubilized in 1% SDS and 0.065M-DTT in 0.1M-Na phosphate, pH 7.2, or 0.0625M-tris/HCl, pH 6.8, at 100°C for 2 min, and analysed on 10% polyacrylamide gels (Section 2.7.2). Two minor polypeptides
Table 1  Immunoprecipitation of $^{125}$I-HB$_s$Ag following treatment with Triton X-100.

<table>
<thead>
<tr>
<th>detergent : protein ratio</th>
<th>% pptn of peak fraction</th>
<th>% pptn of solubilised radiolabel</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 : 1.0</td>
<td>92%</td>
<td>50%</td>
</tr>
<tr>
<td>0.25 : 1.0</td>
<td>92%</td>
<td>26%</td>
</tr>
<tr>
<td>0.5 : 1.0</td>
<td>93%</td>
<td>13%</td>
</tr>
</tbody>
</table>
of 90-100,000 and 60-66,000 daltons and one major polypeptide of 12-14,000 daltons were detected after electrophoresis in either buffer system (Fig. 7).

c. Effect on the serological activity and structure of unlabelled HB$_s$Ag

Purified HB$_s$Ag was incubated for 3-24h with 1-5% Tween 80 or Triton X-100 and titrated immediately in serial 10 fold dilutions in an inhibition DA-RIP assay. None of these treatments had any effect on antigen titre when compared with a control sample assayed at the same time, in the absence of detergent. No antigenic sites on the surface of the 22nm particle capable of reacting with rabbit anti-HB$_s$ were therefore destroyed or exposed by nonionic detergent treatment.

The effect of these agents on particle structure was then determined by separation of detergent-treated HB$_s$Ag on velocity gradients and detection of serological activity by DA-RIP inhibition assay. The relative amount of activity in each gradient fraction was calculated from standard curves of untreated HB$_s$Ag titrated under identical DA-RIP conditions (Fig. 8). The final dilution of HB$_s$Ag detectable by DA-RIP, 1/1000 in this assay, was assigned on arbitrary value of 1 unit of antigen. Next, 1/10 and 1/100 dilutions of HB$_s$Ag then had values of 1000 units, 100 units and 10 units respectively.

Using this standard curve the percentage precipitation of each of the gradient fractions was converted to an equivalent number of antigen units.
Fig. 7. Distribution of radioactivity after PAGE analysis of serologically active material solubilized by Triton X-100 at high detergent:protein ratios. Fractions 22-23 from fig. 6c were precipitated with an excess of rabbit anti-HBs and run on 10% acrylamide gels. Estimated molecular weights x10^{-3} are indicated.

BFB: bromophenol blue marker dye.
Fig. 8. Standard antigen detection curve relating units of antigen activity to inhibition of precipitation of radiolabelled HB₃Ag in a DA-RIP assay.
Incubation at 37°C with 1% or 5% Tween 80 for 3 to 24h increased the heterogeneity of the antigen peak when compared to a control sample incubated under similar conditions in the absence of detergent (Fig. 9). Less than 5% of the total antigenic activity was released as small molecular weight components. Incubation with 1% or 5% Triton X-100 at 37°C for 3h again increased the heterogeneity of the main antigen peak, and increasing amounts of antigenically active low molecular weight material were solubilized as the detergent concentration increased (Table 2). Incubation with 5% Triton X-100 at 37°C for 24 to 48h solubilized more than half of the serological activity of the particle and decreased the sedimentation coefficient of the remaining particulate material to 26S (Fig. 10).

The approximate molecular weight of the serologically active material released from purified HB$_{Ag}$ subtype ad by 5% Triton X-100 was investigated by column chromatography. 300 µl of the pooled gradient fractions 19 & 20 from Fig. 10 were separated on Sephadex G-200 equilibrated with PBS, pH 7.2 containing 0.01% Triton X-100. Antigenic activity in the column eluate was monitored in a DA-RIP inhibition assay using monospecific antisera against the group determinant a, and the subtype determinant d. The former consisted of commercial rabbit anti-HB$_{Ag}$ (Hoechst Pharmaceuticals) at a dilution of 1/1600, and the latter was a rabbit antiserum prepared in this Department by heterologous adsorption of an anti-HB$_{Ag}$/ad antiserum, as described in the Appendix. Serologically active material eluted as a minor peak in the void volume of the column, and a
Fig. 9. Sucrose density gradient profile of purified HB$_g$Ag incubated with 1% Tween 80 at 37°C for 24 h. Gradient fractions were assayed by inhibition DA-RIP. The position of untreated HB$_g$Ag spun under similar conditions is indicated.
Fig. 10. Sucrose density gradient profile of purified HBAg incubated with 5% Triton X-100 at 37°C for 48h. Gradient fractions were assayed by inhibition DA-RIP. The position of untreated HBAg spun under similar conditions is indicated.
Table 2  Proportion of total serological activity solubilised by incubation of HBsAg with Tween 80 or Triton X-100 at 37°C.

<table>
<thead>
<tr>
<th>detergent</th>
<th>concentration</th>
<th>time</th>
<th>% total serological activity released</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80</td>
<td>1%</td>
<td>3h</td>
<td>4.4%</td>
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<td></td>
<td>5%</td>
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<tr>
<td></td>
<td>5%</td>
<td>24h</td>
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<tr>
<td>Triton X-100</td>
<td>1%</td>
<td>3h</td>
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major peak just ahead of the marker protein BSA, suggesting a molecular weight of 70-80,000 daltons (Fig. 11). The distribution of antigenically active material detected by both the anti-\( a \) and the anti-\( d \) antisera coincided exactly, indicating that the group- and subtype-specific antigenic determinants must lie either on the same structural component, or on different components of the same size.

3.2.2. Anionic detergent.

Incubation of \( ^{125}\text{I-}H\text{B}_A\text{g} \) with 1% SDS in tris/HCl buffer at 37°C for 16h or 100°C for 2 min had no effect on the sedimentation coefficient of the antigen and did not solubilise any low molecular weight radiolabelled material. Similarly boiling purified unlabelled HB\( _A\)g with 1% SDS for 2 min increased the heterogeneity of the antigen on a sucrose gradient but did not release any antigenically active material from the particle. Boiling with 1% SDS did however cause a two fold decrease in total antigen titre by DA-RIP. Boiling of a control preparation of antigen in the absence of detergent had no effect on antigen titre.

Incubation with 1% SDS in tris/HCl buffer therefore did not solubilise either radiolabelled protein or serologically active material from 22nm HB\( _A\)g particles.
Fig. 11. Column chromatography profile of pooled fractions 19-20 from fig. 10 separated on a 1.6 x 15 cm column of Sephadex G-200 run at a flow rate of 1.5 ml/hour. 0.5 ml fractions were collected and serological activity detected by DA-RIP using monospecific anti-a (○---○) and monospecific anti-d (●---●) antisera as first antibodies. Vo: void volume.
3.3. **Effects of enzyme treatment**

**a. Sedimentation properties of $^{125}$I-HB$_g$Ag**

Incubation of $^{125}$I-HB$_g$Ag with trypsin, bromelain or phospholipase C at 37°C for 3h had no effect on the sedimentation coefficient of the antigen and released less than 10% of the total $^{125}$I label from the particle. Similarly, phospholipase C treatment of trypsinised antigen isolated by density gradient centrifugation had no effect on structural integrity of the particle.

**b. Serological activity**

Phospholipase C treatment had no effect on the immunoprecipitability of the peak of radiolabelled antigen with either anti-HB$_g$ or anti-whole human serum. Trypsinization selectively destroyed sites on the particle reacting with anti-whole human serum but did not affect precipitation with anti-HB$_g$ in a DA-RIP assay.
3.4. Effects of combined treatments

3.4.1. Nonionic detergent and enzymes.

The radiolabelled peak fraction isolated after velocity gradient centrifugation of $^{125}$I-HB$_s$Ag treated with Triton X-100 (detergent to protein ratio 0.5:1.0, Fig. 6c) was then incubated with 0.1% trypsin or 0.1% bromelain at 37°C for 3h, and the radiolabel re-isolated by gradient centrifugation. Sequential treatment of antigen in this manner did not cause any breakdown in particulate structure. In contrast, simultaneous treatment with a mixture of Triton X-100 and trypsin at 37°C for 4h released the majority of the radiolabel as low molecular weight material at the top of a density gradient, with the simultaneous appearance of a minor radiolabelled peak of 15-20S (Fig. 12).

The reasons for the difference in susceptibility to trypsinization in these two situations is not apparent. It is likely that detergent treatment removes lipid from the particle and exposes polypeptide chains to cleavage by proteolytic enzymes. Isolation of the detergent treated antigen by gradient centrifugation prior to addition of trypsin may allow coating of the particle by a protective layer of detergent molecules hydrophobically bound to the protein in place of the solubilised lipid (Helenius & Simons, 1972).

3.4.2. Anionic detergent and enzymes.

a. Sedimentation properties of $^{125}$I-HB$_s$Ag

Incubation with 1% SDS for 3h at 37°C of the peak of $^{125}$I-HB$_s$Ag isolated by gradient centrifugation after trypsin treatment
Fig. 12. Radioactivity profile of $^{125}$I-HB Ag after incubation with 1% Triton X-100 and 0.1% trypsin at 37°C for 4h followed by centrifugation on a 5-20% linear sucrose gradient.
had no effect on particulate structure of the antigen. This indicated the absence of a trypsin sensitive layer of protein around the outside of the particle protecting an internal layer of lipid, as occurs in the marine bacteriophage PM2 (Franklin, 1974). The converse experiment, namely incubation of $^{125}\text{I-HB}_3\text{Ag}$ with 1% SDS, isolation of the intact SDS treated particle by gradient centrifugation, and treatment of this material with trypsin destroyed the particulate structure of the radiolabelled antigen and released most of the $^{125}\text{I}$-label as low molecular weight material at the top of a velocity gradient (Fig. 13).

**b. Serological activity**

Radiolabelled material released from $^{125}\text{I-HB}_3\text{Ag}$ by SDS and trypsin showed only low levels of precipitation with anti-$\text{HB}_3$ by DA-RIP. However similar treatment of unlabelled antigen released all the serological activity as low molecular weight material. Further characterization of this released material demonstrated the presence of a protease resistant peptide of 5-15,000 daltons containing carbohydrate residues (Burrell et al., 1976).

### 3.4.3. Anionic detergent and reducing agent.

**a. Sedimentation properties of $^{125}\text{I-HB}_3\text{Ag}$**

$^{125}\text{I-HB}_3\text{Ag}$ was incubated at room temperature for 15 min with 1% SDS and 0.065M-DTT in 0.1M-Na phosphate or 0.0625M-tris/HCl buffer. This treatment altered the sedimentation coefficient of the particle from 36S to 25S and released 30-50% of the $^{125}\text{I}$
Fig. 13. Sucrose density gradient profile of SDS treated \( ^{125}\text{I}-\text{HB}_{\text{ag}} \) (1% SDS at 37°C for 3h) after incubation with 0.1% trypsin at 37°C for 3h in FBS, pH 7.2.
label as low molecular weight material. With increased time
or temperature of incubation relatively less of the 25S component
was produced together with increasing amounts of radiolabel
at the top of the gradient (Fig. 14). In most cases heating
at 100°C for 2 min was sufficient to dissociate the particle
completely into low weight components, as confirmed by disc-PAGE
and column chromatography (Section 3.5.2a & 3.6.2). However
with HB₅Ag 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 8M-urea alone, 8M-urea and 1% SDS and 0.065M-DTT,
1% SDS and 1M-DTT at pH 5.6 or 7.2, or incubating overnight at
37°C with 1% SDS and 0.5M-DTT caused some further breakdown
in particulate structure. Labelled material now sedimented
around 5-10S but was still too large to migrate in 10%
acrylamide gels.

b. Recovery of serological activity

For detection of serological activity gradient fractions
containing either the 25S component or the released radiolabel
were assayed by DA-RIP, after dialysis against 10 changes
of 500 vol. of 0.05M-tris, 0.1M-NaCl containing 0.05% Triton
X-100, pH 7.5 to assist renaturation. The 25S component was
precipitated by anti-HB₅ to the same extent as a control sample
of untreated ¹²⁵I-HB₅Ag assayed in parallel. No renaturation
of antigenic activity however occurred with the low molecular
weight material released to the top of the gradient.
Fig. 14. Radioactivity profiles after centrifuging $^{125}$I-HB$_8$Ag through 5-20% linear sucrose gradients following incubation with 1% SDS and 0.065M-DTT under the following conditions: •• room temperature, 15 min; ▲▲ room temperature, 30 min; ■■ 37°, 30 min; ○○ 100°C, 2 min. Untreated $^{125}$I-HB$_8$Ag spun in a similar gradient sedimented at 36S.
3.5. Analysis of HB Ag polypeptides by polyacrylamide gel electrophoresis

3.5.1. PAGE of HB Ag polypeptides detected by Coomassie Blue staining.

Preparations of HB Ag of both subtypes (ad and ay) at different stages of purification were heated to 100°C for 2 min with 1% SDS and 0.065 M-DTT and examined by PAGE using the discontinuous buffer system.

a. HB Ag purified by column chromatography + 2 x CsCl equilibrium gradient centrifugation

PAGE analysis of 22nm HB Ag particle at this stage of purification revealed nine or more polypeptides covering the molecular weight range from 130,000 to 14,000 daltons (Fig. 15a). As described earlier this material gave a faint precipitation line by gel diffusion with antibody to whole human serum.

b. HB Ag purified by column chromatography, 2 x CsCl gradient plus velocity gradient centrifugation

After further purification the relative intensity of peaks 1, 2, 4, 5 & 6 was significantly decreased indicating that these polypeptides probably represented contaminating human serum components of different sedimentation coefficient to HB Ag. These polypeptides are therefore not indicated on Fig. 15b-d. In 10% acrylamide gels two major polypeptides, 3 & 9, of 66-70,000 and 12-14,000 daltons were consistently detected (Fig. 15b), and in some instances two minor polypeptides, 7 & 8, of 23,000 and 17,000 daltons were also seen (Fig. 15c).
**Fig. 15.** Densitometer scan at 580 nm of disc polyacrylamide gels of Coomassie blue stained polypeptides of hepatitis B antigen at different stages of purification. (a) Column chromatography and 2 cycles of equilibrium gradient centrifugation, 10% acrylamide gel. (b) Column chromatography, 2 cycles equilibrium centrifugation followed by density gradient centrifugation, 10% acrylamide gel. (c) Material of same purity as (b), but from different source, 10% acrylamide gel. (d) Identical material to (c) separated on 12.5% acrylamide gel.
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 molecular weight, now
appearing as broad peaks of 26-30,000 and 20-24,000 daltons
respectively (Fig. 15d). Similar results were obtained using
continuous buffer PAGE with the exception that the band of
12-14,000 daltons was not detected at any acrylamide concentration.

It was apparent that gel concentration, buffer conditions,
the extent of solubilisation of HB_Ag prior to PAGE, and the
degree of purity of the preparations, were all likely to be
affecting the number and apparent molecular weights of the
polypeptides detected. These variables were therefore
examined in more detail, using $^{125}$I-HB_Ag purified further by
gel filtration in Sepharose 6B and rate-zonal centrifugation,
as previously described (Section 2.2.1).

3.5.2. PAGE of radiolabelled HB_Ag.

a. HB_Ag labelled with $^{125}$I using chloramine T

Preparations of $^{125}$I-HB_Ag labelled by chloramine T
oxidation were disrupted as above (Section 3.5.1), alkylated
with 0.07M-iodoacetamide and dialysed overnight (Section 2.7.2.)
before PAGE analysis. Using the continuous buffer system
and 10% acrylamide gels most of the radioactivity remained
at the gel origin; minor peaks of 96,000, 79,000, 60,000,
52,000, 45,000, 31,000 and 26,000 daltons 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 either the number or apparent size of the polypeptides seen. Addition of 8M-urea prior to disruption and inclusion of 4M-urea in the acrylamide gels had no effect on the polypeptide profile obtained.

Using a discontinuous buffer system and 10% acrylamide gels there was only a small accumulation of label at the gel origin and two polypeptides of 60-65,000 and 12-14,000 daltons were consistently observed (Fig. 16a). Minor peaks of 23,000 and 17,000 daltons were observed in some batches of 125I labelled HBsAg. The detection of these minor polypeptides correlated with the observation of diffuse stained bands of the same molecular weight after disc-PAGE of unlabelled HBsAg purified from the same plasma source.

The possibility that material larger than 12,000-14,000 daltons 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 different tris/glycine buffers at pH values up to 9.5 (mobility = -15 units).

Incubation for 72h 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.
Fig. 16. Distribution of radioactivity after disc-PAGE of $^{125}$I-labelled HBsAg polypeptides using different acrylamide concentrations. (a) 10% acrylamide. (b) 12.5% acrylamide. (c) 15% acrylamide. Estimated molecular weights $\times 10^{-3}$ are indicated.
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 still resolved as a single sharp peak of 12,000 daltons but an increasing proportion of the radioactivity remained at the gel origin and as background along the length of the gel; no new peaks were detected (Fig. 16b & c). Inclusion of 4M urea in the gel system had no effect on the polypeptide profile obtained.

b. HB\textsubscript{Ag} labelled with \textsuperscript{125}I after disruption

HB\textsubscript{Ag} was disrupted by boiling with SDS and DTT prior to chloramine T radiolabelling, to establish whether there were any buried tyrosine residues inaccessible to labelling in the intact particle. The polypeptide profile of antigen labelled after disruption was compared to that of \textsuperscript{125}I-HB\textsubscript{Ag} of the same purity labelled and then disrupted in the normal way. In both instances disc-PAGE on 10% acrylamide gels revealed two major polypeptides of 60-65,000 and 12-14,000 daltons, and two minor polypeptides of 23,000 and 17,000 daltons. A fifth, minor, polypeptide of 40,000 daltons was detected only in HB\textsubscript{Ag} labelled after disruption. Since this polypeptide was not detected in \textsuperscript{125}I labelled whole HB\textsubscript{Ag} particles, or in stained gels of antigen purified from the same plasma source it may represent a minor, presumably internal, component of the HB\textsubscript{Ag} particle.
Disc-PAGE of HBsAg labelled with $^{125}\text{I}$-ester which attaches $^{125}\text{I}$-labelled groups by amide bonds to free $\text{NH}_2$ groups in proteins, revealed the same basic polypeptide profiles as with HBsAg labelled by chloramine T. Differences in the relative distribution of radiolabel were however apparent. No aggregation of radiolabelled protein at the origin of disc gels occurred with ester labelled HBsAg, and there appeared to be a preferential labelling of the polypeptide of 60-65,000 daltons (Fig. 17).

In all continuous and discontinuous gels internal marker proteins migrated according to the logarithm of their molecular weight.

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 radiolabelled components of 60-65,000 and 12-14,000 could be resolved. These components correlated closely with the two major polypeptides reproducibly detected by Coomassie Blue staining.

The occurrence of the two minor components of 17,000-24,000 daltons and 23,000-30,000 daltons was variable. In batches of purified antigen in which these two polypeptides were detected by Coomassie Blue staining they could also be seen as minor peaks after disc gel analysis of $^{125}\text{I}$ labelled samples of the same antigen. When present these components always
Fig. 17. Distribution of radioactivity after disc-PAGE analysis of HBeAg radiolabelled with $^{125}$I-ester. 10% acrylamide gel. Estimated molecular weights $\times 10^{-3}$ are indicated.
occurred together in approximately 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.

3.5.3. **Serological activity of polypeptides separated by PAGE.**

$^{125}$I-HB$_s$Ag was disrupted by SDS and DTT and separated by disc gel electrophoresis. Polypeptides of 60-65,000 daltons and 12-14,000 daltons were eluted from crushed gel slices and dialysed extensively (Section 2.9.1.). No renaturation of serological activity was detected in either of these polypeptides following DA-RIP assay with either anti-HB$_s$ or anti-whole human serum.

3.5.4. **PAGE of material released from 25S component.**

$^{125}$I-HB$_s$Ag preparations were treated with 1% SDS and 0.065M-DTT at room temperature for 15 min, alkylated with 0.07M iodoacetamide and centrifuged on sucrose velocity gradients as previously described (Section 3.4.3.a). Radiolabelled material released from the particles during formation of the 25S component was analysed by continuous buffer PAGE in 10% acrylamide gels.

Immediate examination of the released material revealed a single component of 83-87,000 daltons, but after boiling for 2 min with SDS and DTT before electrophoresis this large component dissociated to give a single polypeptide of 14,000 daltons (Fig. 18a & b). Mild denaturation of HB$_s$Ag therefore led to the initial release of an homogeneous aggregate of a smaller
Fig. 18. Radioactivity profile of material released during formation of 2S component, following analysis on 10% continuous acrylamide gels. (a) material examined immediately after release from the particle. (b) identical material following disruption with 1% SDS and 0.065M-DTT at 100°C for 2 min. Estimated molecular weights x10^{-3} are indicated.

![Diagram showing radioactivity profile](image-url)
polypeptide which required further heating in the presence of reducing agent for its complete dissociation. Such aggregates may be related to the polypeptides of 82,000 and 90,000 daltons described by Howard & Zuckerman (1974).

Protein released during the 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 14,000 daltons. These results suggest that the major 12-14,000 polypeptide detected by disc electrophoresis 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.

However, in view of the effect of different electrophoretic conditions on polypeptide profiles independent analyses of HB Ag polypeptides were made using column chromatography in various denaturing solvents.
3.6. Analysis of HB Ag polypeptides by column chromatography

3.6.1. Guanidine hydrochloride (GuHCl).

a. Disruption of $^{125}$I-HB Ag

Samples of $^{125}$I-HB Ag were disrupted with GuHCl, DTT, Triton X-100 and EDTA and chromatographed on 60ml columns of Sephadex G-200 or Sepharose 6B, as described in Section 2.8.2. A large proportion of the $^{125}$I-label eluted in the void volume of both G-200 and 6B columns, along with a single peak corresponding to 60-70,000 daltons.

b. Serological activity

Attempts to recover serologically active material by extensive dialysis of either the void volume or the 60-70,000 dalton peak, and monitoring by DA-RIP were unsuccessful. Some precipitation of this labelled material with rabbit anti-HB Ag occurred, but the detection of equivalent levels of precipitation with pre-immune rabbit serum indicated that this represented non-specific aggregation and precipitation of labelled protein during the RIP assay.

3.6.2. Sodium dodecyl sulphate.

$^{125}$I-HB Ag was disrupted and alkylated as for PAGE and chromatographed on Sepharose 6B equilibrated with Na-phosphate or tris/glycine buffer (Section 2.8.1.). Identical results were obtained with both buffer systems; a small proportion of radiolabel eluted in the void volume (peak I) of the column and a minor polypeptide of 60-65,000 daltons (peak II) and a major polypeptide of 15-20,000 daltons (peak III) were detected (Fig. 19).
Fig. 19. Distribution of radioactivity following column chromatography of $^{125}$I-HB Ag after heating at 100°C for 2 min in 1% SDS and 0.065M-DTT. A 1.6 x 15 cm column of Sepharose 6B was eluted with 0.1M-Na phosphate and 0.1% SDS, pH 7.2, at a flow rate of 1.5 ml/hour and 0.5 ml fractions collected. Estimated molecular weights x10^{-3} are indicated. Vo: void volume.
Direct analysis of the 15-20,000 dalton peak from the column by acrylamide gel electrophoresis in the corresponding buffer system (i.e. tris/glycine buffered column — disc gel; Na-phosphate buffered column — continuous gel) revealed a major polypeptide of 12-14,000 daltons plus a range of heavier components from 40,000 to 17,000 daltons (Fig. 20a). Boiling with SDS and DTT prior to electrophoresis increased the proportion of radiolabel in the 12-14,000 dalton peak but did not eliminate any of the heavier components (Fig. 20b).

Examination by disc PAGE of the 60-65,000 dalton peak from a tris/glycine buffered column revealed a major polypeptide of 12-14,000 daltons plus a number of heavier peaks ranging from 100,000 to 17,000 daltons (Fig. 21). Again, further disruption of the sample prior to electrophoresis increased the relative amount of the smallest polypeptide but did not remove any of the larger polypeptides. In contrast to this, examination by continuous PAGE of a similar 60-65,000 dalton polypeptide isolated on an Na-phosphate buffered column revealed only a single broad peak of 80-100,000 daltons (Fig. 22a). When material from this same column fraction was examined by disc gel electrophoresis without any further disruption, it migrated as a single homogeneous peak of 12-14,000 daltons (Fig. 22b). These results are summarised in Table 3.

Column chromatography of $^{125}$I-I-HB Ag in SDS therefore revealed a polypeptide profile similar to that obtained by disc gel electrophoresis. PAGE analysis of radiolabelled peaks separated by column chromatography however revealed a range of additional components that were not detected when identical samples of $^{125}$I-labelled antigen were examined directly by disc PAGE.
Fig. 20. Distribution of radioactivity after continuous PAGE of peak III (fig. 19) isolated by column chromatography in 0.1M-Na phosphate buffer and 0.1% SDS, pH 7.2. (a) Material examined without further disruption, 10% acrylamide gel. (b) Material examined after disruption with 1% SDS and 0.065M-DTT at 100°C for 2 min, 10% acrylamide gel. Estimated molecular weights x10^{-3} are indicated.
Fig. 21. Radioactivity profile after disc PAGE of peak III isolated by column chromatography in 0.15% SDS, 0.025M-tris/HCl, 0.192M-glycine, pH 8.3. 10% acrylamide gel. Estimated molecular weights x10^-3 are indicated.
Fig. 22. Distribution of radioactivity following PAGE analysis of peak II isolated on a 0.1M-Na phosphate buffered Sepharose 6B column. (a) continuous PAGE system, 10% acrylamide gel. (b) disc-PAGE system, 10% acrylamide gel. Estimated molecular weights $x10^{-3}$ are indicated.
Table 3  Polypeptides detected by PAGE of column chromatography

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<th>peak III 15-20,000 daltons</th>
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<td>continuous</td>
<td>discontinuous</td>
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<td>0.1M-Na phosphate + 0.1% SDS</td>
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<td>12-14,000 major</td>
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<tr>
<td>0.025M-tris/HCl &amp; 0.192M-glycine + 0.1% SDS</td>
<td>N.D.</td>
<td>12-14,000 major</td>
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3.7. Analysis of 22nm HBsAg particle structure by electron microscopy

3.7.1. Untreated HBsAg

22nm particles purified by column chromatography and two cycles of equilibrium gradient centrifugation were examined by electron microscopy following staining for 10 min with 2% uranyl acetate, pH 4.5. Spherical particles with diameters ranging from 14-26nm were observed, with 80% of the particles falling within the size range 18-24nm (Fig. 23). No structural subunits were detectable on the surface of the particles. Occasionally hollow ring forms of similar diameter were seen, the penetration of negative stain giving the appearance of a central hole of 4-8nm (Fig. 23). Filamentous forms of HBsAg were only rarely observed in preparations purified in this way.

Except for examination of gradient fractions all incubations with detergent, and/or reducing agent, were performed on antigen pre-adsorbed to carbon coated grids as described in Materials and Methods.

3.7.2. Effects of nonionic detergents

a. Tween 80

Treatment of purified antigen with 1% Tween 80 at 37°C for 3h caused a slight increase in overall particle diameter. The size heterogeneity of the preparation was also increased, which correlated well with the diffuse distribution of antigen on sucrose velocity gradients illustrated in Fig. 9. The majority of the particles now appeared as hollow ring forms with an electron-dense central hole of 4-8nm diameter. The presence of detergent had the effect of obscuring the surface of the particles and giving them a "fuzzy", indistinct outline.
Size distribution of spherical particles

Diameter in nanometers
Fig. 23. Electron micrograph of negatively stained preparation of purified, untreated HB Ag.

Final mag. 180,000 x.

R: hollow ring form.
Examination of peak fractions following sucrose gradient centrifugation of HB Ag treated with 5% Tween 80 at 37° for 24h (Fig. 9) revealed a major population of spherical particles of 16-20nm diameter, plus a minor population of 22-24nm (Fig. 24). Most of the spherical particles were present as irregular hollow ring forms with a central hole of 4-6nm diameter. In some cases the circumference of the particles was broken, giving the appearance of "horseshoe" shapes or incomplete circles. Similar closed ring shaped structures have previously been described by Sutnick and coworkers (1972) following Tween 80 treatment of purified antigen.

b. Triton X-100

The peak HB Ag fractions isolated by velocity gradient centrifugation after treatment with 5% Triton X-100 at 37°C for either 3 or 24h were examined by electron microscopy. A slight decrease in particle diameter, with 80% of the particles now falling within the size range 16-20nm, occurred following short incubation (3h) with Triton. No ring forms were seen, and there was no evidence of particle breakdown. Incubation for 24h did however have an obvious effect on particle structure. Two major populations of particles of 14-16nm and 18-20nm diameter were seen (Fig. 25). The larger of these was similar in size to that observed following shorter incubation periods with Triton, but differed morphologically in that a large proportion of the particles were present as closed or broken ring forms (Fig. 25). The circumference of many ring forms was irregular, and appeared to be breaking down to produce strands 4-8nm in width. The second major population
Size distribution of spherical particles

Diameter in nanometers
**Fig. 24.** Electron micrograph of peak density gradient fraction of HB$_5$Ag after incubation with 5% Tween 80 at 37°C for 24 h.

Final mag. 180,000 x.

R: hollow ring form.

B: broken ring form.
Size distribution of spherical particles

Size distribution of strands
Fig. 25. Electron micrograph of peak density gradient fraction of HB Ag after incubation with 5% Triton X-100 at 37°C for 24h.

Final mag. 180,000 x.

R: hollow ring form 18-20nm diameter.

B: broken ring form 18-20nm diameter.

S: strand 4-8nm wide.

SS: small spherical particles 14-16nm diameter.
of spherical particles (14-16nm) was present either as solid spheres or ring forms, but the relationship of these structures to either the larger spheres or the elongated strands was difficult to determine.

3.7.3. Sodium dodecyl sulphate.

The effect of SDS on 22nm HB Ag particles was markedly dependant on the ionic composition of the buffer solution. Incubation with 1% SDS in PBS, pH 7.2, at 37°C for 3h caused a marked breakdown in particle structure, resulting in an heterogeneous population of approximately spherical particles covering the size range from 8-26nm, with 80% of the particles falling within the limits 10-20nm diameter (Fig. 26). Many of these were present as either broken or closed ring forms, and in most instances the strand forming the circumference of the particle appeared to be made up of small circular, or possibly hexagonal structural units of 4-6nm diameter (Fig. 26). Many of these units appeared hollow, consisting of a strand 1.5-2.0nm in thickness enclosing a circular electron-dense area 2-3nm across. These small ring-like units were also observed in small random aggregates and in linear chains 4-6nm wide of varying lengths. Occasionally larger rod-like structures 16-18nm wide were seen apparently formed by helically coiled chains of the small circular units (Fig. 26).

Treatment with 1% SDS in 0.0625M-tris/HCl, pH 6.8, at 37°C for 3h had little effect on particle structure. Incubation under the same conditions for up to 24h did however cause
Size distribution of spherical particles

Size distribution of small spherical units
Fig. 26. Negatively stained preparation of HB$_6$Ag after treatment with 1% SDS in PBS, pH 7.2, at 37°C for 3h.

Final mag. 180,000 x.

U : 4-6nm subunits present in (1) circumference of ring forms.
(2) chain-like arrays.
(3) rod-like structures formed by helical coiling of chains of subunits.
particle breakdown. Approximately spherical particles, in the form of closed and broken rings covered the size range from 8-26nm, with 80% again falling within the 10-20nm range (Fig. 27). Strands 4-10nm in thickness were observed, either free or as part of the circumference of the rings which appeared to be breaking down. Small spheres of 8-10nm diameter were occasionally seen in association with those strands, and may indicate dissociation of the strands into individual spherical units.

Incubation with SDS in FBS, pH 7.2, therefore had a more rapid and more complete effect on particle structure than incubation in tris/HCl buffer at the same pH. The reasons for this effect of buffering conditions is not known, but Hirschman and coworkers (1973) have described the occurrence of relatively larger numbers of ring forms and uncoiling particles when purified untreated antigen was suspended in Na-phosphate buffer. A synergistic reaction between SDS and salts in the disruption of certain plant viruses including brome mosaic virus and cucumber mosaic virus has been described by Boatman & Kaper (1976). These viruses are however thought to be stabilised predominantly by protein-nucleic acid interactions rather than protein-protein interactions so the applicability of this observation to the disruption of HBsAg particles is difficult to assess.

3.7.4. Sodium dodecyl sulphate and reducing agent.

In an attempt to define the role of disulphide bonding in particle structure the effect of DTT alone, or DTT plus SDS was examined by electron microscopy. Incubation with 65mM-DTT at 37°C for 3h had no apparent effect on either particle size
Size distribution of spherical particles

Diameter in nanometers

%
Fig. 27. Negatively stained preparation of HB Ag after treatment
with 1% SDS in tris/HCl buffer, pH 6.8, at 37°C for 3h.
Final mag. 180,000 x.
R: hollow ring form.
B: ring form breaking down to strand 4-10nm thick.
SS: small spherical particles 8-10nm diameter.
or structure. However, treatment of antigen with a mixture of 1% SDS and 65mM-DTT in 0.0625M tris/HCl, pH 6.3, at room temperature for up to 5 min produced closed and broken ring forms of 6-28nm diameter (Fig. 28). There was some apparent breaking down of the circumference of these structures. Strands 4-10nm wide were also observed, which again seemed in some instances to be dissociating to small spherical units of 8-10nm diameter.

Separation of the above material by density gradient centrifugation led to the recovery of a 25S HB Ag component in the form of solid spheres and closed or broken ring forms of 14-20nm diameter (Fig. 29). The material released to the top of the gradient during the formation of this 25S component was examined after storage at 4°C for 10 days. Large, heterogeneous 27-60nm aggregates of coiled strands 1.5-2nm wide were observed, but there was no evidence of regular arrangement of small ring shaped structural units (Fig. 30). Reaggregation of these strands presumably occurred during storage at low temperature prior to examination.

Sequential treatment with DTT at room temperature for 10 min, followed by washing in distilled water for 60 sec and incubation with SDS in PBS at 37°C for 3h caused complete breakdown of particle structure. Hollow ring shaped units of 6-8nm diameter occurred either singly, or in apparently random aggregates, together with free strands of similar material (Fig. 31). Only occasionally were larger dissociating circular structures seen in these preparations. In contrast, incubation with SDS in
tris/HCl buffer for 3h after reduction caused very little breakdown in structure, which correlated with the relatively greater particle stability to treatment with SDS alone in the same buffer.

The results of the effects of detergent treatment on the structure of the 22nm HB₃Ag particle are summarised in Table 4.
Size distribution of spherical particles

Diameter in nanometers
Fig. 28. Electron micrograph of HB Ag incubated with 1% SDS and 0.065M-DTT in tris/HCl buffer, pH 6.8, at room temperature for 5 min.

Final mag. 180,000 x.

R: hollow ring form.

B: ring form showing breakdown of circumference.

S: strands 4-10nm wide.

SS: small spherical particles 8-10nm diameter.
Size distribution of spherical particles

Diameter in nanometers
Fig. 29. Electron micrograph of 25S component isolated by density gradient centrifugation of HB Ag after treatment with 1% SDS and 0.065M-DTT in tris/HCl at room temperature for 5 min.

Final mag. 180,000 x.
Size distribution of strands

Width in nanometers
Fig. 30 Negatively stained preparation of low molecular weight material released from HB Ag particles during formation of 25S component. Stored at 4°C for 10 days before examination. Final mag. 180,000x.

Arrows indicate coiled strands 1.5-2.0nm wide.
Size distribution of small spherical units

![Graph showing size distribution of small spherical units with diameter in nanometers on the x-axis and percentage on the y-axis. The graph has two bars: one at 8 nanometers with a percentage of 60% and another at 10 nanometers with a percentage of 20%.]
Fig. 31  Electron micrograph of HBsAg after sequential treatment with 0.065M-DTT at room temperature for 10 min followed by incubation with 1% SDS in PBS at 37°C for 3h.
Arrows indicate single subunits 6-8nm diameter.
(1) represent only a small proportion of total structures seen.
(2) occasionally seen in linear or helical arrangement.
(3) not clearly distinguishable on photographs.
(4) occur as heterogeneous 27-60nm aggregates after storage.
<table>
<thead>
<tr>
<th>particle structure</th>
<th>treatment</th>
<th>Stage of particle breakdown</th>
<th>I</th>
<th>II</th>
<th>III</th>
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<td>Closed ring forms 16-24nm diam.</td>
<td>Broken ring forms</td>
<td>Strands 4-10nm thick</td>
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<tr>
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<td>37°C 3h</td>
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3.7.5. **Incubation at high pH.**

Samples of purified HBsAg were incubated in 0.1M-carbonate-
bicarbonate buffer, pH 10.6, at 37°C for up to 5 days and samples
removed at intervals for electron microscopic examination.

After 16 hours incubation the majority of particles were
present as spheres of slightly larger diameter than the control
population. 60% of these particles fell within a diameter range
of 20-28nm but occasional larger spheres up to 38nm in diameter
were also seen (Fig. 32a). Most of the particles appeared
solid but in some instances the central region of the particle
had broken down to produce closed ring forms with a less dense
interior. Occasional filaments were also seen which in many
instances showed indentations along the surface suggesting that
they had arisen by end-to-end association of spheres. In
contrast, random clumps of spheres were rare (Fig. 32a).
The filaments were generally solid but some breakdown and stain
penetration along the long axis was occasionally seen (Fig. 32b).

After 48h incubation the proportion of spherical particles
present as closed ring forms had increased. Breakdown of the
material in the centre of these particles had progressed to a
stage where ring forms had a small central or eccentric patch
of material inside the lumen of the ring. In some instances
this central material seemed to be attached to the perimeter
of the particle at at least one point (Fig. 33a). Up to eight
closed ring forms were seen lined up end-to-end to form slightly
curved filaments (Fig. 33b). In some cases the circumference
of each ring form remained recognisable but in others the
filaments were made up of one continuous strand. Stain penetration
along the long axis of these filaments was observed and in one
instance the strand making up the outer layer of the filament
Size distribution of spherical particles

Diameter in nanometers
Fig. 32. Negatively stained preparation of HB$_3$Ag following incubation at pH 10.6, 37°C for 16h.

Final mag. 180,000 x.

a. R: ring form with less dense interior.

F: filament showing indentations along surface.

b. F: filament showing stain penetration along long axis.
Fig. 33 Negatively stained preparation of HB$_{Ag}$ incubated at pH 10.6, 37°C for 48h.
Final mag. 180,000x.

a. ring forms containing variable amounts of internal, electron dense material.
b. filament made up of eight spherical particles joined end to end.
c. filament showing central stain penetration and breakage of the outer strand on one side.
appeared to be broken on one side (Fig. 33c).

After 5 days at high pH most of the spherical particles were present as closed ring forms with varying amounts of internal material and only occasional filaments were seen. In many instances the strand making up the circumference of a ring shaped particle appeared to be made up of small hollow units 4-6 nm in diameter (Fig. 34).

Broken ring forms were only occasionally seen in these preparations.

3.7.6. Incubation at high temperature.

Treatment of purified antigen in PBS at 100°C for 1-5 min had a very marked effect on particle structure. The spherical particles increased in diameter to 22-30 nm and associated end-to-end to form solid filaments. After boiling for 1 min short filaments, some of which showed indentations at regular intervals along the surface, were observed (Fig. 35a). Increasing the time of boiling led to an increase in both the total number and the average length of these filaments. Bending, and occasional formation of very large curved forms indicated that the filaments were flexible rather than rigid structures (Fig. 35b); branching of filaments was also seen, but random clumps of particles were never observed. There was no evidence to suggest partial breakdown to ring forms or smaller structural units before the reassociation of spherical particles into filaments.
Fig. 34  Negatively stained preparation of HB$_2$Ag incubated at pH 10.6, 37°C for 5 days.
Final mag. 180,000x.
Ring shaped particles showing variable amounts of internal material.
Arrow indicates small, 4-6nm subunits in circumference of
Fig. 35  Electron micrograph of HB Ag heated to 100°C for 1-5 min.

a. 100°C for 1 min: short filaments showing indentations along the surface. Final mag. 180,000x.

b. 100°C for 5 min: long, flexible filaments. Final mag. 48,000x.
3,8. **Stages in the dissociation of 22nm HB Ag particles**

The results presented here of the disruption of 22nm HB Ag particles allowed the identification of several intermediate structures between solid 18-24nm spheres and completely dissociated particles. The extent of disruption by any particular set of denaturing conditions differed, but many of the intermediate structures appeared to be common to more than one treatment. On this basis the following sequential dissociation pattern, which involves at least three stages, is suggested.

**Stage I.** Production of ring forms from solid spheres.

Ring forms produced by incubation at high pH were of slightly larger diameter than solid spheres and frequently contained a central or eccentric patch of material within their lumen. Closed or broken hollow ring forms of variable diameter from 10-22nm were observed after treatment with both nonionic and anionic detergents, or with a combination of anionic detergent and reducing agent. Evidence that these ring forms represented an early stage of particle breakdown came from two sources. (i) Similar closed ring structures were occasionally observed in control preparations of untreated antigen. (ii) The 25S component, which was a reproducible early stage in breakdown, also had a closed or broken ring conformation.

**Stage II.** Strands 4-6nm wide and variable length were commonly seen either free, or in association with broken hollow ring forms. Following incubation with SDS in PBS these strands appeared to be composed of hollow ring-like units of 4-6nm diameter. Random, linear and regular two-dimensional aggregates of these smaller rings were also seen. Units of similar
diameter and appearance were also seen in the strands making up the circumference of the 20-28nm closed ring forms (Stage I) produced by incubation at high pH.

Stage III. Reduction of antigen particles prior to SDS treatment caused dissociation of the above aggregates to their individual 4-6nm ring shaped units made up of a fine strand 1.5-2nm wide enclosing a central electron dense area of 2-3nm. The material released from the particle during production of the 25S component formed large aggregates of fine strands of similar width.

As can be seen from the electron micrographs, the various stages of particle dissociation outlined above were not stable end-products of a particular type of treatment; within a small area of the grid structures representative of more than one of the postulated disruption stages could usually be seen.
3.9. **Structural models for HB$_{Ag}$ particles**

3.9.1. **Components.**

It is proposed that the 4-6nm hollow ring-like forms described above (Stage III) represent basic structural units of the HB$_{Ag}$ particle. These rings could be seen on the surface of relatively intact particles (Stage I), as linear strands and lattice formations after partial particle dissociation (Stage II), and as single isolated units after extensive disruption (Stage III).

Partial breakdown of particles allowed the identification of more diffusely staining central material. This was very variable in both amount and appearance and occurred as a central dot with fine strands radiating out to the inner surface of the outer strand (Fig. 36a), as a central solid or ring shaped structure (Fig. 36b), or as more amorphous eccentrically situated material (Fig. 36c). Evidence for continuation of a surface strand to form the central material was occasionally seen (Fig. 36d) but it was not usually possible to establish whether this central material was made up of the same morphological units as the outer strands.

3.9.2. **Conformation.**

a. **Spherical particles**

In relatively intact particles where stain penetration still allowed identification of surface subunits, two major patterns were seen. In one, up to 12 ring shaped units were seen arranged around the periphery of the particle (Fig. 37a), and in the other, 2 or 3 rows of subunits appeared to cross the particle transversely (Fig. 37b). Many particles showed a curved eccentric row of
Fig. 36  Electron micrographs of spherical HbAg particles showing varying amounts of internal breakdown.

Final mag. 180,000x.
Fig. 37 Negatively stained preparation of spherical HBAg particles showing arrangement of individual subunits. Final mag. 180,000x.
subunits; this appearance suggested strands wound parallel to the equator, in particles lying tilted to the vertical axis.

b. Filaments

Filaments produced by boiling had similar staining properties as untreated preparations whereas those formed after alkali treatment frequently showed considerable stain penetration. The marked tendency for particles to aggregate in linear chains rather than as random clumps suggested some form of two-dimensional symmetry. Two types of linear aggregation were seen. In one, adjacent particles were flattened at their points of contact (Fig. 38a); two or three rows of hollow 4-6nm ring-like subunits were sometimes seen crossing the filament transversely (Fig. 38b), suggesting that aggregation of the spherical particles was occurring away from the equatorial region, i.e. at the poles. In the other type, which occurred only after alkali treatment, closed or broken ring forms (Stage I) lying adjacent to one another appeared to be joining at their circumferences (Fig. 39); in this type of aggregation central material was often retained within the hollow filament.

3.9.3. Conclusions.

A possible structural model of the 22nm HB 5 Ag particle is represented diagrammatically in Fig. 40. Individual 4-6nm ring shaped subunits (a) (Stage III) can assemble in a linear fashion to form chain like arrays (b) (Stage II). These chains, containing approximately 12 individual subunits can then join up in a circular manner to form the frequently observed hollow ring shaped particles (c) (Stage I). In some instances the
Fig. 38  Electron micrographs of filaments produced by boiling or incubation at high pH.
Final mag. 180,000x.

Fig. 39  Electron micrographs of filaments produced by incubation at high pH.
Final mag. 180,000x.
centre of these particles showed stain penetration and therefore appeared hollow, whereas in others variable amounts of amorphous material was visible within the particles. Neither the chemical nature nor the source of this internal material, whether of host or viral origin, has yet been established. Association of bands of 4-6nm subunits either by stacking one on top of another (di) or by spiral winding (dii) may give to the complete 22nm HB\textsubscript{Ag} particle as normally observed. Heterogeneity of particle size may be due to the enclosure of varying amounts of internal material by bands which vary in length, and possibly also in number. The size of the assembled particle is therefore not dependent on a fixed geometrical pattern of interlocking subunits as occurs in viruses of similar dimensions which possess icosahedral symmetry. Differences in surface properties between the equator and the poles of the particles may allow linear aggregation to occur under certain conditions, but it is not yet clear whether the individual subunits are arranged round the particle in a stacked or spiral configuration.
Fig. 40. Diagrammatic representation of possible structural model of spherical 22nm HB$_2$Ag particle.
DISCUSSION
4.1. Polypeptide composition of HB Ag

4.1.1. Basis of the SDS-PAGE system of polypeptide molecular weight determination.

The use of SDS-PAGE for the separation of polypeptides of different sizes was based on the experimental observation of Shapiro and coworkers (1967) that the relative migration of a polypeptide under these conditions was directly proportional to its molecular weight. Subsequently equilibrium dialysis studies established the theoretical basis of this observation by demonstrating that the weight of SDS bound per gram of protein (1.4g SDS per g protein) was identical for a wide range of hydrophilic proteins (Pitt-Rivers & Impiombato, 1968). The large amount of bound SDS was sufficient to overcome the intrinsic charge on most protein molecules and the constant ratio of bound SDS to protein produced molecules with a total negative charge proportional to their size (Reynolds & Tanford, 1970a). Hydrodynamic studies showed that the SDS-protein complexes assumed a rod-shaped configuration in which the short axis was constant and the long axis varied in proportion to the molecular weight (Reynolds & Tanford, 1970b).

The original SDS-PAGE technique of Shapiro used a continuous buffer system containing 0.1M-Na phosphate, pH 7.2, throughout. Later, the discontinuous PAGE system using tris/HCl buffers at different molarities and pH values (Ornstein, 1964) was adapted by Laemmli (1970) for use in the presence of SDS. This latter system gave clearer resolution of individual protein bands due
to the "stacking" of polypeptides in a low acrylamide concentration gel at pH 6.8, before migration into a high acrylamide concentration resolving gel at pH 8.8 (Fraenkel-Conrat & Rueckert, 1967). Apart from the improved polypeptide resolution of the discontinuous system the basis of separation of protein-SDS complexes by both PAGE systems was thought to be identical (Laemmli, 1970).

However, at least three different experimental situations have been described in which the direct relationship between polypeptide molecular weight and distance migrated in an SDS acrylamide gel appeared to break down.

(a) Three enzymes, glucose oxidase, papain and pepsin, were found to bind little or no SDS (Nelson, 1971) and hence represented exceptions to the general rule for hydrophilic proteins. Conversely, certain hydrophobic proteins including the envelope protein of Semliki Forest virus (Simons & Kaariainen, 1970), and the apoprotein component of human plasma low density lipoprotein (Simons & Helenius, 1970) bound very large amounts of SDS. These proteins migrated anomalously on SDS gels when compared to hydrophilic proteins used as molecular weight markers.

(b) SDS-PAGE has given spurious molecular weight estimates for polypeptides containing attached carbohydrate residues. Decreased binding of SDS to the oligosaccharide side chain relative to the protein backbone resulted in glycoproteins having a lower charge to mass ratio than standard polypeptides. The effect of this on the SDS-glycoprotein complex was greater at low acrylamide concentrations where the sieving effect of the gel was least and resulted in a decrease in apparent molecular weight of the
glycoprotein as the acrylamide concentration increased
(Segrest et al., 1971; Bretscher, 1972). A second explanation
for the low mobility of glycoproteins in SDS-PAGE, namely that
the oligosaccharide side chain extends laterally from the
SDS-protein rod with a consequent increase in Stokes’ radius,
has been suggested by Tanford (1973). Variations in the ratio
of protein to carbohydrate moieties in individual glycoprotein
molecules may also occur, thereby leading to heterogeneous bands
in acrylamide gels (Sokol et al., 1971).

(c) Although in most instances the intrinsic charge on a protein
molecule is completely overcome by the large amount of bound
dodecyl sulphate, some exceptions to this rule do exist. The
histones, although they bind the same amount of SDS as other
proteins, carry a high positive charge which reduces the net
negative charge of the bound SDS (Williams & Gratser, 1971; Banker
& Cotman, 1972). Variations in intrinsic charge were also thought
to account for minor differences in molecular weight between
the structural polypeptides of different strains of cucumber mosaic
virus (Tung & Knight, 1971) and tobacco rattle virus (Chabrial
& Lister, 1973) and between the \( \alpha_1 \) and \( \alpha_2 \) chains of collagen
(Svjetkova, Deyl & Adam, 1973), which were detectable by SDS-PAGE
but not by other techniques.

4.1.2. Discrepancies between discontinuous and continuous
gel profiles.

Marked discrepancies between the continuous and disc PAGE
profile of HB\(_8\)Ag were observed in this work (Section 3.5.2.).
In the disc system there was little aggregation of \(^{125}\)I-labelled
material at the gel origin and two major polypeptides of 60-65,000
and 12-14,000 daltons were detected whereas in the continuous system the majority of the radiolabel remained as non-migrating material at the top of the gel. Seven peaks of $^{125}$I-label covering the molecular weight range from 96,000 to 26,000 daltons were detected in the continuous system, but these represented only a fraction of the total label applied to the gel.

Discrepancies have been noted between continuous and discontinuous PAGE molecular weight estimates of a structural protein of Bacillus subtilis phage \( \Phi 29 \) (Camacho et al., 1975) and between discontinuous PAGE profiles of foot and mouth disease virus polypeptides using SDS from different commercial sources (Swaney, van de Woude & Bachrach, 1974). In each case altering the conditions of PAGE led to inversion of the relative mobilities of two polypeptides of similar size. However, neither study reported aggregation of polypeptides in the continuous gel system, which was a marked feature in the present work.

Variable, minor levels of aggregation of Newcastle disease virus polypeptides at the origin of continuous acrylamide gels have been observed (Mountcastle, Compans & Choppin, 1971) but no explanation of this finding was suggested.

4.1.3. Additional evidence for reaggregation and/or lack of dissociation of HB$_{Ag}$.

The experimental observations in this work suggested that both incomplete dissociation and a tendency towards reaggregation were significant problems in the investigation of HB$_{Ag}$ polypeptides.
a. Reaggregation.

(i) The absence of a significant peak in the void volume following SDS-column chromatography of $^{125}$I-HB$_g$Ag in both continuous and discontinuous buffers (Section 3.6.2.) indicated that little high molecular weight material remained after boiling with SDS and reducing agent. The accumulation of radiolabel at the origin of continuous gels must therefore have occurred through reaggregation, rather than simple lack of dissociation of particles following this treatment.

(ii) The multiplicity of radiolabelled peaks detected by disc PAGE in peaks II & III of the SDS-column chromatography profiles indicated that reaggregation of separated polypeptides during storage and laboratory investigation was a major problem.

b. Lack of dissociation.

(i) The stepwise solubilisation of $^{125}$I-HB$_g$Ag particles following heating in SDS and reducing agent, and the stability of the 25S component (Section 3.4.3.) indicated that under certain conditions HB$_g$Ag was markedly resistant to the standard dissociating techniques used for the polypeptide analysis of virus particles and hydrophilic proteins (Shapiro & Maizel, 1969; Weber & Osborn, 1969).

(ii) The release from the particle during the formation of the 25S component, of a polypeptide complex of 83–87,000 daltons which could be dissociated to a single major peak of 12–14,000 daltons following further heating in the presence of reducing agent (Section 3.5.4.) demonstrated the existence of stable aggregates of smaller polypeptides.
Finally, the observation that a peak of $^{125\text{I}}$-label with an apparent molecular weight of 60-65,000 daltons (peak II) by SDS-column chromatography in phosphate buffer (Section 3.6.2.) migrated with an apparent molecular weight of 80-100,000 daltons by continuous PAGE and 12-14,000 daltons by disc PAGE can be taken as evidence for either reaggregation or lack of dissociation during continuous buffer PAGE and column chromatography.

Problems of reaggregation and non-dissociation in acrylamide gel systems have been observed with certain hydrophobic proteins including bovine cell membrane (Maddy & Dunn, 1973; Green et al., 1974) and brain myelin proteins (Katzman, 1971; Morell, Wiggins & Grey, 1975). The presence of relatively large amounts of lipid in the HB$_{Ag}$ particle (Jozwiak et al., 1971) suggests that certain regions of the polypeptide chains are likely to contain a high proportion of hydrophobic amino acids. Hence their behaviour on polyacrylamide gels is more likely to be analogous to hydrophobic membrane proteins than to the predominantly hydrophilic proteins usually examined by this technique.

The results in this work clearly demonstrated the anomalous behaviour of HB$_{Ag}$ polypeptides following dissociation and analysis in phosphate buffer. On the other hand, immediate disc PAGE produced consistent results in which no evidence of reaggregation or incomplete dissociation was demonstrated. The reliability of this latter technique is therefore considered further in the following section.

4.1.4. Discontinuous PAGE profile of HB$_{Ag}$ polypeptides.

a. General considerations

Two major polypeptides were detected by disc PAGE of purified the unlabelled HB$_{Ag}$ and in/fame material after radiolabelling with
The larger of these migrated with an apparent molecular weight of 65-70,000 daltons before radiolabelling and 60-65,000 daltons after attachment of $^{125}\text{I}$ by chloramine T or $^{125}\text{I}$-ester. The reasons for this decrease in apparent molecular weight following iodination are not clear and the molecular weight of this polypeptide can only be defined as lying somewhere between 60,000 and 70,000 daltons.

Two minor polypeptides of 17-24,000 daltons and 23-30,000 daltons were detected in unlabelled and $^{125}\text{I}$-labelled antigen from certain sources, and a fifth, minor polypeptide of 40,000 daltons was detected only in antigen which had been disrupted before iodination. Thus the major polypeptides detected by Coomassie blue staining were accessible to radiiodination using chloramine T.

The value of 12-14,000 daltons for the smaller of the two major polypeptides must be considered an estimate since the relationship between molecular weight and mobility in SDS solutions has been found to break down for polypeptides of less than 14,000 daltons (Williams & Gratzer, 1971). Below this size, rod shaped protein-SDS complexes begin to approximate spheres and no longer separate on acrylamide gels in direct proportion to their molecular weight (Trayer et al., 1971; Weber & Osborn, 1975).

b. Variations in profile

1. Relative amounts of individual polypeptides.

The 60-70,000 dalton polypeptide represented a greater proportion of the total protein in both unlabelled HB$_s$Ag and in
antigen labelled by $^{125}$I-ester (Section 3.5.1. & 3.5.2a) than it did in material iodinated by chloramine T oxidation (Section 3.5.2a). These results may suggest an internal localisation of the tyrosine residues of this polypeptide since Montelaro & Rueckert (1975) have demonstrated that at low iodide concentrations, chloramine T iodination labelled only the external polypeptides of intact Rous associated virus, a typical enveloped virus. However the labelling procedure used by these authors differed markedly from that used in the present work so no direct correlation with these results was possible.

ii. Effect of resolving gel concentration.

The resolving gel concentration significantly affected both the proportion of material migrating with a molecular weight of 12-14,000 daltons (Section 3.5.1. & 2.) and the apparent molecular weights of the two minor polypeptides of 17-24,000 and 23-30,000 daltons. Neither of these variations in relative amount or apparent molecular weight of these polypeptides correlated with any of the well characterised SDS-PAGE anomalies described earlier (Section 4.1.1.a-c).

4.1.5. Comparison with published polypeptide profiles of HB$_3$Ag.

Variations in the total number and molecular weight of the individual polypeptides of 22nm HB$_3$Ag particles have been described using purified antigen from the same source (Gerin et al., 1971; Gerin, 1972; Shih & Gerin, 1975), antigen from different sources but of the same subtype (Shih & Gerin, 1975) and between antigens differing in major subtype (Shih & Gerin, 1975; Chairez et al., 1975). Most workers have however detected between five and nine separate polypeptides, a surprisingly large number in view of the
small size of the genome of the probable virus candidate of hepatitis B, the Dane particle (Section 1.5.2.). This apparent discrepancy between the coding capacity of the genome and the total number of polypeptides detected could be explained in one of four possible ways.

a. Association of host proteins with HB$_Ag$

Although there is immunological evidence of an association between host serum components and purified 22nm HB$_Ag$ particles (Section 1.4.3d) it is not clear yet whether this represents incorporation into the particle of the host components themselves, or whether the HB$_Ag$ polypeptides carry antigenic sites which cross-react with antibodies to human serum proteins. Variations in the method of purification (Section 1.4.1c) and in the procedures used for assessing the purity of a 22nm HB$_Ag$ particle preparation could have marked effects on the amount of contaminating host material and hence on the polypeptide profile obtained. Analysis of HB$_Ag$ polypeptides at various stages of purification revealed a decrease in the amount of number of high molecular weight polypeptides (Section 3.5.1b) indicating that these probably represented associated serum proteins.

b. Estimates of the coding capacity of the hepatitis B virion

The circular double-stranded DNA molecule of $1.6 \times 10^6$ daltons found as an internal component of the Dane particle (Section 1.5.2.) contains sufficient nucleic acid to code for approximately 100,000 daltons of unique amino acid sequence. The total number of polypeptides detected in the various particulate forms of HB$_Ag$
therefore far outweighs the expected coding capacity of the genome. However, it has been demonstrated that a large proportion of the Dane particles in serum contain relatively small amounts of nucleic acid as determined by electron microscopy (Gerin, Ford & Purcell, 1975), and that the $1.6 \times 10^6$ dalton circular DNA molecules show considerable length heterogeneity (Robinson, 1974). If the latter molecular weight estimate was based on a preparation containing a large number of defective Dane particles a more accurate estimate of the true size of the hepatitis B genome may follow from the purification and analysis of the "heavy" subpopulation of Dane particle cores.

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**c. Polypeptide cleavage**

Cleavage of a high molecular weight polypeptide could occur either by specific intra-cellular post-translational cleavage as happens during the maturation of enteroviruses (Summers & Maisel, 1968), or non-specifically through exposure to enzymes during circulation in the host and laboratory storage. The minor polypeptides of 17-24,000 and 23-30,000 daltons described in this study (Section 3.5.1. & 2.) always occurred together in approximately equal amounts when present, and thus may have arisen by cleavage(s) of a larger molecule. Variable proteolytic degradation of human erythrocyte membrane proteins due to the action of leucocyte proteinases has been recognised (Fairbanks, Steck & Wallach, 1972); it is reasonable to expect that the proteins of HBsAg prepared from donations of blood may be susceptible to similar proteolytic action. Although evidence for the occurrence of polypeptide cleavage could be obtained from
trypic peptide analysis of HB₃Ag, in vitro cultivation of virus and pulse-chase experiments would provide a more direct demonstration of specific post-translational cleavage.

d. Reaggregation or incomplete dissociation of polypeptides

Dreesman and coworkers (1975) have suggested that the polypeptides of HB₃Ag may be comprised of polymers of smaller polypeptides with similar properties. The results described in the present work have indicated that both aggregation and incomplete dissociation of HB₃Ag were frequent occurrences.

The observation by Garin (1974) that the two polypeptides of 23,000 and 30,000 daltons had an essentially identical amino acid composition, and the demonstration that most, if not all, of the polypeptides of the 22nm particle carry both group and subtype antigenic specificities (Dreesman et al., 1975; Shih & Gerin, 1975) could be explained by either of the last two alternatives described above.

4.1.6. Summary

The present study clearly demonstrated that incomplete dissociation and reaggregation of HB₃Ag polypeptides, and discrepancies between different electrophoretic techniques, have produced major problems in the analysis of HB₃Ag polypeptides by SDS-PAGE. Column chromatography in SDS, in which separation is dependant on the same hydrodynamic properties of the protein-SDS complex (Fish, Reynolds & Tanford, 1970) is open to the same criticism. When these factors were allowed for as far as possible two major polypeptides of 60-70,000 and 12-14,000 daltons were consistently detected. However in view of the limitations of the above techniques for examining this material these estimates should at present be considered as provisional.
4.2. Effect of denaturing agents on 22nm HB_Ag particles

4.2.1. Comparison between unlabelled and $^{125}$I-labelled HB_Ag.

Solubilization of HB_Ag by Triton X-100 was the only instance in which marked discrepancies were seen between the behaviour of unlabelled and iodinated antigen. With the latter material no breakdown of antigen to components of lower sedimentation coefficient was observed (Section 3.2.1.). Disruption of unlabelled antigen was detected by DA-RIP assay of serologically active material whereas iodinated antigen was monitored by counting gradient fractions containing radioactively labelled protein. A direct comparison of the effect of Triton X-100 in these two situations therefore presupposes that the serologically active material is protein, or protein-associated, in nature. Published work on the isolation of antigenic and immunogenic components of HB_Ag has indicated that this is probably the case (Shih & Gerin, 1975; Dreesman et al., 1975; Burrell et al., 1976).

Two alternative explanations can be suggested for the difference in behaviour between iodinated and non-iodinated antigen.

a. Structural alterations during iodination

In vivo enzyme catalysed iodination of rat thyroid proteins has been suggested to cause interchain disulphide bond cross-linking (Edelhoch, Carlomango & Salvatore, 1969), but any extrapolation of this observation to the in vitro chloramine T oxidised iodination procedure described in the present study is difficult. Oxidation and oxidative cleavage of simple tryptophan peptides has been observed during chloramine T catalysed iodination (Alexander, 1974). Though this observation is of interest due
to the very high tryptophan content of the HBsAg particle (Rao & Vyas, 1974) it is thought unlikely that it could be
directly extrapolated to larger peptides and proteins possessing
secondary structure (Alexander, 1974).

b. Selective labelling of HBsAg

The chloramine T labelling procedure involves exposure
of highly purified HBsAg to an oxidising agent, followed by a
reducing agent and further purification steps. These manipulations
may result in partial destruction of the antigen preparation.
\(^{125}\)I-labelled HBsAg may therefore represent the most structurally
stable subpopulation of 22nm particles. A close comparison of
the biophysical properties of unlabelled and \(^{125}\)I-labelled HBsAg
from the same source was not attempted during the course of
this work, but selective labelling by chloramine T oxidation
of the subpopulation of particles with the lowest pH value has
been reported by Chaires and coworkers (Chaires et al., 1975b;
Hollinger et al., 1975).

4.2.2. Differences in the effects of various denaturants
on HBsAg structure.

a. Triton X-100, Tween 80 and sodium dodecyl sulphate

Purified HBsAg showed marked differences in its susceptibility
to the three detergents used in this study, only Triton X-100
producing a stable component of lower S value and releasing
relatively large amounts of serologically active material.
A comparison of the electron micrographs after prolonged incubation
with Triton X-100 (section 3.7.2b) or shorter incubation periods with
SDS in PBS (Section 3.7.3) suggested however that the degree of particle breakdown was similar in both cases. The absence of solubilized serologically active material following gradient centrifugation of SDS-treated unlabelled HBsAg may therefore have been due to denaturation of the released material. As noted earlier (Section 3.2.2.) a two-fold drop in total antigen titre occurred after this treatment.

Sodium dodecyl sulphate, at similar concentrations to those used in this study has been described to solubilize and dissociate cell membranes into individual protein-detergent and lipid-detergent complexes (Triplett et al., 1972). Conformational changes in the protein brought about by the binding of large numbers of SDS molecules was thought to account for the observed tendency of this detergent to inactivate biological activity (Reynolds & Tanford, 1970a; Kirkpatrick & Sandberg, 1973). In contrast the strong tendency of Triton X-100 to form micelles effectively limits the monomer detergent concentration in solution so cooperative binding does not occur and biological activity is largely preserved (Makino et al., 1973).

The reasons for the marked difference in the extent of particle solubilization between the two non-ionic detergents Triton X-100 and Tween 80 are not clear at present. Differences in the rate and extent of lipid and protein solubilization from erythrocyte membranes by various detergents have however been described (Kirkpatrick, Gordesky & Marinetti, 1974) so this may be a common phenomenon.
b. Triton X-100 or sodium dodecyl sulphate with reducing agent

Components of similar sedimentation coefficient (25-26S) were produced following prolonged incubation with Triton X-100 (Section 3.2.1c) or short incubation with SDS and reducing agent (Section 3.4.3c). Electron micrographs of antigen disrupted by these two procedures (Fig. 25 & 28) also showed morphological similarities. On this basis, the 83-87,000 dalton radiolabelled component isolated by continuous PAGE after treatment of $^{125}$I-HB$_3$Ag with SDS and reducing agent (Section 3.5.4c) may be similar to the serologically active component of 70-80,000 daltons isolated by column chromatography of material solubilised from HB$_3$Ag by Triton X-100 (Section 3.2.1c). Dissociation of the 83-87,000 dalton radiolabelled component produced a single major polypeptide of 12-14,000 daltons detected by continuous PAGE (Section 3.5.4c). A similar direct demonstration of the dissociation of the 70-80,000 dalton unlabelled component was not carried out. However, the small amount of radiolabelled serologically active material released from $^{125}$I-HB$_3$Ag at high concentrations of Triton X-100 migrated on both continuous and discontinuous gels as a single polypeptide of 12-14,000 daltons after boiling with SDS and DTT (Section 3.2.1b). These results suggest that in both instances the high molecular weight component initially released from the particle represented an aggregate of smaller polypeptides. Since the 12-14,000 dalton polypeptide was never observed prior to extensive treatment with reducing agent it is likely that the relatively stable aggregates of this material were maintained by disulphide bonds.
4.3. **Antigenic and immunogenic subunits of HB Ag**

4.3.1. **Antigenically active subunits.**

Serological activity, monitored by HAI and RIA was still detectable in a polypeptide of 6,000 daltons produced by ultrasonication in urea and reducing agent (Rao & Vyas, 1973). In the present study the smallest component in which activity was detected had an apparent molecular weight of 70-90,000 daltons and could be dissociated into a polypeptide of 12-14,000 daltons. Although the evidence presented here indicated that these 12-14,000 dalton polypeptides were arranged into larger morphological structures through interchain disulphide bonds it was not possible to establish whether such bonds were involved directly in the serologically active site.

4.3.2. **Immunogenic subunits.**

Antisera to individual polypeptides have been produced in guinea pigs which reacted with native HB Ag (Shih & Gerin, 1975; Dreesman et al., 1975) suggesting that these components may retain, or acquire by renaturation, the ability to induce antibody. Both groups of workers found that antibodies induced by polypeptides from ad subtype antigen reacted to a higher titre with the homologous (ad) antigen subtype, whereas av polypeptides induced antibodies which reacted better with the heterologous (ad) subtype. Dreesman and coworkers (1975) have suggested that these results indicated that the y determinant was conformation dependant. Schober and coworkers (personal communication, 1973) have demonstrated a selective inactivation of the subtype determinant y at concentrations of reducing agent which had no effect on the
group-specific \( a \) activity. However, the demonstration that both the group-specific \( a \) and subtype-specific \( a \) determinants on purified antigen were more immunogenic than the subtype-specific \( y \) determinant (Gerin, Faust & Holland, 1975) also has to be taken into consideration.

A comparison of the antibody titration curves and relative binding efficiencies of antibodies produced by three polypeptides from \( ad \) antigen of 72,000, 30,000 and 23,000 daltons indicated that the two heavier polypeptides induced antisera which most closely resembled that produced by injection of native \( ad \) antigen (Shih & Gerin, 1975). The high immunogenicity of the component of 72,000 daltons therefore correlated directly with the antigenicity of the 70-80,000 dalton component isolated in the present work by treatment of antigen with Triton X-100 (Section 3.2.1e).
4.4. Quaternary structure of the 22nm particle

The components of varying size and state of aggregation produced by disruption of HB Ag with the various denaturing agents described earlier (Section 3.2 & 3.4) were examined by electron microscopy in an attempt to visualize directly the basic structural units of the 22nm particle.

4.4.1. Identification of basic structural units by electron microscopy.

The component of 83-87,000 daltons solubilised by incubation with SDS and DTT (Section 3.5.4) appeared in the electron microscope as large aggregates of 1.5 - 2nm thick strands of varying length (Fig. 30). It was therefore not possible to establish the unit size of these polypeptide chains or their relationship to the 4-6nm ring-like subunits of the particle. The thickness of the strands did however correspond closely to the value of 1.8nm for the width of a reduced rod-shaped protein-detergent complex determined by hydrodynamic studies (Reynolds & Tanford, 1970b). From the present study the circumference of a ring-shaped unit of 4-6nm diameter can be calculated as 12.6-19nm. Hydrodynamic studies have indicated that rod-shaped SDS-protein complexes of reduced BSA (mol. weight 69,000 daltons) and reduced ovalbumin (43,000 daltons) had mean lengths of 25.0nm and 15.7nm respectively (Reynolds & Tanford, 1970b). If these two methods for the estimation of the length of SDS-protein complexes are comparable this would indicate that the ring-shaped units are of the same order of magnitude as the serologically active aggregates isolated by gradient centrifugation of Triton-disrupted antigen. However, a direct comparison between the hydrodynamic properties of such a ring-shaped structure and those
of a rod shaped protein-detergent complex is probably not possible. A more critical evaluation of the relationship between molecular weight and length of SDS-protein complexes for standard proteins is therefore necessary to establish the general validity of this technique.

4.4.2. Types of bonding.

Previous workers (Section 1.7) have described complete particle breakdown following treatment with agents capable of disrupting either noncovalent (Traavik et al., 1973) or covalent bonds alone (Brzosko et al., 1972; Hirschman et al., 1973). However in most instances particle dissociation only occurred after treatment with reducing agent, in the presence of SDS (Gerin et al., 1972; Dreesman et al., 1972) or high concentrations of urea (Rao & Vyas, 1973) combined with high temperature or ultrasonication. Since reducing agents in the absence of denaturants cleave only the most accessible disulphide bonds (Bewley & Li, 1969) this appeared to indicate the importance of non-covalent bonding in stabilizing particle structure and preventing cleavage of disulphide bonds buried within the particle.

In the present study incubation with reducing agent alone had no effect on particle structure (Section 3.7.4) but did cause marked aggregation when particles were treated in suspension. Adsorption of purified antigen to electron microscope grids prior to exposure to reducing agent overcame this problem, but it is possible that this factor may have accounted for the apparent loss of particles described by Hirschman and coworkers (1973).

On the basis of the experimental observations reported here the following scheme of bonding forces stabilizing the quaternary structure of the 22nm particle is suggested.
(1) Material in the centre of the 22nm particle is held in place by non-covalent bonds since hollow ring forms containing a central hole 4-6nm in diameter were produced by prolonged incubation at high pH (Section 3.7.5.) and treatment with nonionic (Section 3.7.2.) and anionic detergents (Section 3.7.3.). Loss of this central material appeared to represent the first stage in particle breakdown.

(2) The detection of broken ring forms and associated linear strands following treatment with nonionic and anionic detergents (Section 3.7.2. & 3.7.3.) in the absence of reducing agent indicated that the units making up the circumference of these ring forms were held together by non-covalent bonds.

(3) Treatment with reducing agent was essential for the production of the major polypeptide of 12-14,000 daltons consistently detected in this work (Section 3.5.2. & 3.5.4.). The aggregate of 80-100,000 daltons released from the particle by a number of treatments therefore appeared to be made up of several of these small polypeptides held together by disulphide bonds (Section 3.5.4.). As suggested above this aggregate may correspond to the 4-6nm ring like units which made up the circumference of the large ring form of the 22nm particle (Section 3.7.3. & 3.7.5.).

The quaternary structure of the 22nm spherical particle is therefore maintained by a combination of non-covalent and covalent bonding. As confirmed in this study, complete particle breakdown was only achieved after treatment with a combination of agents capable of disrupting both these groups of bonding forces (Section 3.5.1.).
4.4.3. **Formation of filaments.**

The production of filaments after boiling (Section 3.7.6.) or incubation at high pH (Section 3.7.5.) appeared to occur through association and fusion of spherical 22nm particles. Such a mechanism is not analogous to other viruses possessing tubular forms which normally dissociate to smaller components or individual capsomeres before polymerisation (Ralph & Bergquist, 1967). The relationship of these filaments to those found naturally in serum was difficult to determine. A demonstration of the serological activity of this material by immune electron microscopy was not carried out, but titration by inhibition RIP showed that there was no loss of activity from the boiled antigen preparation (Section 3.2.2.).

Among viruses of approximately similar size range to the 22nm HBsAg particle a few instances of naturally occurring and experimentally produced filaments have been described. Both wide and narrow filaments, the former of similar diameter to the spherical virus particle, have been found in several members of the Papovavirus Group (Howatson, 1973; Hull, 1976). It has been suggested that, in the absence of nucleating agents, the filamentous forms of these particles represented the most thermodynamically stable arrangement of capsomeres (Finch & Crawford, 1974). In contrast, dissociated capsomeres of broad bean mottle virus (BBMV) and polyoma virus produced by incubation at high pH were found to aggregate in linear arrays in the presence of either calf thymus or viral DNA (Bancroft, Hiebert & Bracker, 1969; Friedman, 1971). Therefore, in both instances the presence
of core material appeared to play a significant role in determining the ultimate quaternary arrangement of the individual capsomeres.

In the present study no disaggregation of spherical particles was observed and no extraneous nucleating agents had been deliberately added to the particle suspension prior to or during treatment. The barely detectable production of filaments following addition of RNA to purified preparations of 22nm HB$_3$Ag particles observed by Hirschman (1976) suggested however that similar nucleation processes may be possible.

The variable presence of naturally occurring filaments observed with HB$_3$Ag may therefore be a reflection of variations in the occurrence of nucleating agents, as yet unidentified, either inside the liver cell or possibly in the blood-stream. A similar analogy between in vivo formation of double shelled particles of cucumber chlorotic mottle virus (CCMV) by aggregation around synthetic polynucleotides, and the naturally occurring double shelled 42nm Dane particle was originally suggested by Cossart & Field (1970) and Almeida and coworkers (1970). However, the above examples all refer to non-lipid containing capsids where the thermodynamic relationship between capsomeres is likely to be less complex than in the example studied here.

The structural model proposed in Section 3.9 is unique in that it suggests that the spherical HB$_3$Ag particle may possess helical rather than icosahedral symmetry. Although filament formation has been observed with BBMV, CCMV and polyomavirus, all of which show typical icosahedral symmetry, these filaments were built up from individual disaggregated capsomeres rather than by fusion of complete spherical particles as appeared to
occur with HB$_s$Ag (Section 3.7.5. & 6.). Helical symmetry within the 22nm particle may therefore favour the latter type of filament formation.

### Relationship to particles detected in the liver.

The apparent absence in the cytoplasm of human hepatocytes of spherical 22nm particles, the most predominant morphological form found in the circulation (Section 1.10.1.) is difficult to explain. Brzosko and coworkers (1975) have suggested that this observation may have been an artefact due to the preferential destruction of particles during the processing and embedding of liver tissue for thin sectioning. This is however incompatible with the demonstration that these particles were also absent from negatively stained homogenates of liver from the same source (Huang et al., 1974).

The conversion of spheres to filaments demonstrated in the present work suggests that the spherical particle may be the basic unit of thermodynamic stability of HB$_s$Ag protein, and that filaments are formed by linear aggregation of these spheres. Polymerisation within the hepatocyte cytoplasm may initially produce the filamentous structures observed by a number of workers (Huang et al., 1974; Gerber & Hadziyannis, 1974) which later break down to more stable spherical particles. Similarly, evidence of breakdown of filaments to spherical particles has been observed in negatively stained preparations of HB$_s$Ag positive human serum examined by electron microscopy (Traavik et al., 1973; Muscatello et al., 1973).
The "owls eye" particles described by Stein and coworkers (1972) within the smooth endoplasmic reticulum of human hepatocytes (Section 1.10.2.) have been interpreted as representing cross-sections of the abundant filamentous material found in the same site (Gerber et al., 1974a). The apparent absence of "owls eye" particles in negatively stained liver homogenates would tend to support this interpretation. The observation in the present work of similar spherical particles containing varying amounts of central or eccentric electron-dense material (Section 3.7.5.) may however indicate that these structures in the liver represent incompletely assembled 22nm particles or filaments.
GENERAL DISCUSSION
5.1. The role of lipid in particle structure

The role of lipid in the structure of the 22nm particle has not been directly examined in this work. Although both the 22nm particle and the outer coat of the Dane particle carry surface antigen specificity they differ markedly in both electron microscope appearance and sensitivity to nonionic detergents. Incubation with low concentrations of Tween 80 released the Dane particle core from its outer envelope (Almeida et al., 1971) indicating that this envelope may be assembled along similar lines to other, well characterized, enveloped viruses.

In most instances lipid containing viruses are thought to contain outer envelopes formed by modification of a cellular membrane during virus maturation and budding (Blough & Tiffany, 1973). The virus envelope therefore consists of a bilayer of lipid molecules orientated with their polar head groups towards the outside and their non-polar fatty acid chains pointing inwards towards one another (Singer & Nicolson, 1972). Virus structural proteins are embedded in this lipid bilayer to varying extents, with their highly polar ends containing ionic amino acids and covalently bound saccharide residues situated on the outside of the membrane, and their non-polar regions buried in a non-aqueous environment within the membrane (Lenard & Compans, 1974). From electron microscopic evidence it has been suggested that in certain groups of viruses envelope formation can sometimes occur through spontaneous de novo association of lipid and protein within the cell cytoplasm (Blough & Tiffany, 1973). However the evidence for this is not well established (Fenner et al., 1974) and may be a reflection of the area of the cell sampled by thin sectioning.
The only other well described form of naturally occurring lipid protein association is that which occurs in the serum lipoproteins (Scanu, 1972). In the model proposed by Pollard and coworkers (Pollard et al., 19??) analogies were drawn between the structure of small icosahedral viruses and that of human serum low density lipoprotein (LDL). On the basis of electron microscopic observations LDL was thought to be made up of twenty protein subunits occupying the vertices of a dodecahedron with phospholipids arranged between the vertices and neutral lipid sequestered within the hydrophobic core.

The stability of both cell membranes and serum lipoproteins has been shown to be dependant on both lipid-lipid and lipid-protein interactions (Singer & Nicolson, 1972; Scanu, 1972). The ease of delipidation of LDL following extraction with a range of organic solvents (Scanu, 1972) and the disruption of cellular membranes by detergent treatment (Kirkpatrick et al., 1974) contrasts markedly with the stability of the 22nm HB$_5$Ag particle to similar treatment (Section 1.7.). Present evidence therefore suggests two alternative explanations for the presence of lipid in the 22nm particle.

Firstly, the lipid may be essential for structural integrity of the particle but arranged in an unusual conformation stabilized by strong hydrophobic bonding, possibly associated with polypeptides containing large amounts of the amino acid tryptophan (Rao & Vyas, 1974). Secondly, the lipid may be simply adsorbed to or inserted around the essential protein skeleton of the 22nm particle.

In either case the type of lipid-protein association is likely to have unusual features and variations in both the amount and the relative composition of the lipid component of particles.
purified from different sources would be expected. An examination of the composition and conformation of lipid in a number of purified preparations of 22nm particles would therefore seem to be an important area for future investigation.

5.2. The role of hepatitis B antigen in pathogenesis

5.2.1. Defective interfering particles.

Two categories of potentially interfering situations may exist in hepatitis B infection. Firstly, there are the 22nm spherical particles and associated filaments which share an antigenic determinant, HB Ag, with the Dane particle. If this determinant, or possibly another as yet unrecognized shared specificity, is associated with attachment of virus to the host cell prior to replication, the blocking of attachment sites by large numbers of 22nm particles may physically hinder the infection of susceptible cells by Dane particles (Gerin, Ford & Purcell, 1975). Secondly, the high proportion of Dane particles containing an incomplete genome may interfere with intracellular replication of complete viral nucleic acid molecules in a manner analogous to other, well characterized, defective interfering viruses (Huang, 1973; Gerin, Ford & Purcell, 1975).

The presence of large numbers of potentially interfering particles in hepatitis B may therefore explain some of the problems which have so far prevented the successful \textit{in vitro} cultivation of the virus, and may account for certain pathological features of the disease including the long latent period and the tendency towards chronic asymptomatic carriage of the virus.
5.2.2. **Immune responses to "self" and viral antigens.**

The detection of large amounts of antigenic material inside histologically normal hepatocytes and free in the circulation would seem to indicate that hepatitis B virus is largely non-cytocidal in nature (Edgington & Chisari, 1975). In contrast, the temporal association between development of the immune response and clinical manifestations of hepatitis in an individual, and the demonstration that the pathologic lesions in the liver caused by hepatitis B infection are characterized by a predominantly mononuclear cellular infiltration (Petersen, Dienstag & Purcell, 1975) would suggest that immunological factors may play a role in the pathogenesis of the infection (Le Bouvier & McCollum, 1970). The detection of components in the 22nm particle which cross-react with antisera to various serum proteins (Section 1.4.3.d) is therefore of interest in relation to the possibility that humoral and cell mediated immune responses to both viral and hepatocellular antigens may be important in the pathogenesis of hepatitis B (Popper, 1975). Now that certain liver cell antigens have been purified and characterized (Meyer zum Buschenfeld & Miescher, 1972; Mihas, Herschowitz & Saccomani, 1976) it would seem important to determine the association, if any, of these antigens with any of the particulate forms of hepatitis B.

The possibility of stimulation of autoimmune responses following injection of purified preparations of 22nm particles may be a serious disadvantage in the use of such material for vaccine purposes (Zuckerman & Howard, 1975; Melnick et al., 1976). Analysis of the structure and antigenicity of the 22nm particle, such as described in the present work should in theory allow the
production of a subunit vaccine for hepatitis B with considerably reduced risks of either accidental infection on the one hand, or stimulation of autoimmune responses on the other.
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Appendix

Preparation of monospecific anti-HB$_d$ subtype $d$ antiserum

Monospecific antiserum to the $d$ subtype specificity of HB$_d$Ag was prepared by staff of the Hepatitis Reference Laboratory according to the following procedure. The immunogen consisted of 0.6ml of saline containing 70 $\mu$g of HB$_d$Ag/$ad$ purified by column chromatography and equilibrium gradient centrifugation, as described in Section 3.1. New Zealand White rabbits were injected subcutaneously with antigen in the presence of Freunds complete adjuvant (FCA), and boosted 2 months and 3 months later with the same amount of antigen in FCA, and Freunds incomplete adjuvant (FIA) respectively. One month later, a total of 4 months after the initial injection, the rabbits were bled out.

Dilutions of this rabbit antiserum from 1/100 to 1/102,400 in carrier pre-immune rabbit serum 1/160 were reacted with $^{125}$I labelled $ad$ and $ay$ antigen, in the presence of RIP buffer or a 1/100 dilution of serum containing HB$_d$Ag/$ad$ or HB$_d$Ag/$ay$. When titrated in RIP buffer the rabbit anti-$ad$ antiserum showed a high affinity reaction with $^{125}$I labelled $ad$ antigen, which was reduced in the presence of both unlabelled $ad$ and $ay$ antigens (Fig. 1a). However titration of the antiserum with $^{125}$I labelled $ay$ antigen gave low levels of precipitation even in RIP buffer, indicating that the anti-$g$ antibodies present were of low affinity (Fig. 1b).

Antibodies to both normal human serum and to the group-specific $g$ determinant were then absorbed from the antiserum by incubation of 2.5ml of a 1/50 dilution of rabbit antiserum with 2.5ml of a 1 in 2.5 dilution of HB$_d$Ag/$ay$ human serum at 4°C for 2 days.
Antigen-antibody complexes were removed by centrifugation at 20,000 rev/min for 2h in a SW50.1 rotor at 4°C. The supernatant fluid, now at a dilution of 1/100, was then titrated from 1/100 to 1/102,400, in carrier pre-immune rabbit serum 1/160, with ¹²⁵I labelled ad and av antigens. This adsorption procedure removed all the anti-a activity from the antiserum as shown by its lack of precipitation with ¹²⁵I labelled av antigen, whereas the reaction with ¹²⁵I labelled ad antigen was only slightly reduced (Fig. Ic). This adsorbed antiserum was used as a source of monospecific anti-a antibody.
Fig. 1. Standard antibody dilution curves of monospecific rabbit anti HB₅ subtype d antiserum. (a) pre-absorption serum titrated against ¹²⁵I-labelled ad antigen in RIP buffer ( ), 1 in 100 dilution of serum containing HB₅ Ag/ad ( ), 1 in 100 dilution of serum containing HB₅ Ag/av ( ). (b) pre-absorption serum titrated against ¹²⁵I-labelled av antigen in RIP buffer ( ), 1/100 HB₅ Ag/ad ( ), 1/100 HB₅ Ag/av ( ). (c) post-absorption serum titrated against ¹²⁵I-labelled ad ( ) and ¹²⁵I-labelled av ( ) antigens in RIP buffer.
Tryptic Cleavage of Antibody Binding Sites from Hepatitis B Surface Antigen Particles

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SUMMARY

The sedimentation of radiolabelled 22 nm hepatitis B surface antigen particles was unaffected by treatment with either trypsin or SDS alone, but combined treatment disrupted the particulate nature of the radiolabelled material. Considerable antibody binding activity by the group-specific determinant (a) was preserved after combined SDS and trypsin treatment but was released from the bulk of the radiolabelled protein; gel filtration indicated an approximate mol. wt. of 5000 to 15000 for the released antibody binding material. This material was precipitated by concanavalin A, suggesting the presence of carbohydrate. Its serological activity was remarkably resistant to boiling and to proteolytic digestion, but was partially sensitive to treatment with 0·01 M-periodate or with mixed carbohydrases and neuraminidase, and was greatly reduced by treatment with reducing agent. These data suggest that the stability of the a determinant is due to the structure of the antibody binding site itself, rather than to involvement in the quaternary structure of the particle, and that intact disulphide bonds and carbohydrate, closely related to the antibody binding site, are necessary for the full expression of serological activity.

INTRODUCTION

There is now abundant epidemiological evidence that circulating hepatitis B surface antigen (HBsAg) in man is uniquely associated with hepatitis B virus (HBV) infection. This antigenic complex consists of a major group-specific determinant a, and at least two sets of mutually exclusive subtype determinants, d/y and w/r (Le Bouvier, 1971; Bancroft, Mundon & Russell, 1972), allowing for four possible phenotypic expressions adw, adr, ayw and ayr. Epidemiological data indicate that these phenotypes are likely to represent the expression of distinct genotypes of HBV, and thus that the antibody binding sites involved are likely to represent, or be closely related to, virus gene products (Le Bouvier, 1972). HBsAg occurs on the surface of three virus-like particle types circulating in the plasma; heterogeneous approximately spherical 22 nm forms which usually predominate, filamentous forms of similar diameter and varying lengths, and a third more complex 42 nm particle composed of an inner core and an outer lipoprotein envelope. There is evidence that passively administered antibody to HBsAg (anti-HBs), and active immunization with heat-inactivated HBsAg-positive material may confer a measure of immunity to subsequent challenge with HBV (Krugman & Giles, 1973).

Attempts to define the biochemical nature of the HBsAg antigenic sites were based
initially on the effect of various chemical and enzymatic treatments on the serological activity of 22 nm particles. Kim & Bissell (1971) reported that such activity was unaffected by treatment with 8 M-urea, acid or alkali, sodium dodecyl sulphate (SDS) up to 0.5%, most proteolytic enzymes, and heating for 6 h at 56 °C. On the other hand, activity was markedly decreased after treatment with 2-mercaptoethanol or dithiothreitol (Sukeno et al, 1972; Vyas, Rao & Ibrahim, 1972; Dreessman et al, 1973; Imai et al, 1974), although considerable activity was regained upon reoxidation unless the reduced sulphhydrol groups were alkylated with iodoacetamide. Vyas et al. (1972) proposed that the conformation of the antigenic site was dependent upon disulphide bonds of the protein moiety. A progressive loss in serological activity was seen after oxidation with 0.01 M-periodate, suggesting that the integrity of the carbohydrate moiety was necessary for antibody binding (Burrell et al, 1973). More recently, Rao & Vyas (1974a) reported a loss in serological activity after combined treatment with 8 M-urea and 5 M-guanidine, and after succinylation of the ε-NH₂ group of lysine. These workers suggested that the conformational disturbance induced by disruption of covalent (disulphide) or non-covalent bonds might lead to masking of antigenic sites in intact particles without necessarily reacting with the sites themselves.

Several reports have described the release of serologically active material from 22 nm particles. After ultrasonication in the presence of 8 M-urea and 2-mercaptoethanol, protein subunits of mol. wt. 80000, 12000 and 6000 were isolated, which retained serological activity by passive haemagglutination inhibition (Rao & Vyas, 1973). In further similar studies, a peptide of mol. wt. 6000 was isolated which retained serological activity in a 'sandwich'-type solid phase radioimmunoassay (Ausria); amino acid analysis revealed a high content of tryptophan, glycine and serine, while cysteine/cystine and methionine were not detected (Rao & Vyas, 1974b). However, no data were presented on the affinity of antibody binding by this material, nor whether it contained only group-specific, subtype-specific or multiple determinants. Dreessman et al. (1973) reported the release of a serologically active fragment (mol. wt. 4000 to 12000) from HBsAg, after treatment with 8 M-urea and 0.1 M-dithiothreitol followed by 0.3 M-HCl; further characterization of this material has not been reported.

An alternative approach to define serologically active components of HBsAg has been the isolation of constituent polypeptides and glycoproteins by SDS-polyacrylamide gel electrophoresis and their use as immunogens in experimental animals. At least seven polypeptides have been described, ranging in size from 16000 to 120000 (Dreessman et al, 1975; Shih & Gerin, 1975; I. Gordon, 1975, personal communication); some, but not all, contained carbohydrate detectable by PAS staining of acrylamide gels. Dreessman et al. (1975) reported that guinea pigs immunized with many of these isolated polypeptides or glycoproteins produced specific antibody that reacted with native HBsAg in radioimmunoprecipitation assays; their results suggested the production of both group-specific and subtype-specific antibody. In similar work, Shih & Gerin (1975) found that all the components examined produced a humoral response to native HBsAg. On the other hand, I. Gordon (1975, personal communication) reported that, of 7 polypeptides examined, only one of 22000 mol. wt. produced specific anti-HBs in guinea pigs, whereas all polypeptides elicited a delayed hypersensitivity response when the animals were subsequently challenged with purified HBsAg. This work suggests that protein or glycoprotein moieties contain antigenic sites that can function as HBsAg-specific immunogens; however, evidence that HBsAg polypeptides can undergo incomplete dissociation and re-aggregation when treated with SDS and reducing agent (Mackay & Burrell, 1976) makes it difficult to attribute serological activity to determinants residing on material of clearly identifiable mol. wt.
Cleavage of HBsAg antibody binding sites

In the present report, evidence is presented that the major anti-a binding site of HBsAg can be cleaved from the bulk of the protein moiety; preliminary characterization of the released material indicates that it contains disulphide residues and carbohydrate, and that both appear to play a role in antibody binding activity. Gel-filtration suggested an approximate mol. wt. for the released material of 5000 to 15000. Further characterization of this material should lead to an immunochemical description of the antibody binding site involved.

METHODS

Purification of HBsAg. HBsAg was purified from separate samples of plasma from different antigen-positive blood donors as previously described (Burrell, 1975). Briefly, 15 to 30 ml samples of plasma were fractionated on Sepharose 6B, and HBsAg-positive fractions were pooled, concentrated and banded twice in discontinuous sucrose/caesium chloride equilibrium density gradients. The HBsAg-positive fractions were pooled and stored at 4 °C in phosphate buffered saline (PBS) containing 0.1 % sodium azide. Such preparations contained densely packed 20 to 25 nm HBsAg particles and occasional filamentous forms, and usually had an optical density at 280 nm of 0.7 to 2.0 units.

Radiolabelling of HBsAg. Preparations of purified HBsAg were iodinated with 125I by the chloramine T method, using a modification of the procedure previously described (Burrell et al. 1973). To 20 μl of a sample containing antigen (6 to 15 μg protein) were added, successively, 1 mCi of 125I (carrier-free, Radiochemical Centre, Amersham) and 20 μl of chloramine T (5 mg/ml). After 1-5 min at room temperature, 20 μl of 0.5 ml PBS containing 0.5 % bovine serum albumin (BSA) were added, and the 125I-labelled HBsAg was isolated by fractionation through a 1.0 x 20 cm column of Sepharose 6B followed by rate zonal sedimentation in a 5 to 20 % sucrose density gradient. The immunological integrity of such preparations was established by standard antiserum dilution radioimmunoprecipitation curves using rabbit antibody to HBsAg (anti-HBs), as previously described. A labelling efficiency of 5 to 25 % was routinely obtained, yielding preparations with a sp. act. of 10 to 50 μCi/μg.

Radioimmunoprecipitation assay. Double antibody radioimmunoprecipitation (RIP) assays were performed as previously described. Except where specified, samples to be assayed (100 μl) were incubated at 4 °C for 16 h in the presence of rabbit anti-HBs (Hoechst Pharmaceuticals, Hounslow, Middlesex) at a dilution of 1/1600 containing 1/200 carrier non-immune rabbit serum (50 μl). After the addition of 125I-labeled HBsAg (50 μl containing approx. 50 cts/s of 125I and 1 ng of HBsAg protein) and further incubation at 4 °C for 16 h, donkey anti-rabbit IgG (100 μl of a 1/27 dilution, Wellcome Reagents Ltd, Kent, England) was added, and the samples incubated again at 4 °C for 16 h. After centrifuging for 30 min at 2500 rev/min at 4 °C, the percentage of 125I-HBsAg precipitated was determined. The diluted used throughout was PBS containing 0-5 % BSA and 0-02 % sodium azide (RIP buffer). Sucrose gradient fractions containing SDS were examined for HBsAg serological activity by diluting 50 μl samples to 200 μl with RIP buffer and assaying as described above. Under these conditions the presence of sucrose and SDS did not interfere with the RIP assay.

Enzymes and reagents. The following enzymes and reagents were used; trypsin (2 x crystallized, salt-free, Koch-Light Laboratories, Colnbrook, England); pronase (B grade, Calbiochem Ltd, Hereford, England); mixed glycosidases from Turbo cornutus (Miles Laboratories, Slough, England); neuraminidase from Clostridium perfringens (Type VI, Sigma Chemical Company, Surrey, England); pepsin (BDH Chemicals Ltd, Poole, England); soybean
Fraction number

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Fig. 1. Radioactivity profiles after centrifuging of 125I-labelled HBsAg through 5 to 20% linear sucrose gradients in RIP buffer for 3.5 h at 35,000 rev/min in a Spinco SW36 rotor. Samples of 500 µl were applied after treatment with (A) SDS and trypsin (●—●), (B) SDS alone (○—○), (C) trypsin alone (▲—▲) or (D) no treatment (△····△). Gradients (A) and (B) contained 0.001% SDS. There was complete recovery of the radiolabel applied to each gradient.

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trypsin inhibitor (2 × crystallized, Sigma); concanavalin A (2 × crystallized, Miles Laboratories). SDS (Specially pure, BDH Chemicals Ltd) was made up as a 10% stock solution in distilled water and diluted for use.

To examine the effects of various treatments on the serological activity of native antigen and the released antibody-binding material, the following conditions were used: pepsin (0.1% in 0.1 M-glycine, pH 2.5); pronase (0.1% in RIP buffer); periodate (0.01 M in 0.1 M-phosphate buffer, pH 7.2 and 5.7, or 0.2 M-acetate buffer, pH 4.5); mixed glycosidases (0.1%) and neuraminidase (0.05%) in 0.05 M-acetate buffer, pH 5.5; 0.1 M-dithiothreitol in 0.6 M-tris, pH 8.2, for 1 h at 37°C, followed by 0.1 M-iodoacetamide for 1 h at 4°C in the dark and overnight dialysis in PBS.

RESULTS

Solubilization of radiolabelled HBsAg

Samples of radiolabelled HBsAg (20 µl or 50 µl) were incubated for 30 min at 37°C with or without 1% SDS; each sample was then diluted tenfold with RIP buffer, divided into two equal portions and 1% trypsin was added to one of each pair to give a final concentration
Cleavage of HBsAg antibody binding sites

Table 1. Immunoprecipitation of $^{125}$I-labelled HBsAg after various treatments and isolation of radioactive peaks on sucrose gradients

<table>
<thead>
<tr>
<th>Treatment</th>
<th>7</th>
<th>11</th>
<th>15</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>56.0%</td>
<td>25.3%</td>
<td></td>
<td>35%</td>
</tr>
<tr>
<td>C</td>
<td>61.9%</td>
<td></td>
<td>39%</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>71.4%</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* Only those fractions from each gradient corresponding to radioactive peaks were examined.
† Percentage of radioactivity precipitated by anti-HBs.

of 0.1%. After further incubation from 2 to 16 h at 37 °C, each sample was made up to 1.0 ml with RIP buffer containing 0.05% soybean trypsin inhibitor. The treatments were identified as follows: (A) trypsin and SDS, (B) SDS alone, (C) trypsin alone, (D) neither reagent. Each sample was then examined by rate zonal sucrose gradient sedimentation.

No alteration in sedimentation properties of radiolabelled HBsAg was seen after treatment with either trypsin or SDS alone, in comparison to untreated material; however, a small proportion of radioactivity was released from the particles with either treatment. In contrast, combined treatment with trypsin and SDS abolished the major HBsAg radiolabelled peak, resulting in the appearance of low mol. wt. material at the top of the gradient, and a small shoulder of intermediate sedimentation (Fig. 1). The gradient fractions corresponding to these peaks were then diluted to contain approx. equivalent concentrations of radioactivity. Samples of 0.5 ml were incubated with 50 µl of rabbit anti-HBs (1/100) at 4 °C overnight, and then with donkey anti-rabbit IgG as described in Methods (Table 1). The major radiolabelled peaks after trypsin or SDS treatment alone retained immunoprecipitability comparable to that of untreated antigen. Released radiolabelled material at the top of the gradients was not precipitable, whereas the shoulder of intermediate sedimentation after combined trypsin and SDS treatment was partially precipitable; in other experiments using longer periods of trypsinization, this intermediate shoulder was abolished, and with some preparations a considerable reduction in immunoprecipitability of the major radiolabelled peak was seen after trypsinization alone (treatment C).

Assay of antigenic determinants

In the above type of experiment, serological activity would only have been detected if it had remained bound to radiolabelled material. Accordingly, a number of similar experiments were done using unlabelled purified HBsAg from different donors. The antibody binding capacity of each preparation after treatments A to D was assayed by a competitive double-antibody radioimmunoprecipitation assay. Relative measurement of antibody binding activity was possible by relating activity to the inhibition of precipitation of radiolabel observed, using a standard dilution curve of untreated HBsAg in similar assays; differences equivalent to greater than one serial twofold dilution were considered significant (Fig. 2). The degree of denaturation of antibody binding sites after various treatments was assessed by comparing the slope of dilution curves with that for untreated HBsAg (Fig. 2).

When identical samples of purified HBsAg from any one donor were treated with SDS and trypsin following procedures A to D above and assayed by double antibody RIP, serological activity was largely preserved. Some variability was noted between experiments with greatest losses usually occurring after combined trypsin and SDS treatment (A), but rarely
Fig. 2. RIP antigen dilution curves. Samples of purified HBsAg were treated (A) with SDS and trypsin (— — — — ), or (D) no treatment (· · · · · · ), and serial dilutions (100 μl) were assayed for anti-HBs binding activity by RIP. A standard antigen preparation (○ — ○ ), and 6 negative controls (100 μl of RIP buffer), were assayed similarly. The insert shows the mean value and 5× standard deviation range obtained for the negative control assays.

Table 2. Antibody-binding activity of purified HBsAg remaining after various treatments, in comparison to an untreated sample assayed in parallel

<table>
<thead>
<tr>
<th>Experiment</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>25</td>
<td>75</td>
<td>100</td>
</tr>
</tbody>
</table>

* Samples of purified HBsAg were treated with SDS and trypsin (A), SDS alone (B), trypsin alone (C) or untreated (D) and assayed for antibody-binding activity by competitive double antibody RIP. The figures represent the percentage of activity remaining after each treatment in comparison to the untreated sample (D), by reading relative antibody-binding activities from a standard antigen dilution curve.

exceeding a fourfold loss when compared to the untreated (D) sample (Table 2). The degree of loss seen was not related to the source of the antigen under study. Since the slope of antigen dilution curves of treated HBsAg (A) was similar to that of untreated antigen (D; Fig. 2), it was inferred that partial denaturation of antibody binding sites was not a marked feature after such treatments.

We feel that serological activity detected in this work involved the group specific α determinant, since (1) similar results were obtained when the unlabelled antigen under study, and the labelled antigen in the assays, were of the same or opposite major subtypes (ad and ay), and (2) the rabbit anti-HBs used as first antibody in the RIP assays contained detectable anti-α activity only, at the dilution (1/1600) used in the assay.
Release of serological activity from HBsAg particles

Unlabelled HBsAg particles were treated as described above (treatments A to D), analysed on rate-zonal sucrose gradients, and the fractions assayed for antibody binding activity by competitive double-antibody RIP. After SDS treatment alone (B), most of the antibody-binding activity sedimented in the same position as untreated antigen, while more rapidly sedimenting antigenic material was also usually seen (Fig. 3). After trypsin treatment alone (C), activity coincided either with the position of untreated antigen, or remained at the top of the gradient; since similar treatment of radiolabelled HBsAg always preserved the major particulate radioactive peak but in some cases reduced its immunoprecipitability (see above), we interpreted this result as reflecting the variable release of antibody binding sites from the surface of HBsAg particles after treatment with trypsin alone.

After trypsin and SDS treatment (A), antibody-binding activity was recovered only at the top of the gradients. Since radiolabel solubilized by such treatment was not immunoprecipitable (Table 1), this finding demonstrated the reproducible release of HBsAg antibody binding sites from the bulk of the radiolabelled moiety of the particles.

Preliminary characterization of antibody binding component

Previous work using intact HBsAg particles has shown a loss of serological activity after treatment with reducing agents (Sukeno et al. 1972; Vyas et al. 1972) and with 0.01 M-periodate (Burrell et al. 1973). However, since conformational changes affecting the whole particle may have accounted for such effects, we examined further the nature of the antibody binding component released from HBsAg particles by SDS and trypsin treatment.
Table 3. Comparison of the effects of various treatments on the antibody-binding activity of native antigen and the released material, determined by competitive RIP assay

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Percentage of serological activity remaining after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native antigen†</td>
</tr>
<tr>
<td>Pepsin, 3 h × 37 °C</td>
<td>50</td>
</tr>
<tr>
<td>Pronase, 16 h × 37 °C</td>
<td>100</td>
</tr>
<tr>
<td>Periodate, 3 h × 37 °C</td>
<td>100</td>
</tr>
<tr>
<td>pH 7-2</td>
<td>20</td>
</tr>
<tr>
<td>Periodate, 16 h × 4 °C, pH 4-5</td>
<td>50</td>
</tr>
<tr>
<td>Reduction and alkylation</td>
<td>1</td>
</tr>
<tr>
<td>Reduction and dialysis</td>
<td>10</td>
</tr>
<tr>
<td>Glycosidases and neuraminidase, 24 h × 37 °C</td>
<td>100</td>
</tr>
<tr>
<td>Boiling 5 min</td>
<td>10</td>
</tr>
<tr>
<td>Boiling 20 min</td>
<td>50</td>
</tr>
<tr>
<td>Boiling 40 min</td>
<td>25</td>
</tr>
</tbody>
</table>

* Detailed procedures are given in Methods.
† A preparation of HBsAg was divided in two, and one portion treated with SDS and trypsin to release antibody-binding sites.
‡ Percentage of serological activity of released material was determined by competition with native antigen.

Identical samples of purified HBsAg were treated at 37 °C with (A) or without (D) SDS and trypsin as described above; gradient analysis of a portion of each preparation for antibody binding activity confirmed that, with preparation A, all antibody binding sites had been released from intact HBsAg particles, as shown in Fig. 3. The effect of various treatments on antibody binding activity by each preparation was then examined by double antibody RIP; preparation (A) contained final concentrations of 0.05 % SDS, 0.05 % trypsin and 0.05 % trypsin inhibitor in RIP buffer. In Table 3 the antibody-binding activity remaining after the subsequent treatment shown has been expressed as a percentage of that of an identical untreated sample, by comparing relative activities from antigen dilution curves as described above (Fig. 2). It was apparent that antibody binding activity was remarkably stable to proteolytic enzymes and boiling, whereas mixed glycosidases or periodate treatment produced a small and variable reduction in antibody binding activity. A marked fall in activity occurred after reduction and alkylation. When the preparations were reduced with dithiothreitol and dialysed for 3 days against successive changes of PBS without alkylation, significant activity was regained by the preparation of native antigen, but not by the released component. With this exception, no reproducible differences were seen between native antigen and the released component, suggesting that involvement of antibody binding sites in quaternary particle structure was not of major importance in determining their susceptibility to such treatments.

Preparations of native antigen and the released component were diluted 1/4 in PBS containing 0.001 M-CaCl₂ and 0.001 M-MnCl₂ and concanavalin A was added to 0.1 %. After 4 h at 4 °C, the preparations were centrifuged for 30 min at 2000 rev/min and the supernatant fluids assayed by RIP. Antibody-binding activity was totally removed from each preparation by this treatment, whereas control samples of RIP buffer treated in the same way did not interfere with the RIP assay. This provided evidence that both native antigen and the released component contained terminal α-D-mannopyranosyl, α-D-glycopyranosyl or β-D-fructofuranosyl residues (Goldstein & So, 1965).
Cleavage of HB$_{Ag}$ antibody binding sites

An approximate estimate of the mol. wt. of the released material was obtained by gel filtration in a 1.6 x 15 cm column of Sephadex G-200 equilibrated with PBS containing 0.05% SDS (Fish, Reynolds & Tanford, 1970). Internal marker proteins (2 mg ovalbumin, 2 mg lysozyme and 2 mg insulin in a total vol. of 300 μl) were incubated at 37 °C for 30 min in the presence of 6 mg SDS in an attempt to achieve binding of SDS comparable to that of the SDS-treated HB$_{Ag}$; a 200 μl sample of the released material was then added, together with 100 μl of 0.04% bromophenol blue. The total sample was applied to the column and eluted at a flow rate of 1 ml/h with PBS containing 0.05% SDS; the position of the marker proteins was located by extinction at 280 nm, and 0.33 ml fractions were assayed by RlP. Antibody binding activity eluted as a single peak in the region of the lysozyme and insulin markers, suggesting a mol. wt. in the range of 5000 to 15000.

DISCUSSION

It has previously been reported that the serological activity of HB$_{Ag}$ assayed by gel diffusion, was destroyed by treatment with 1.0% SDS followed by 0.05% trypsin, but was relatively resistant to either treatment alone (Kim & Bissell, 1971). Our results extend these observations, and demonstrate that combined SDS/trypsin treatment leads to breakdown of $^{125}$I-labeled HB$_{Ag}$ particles. After treatment with SDS and trypsin and in some experiments trypsin alone, significant binding activity for antibody to the α determinant was preserved but was cleaved from the radiolabelled material, which is likely to be largely protein (Mackay & Burrell, 1976). It has previously been proposed that an external lipid shell may protect the protein moiety of HB$_{Ag}$ from proteolytic degradation (Le Bouvier & McCollum, 1970; Kim & Bissell, 1971); our findings are compatible with this interpretation, and suggest that with some preparations of HB$_{Ag}$ this protection may not be complete.

The released antibody binding material was precipitated by concanavalin A, suggesting the presence of carbohydrate; it is also likely to have contained amino-acid sequences since tryptic digestion was necessary for its release, and since its serological activity was susceptible to reducing agent. Serological activity was remarkably stable to boiling and to proteolytic digestion, which suggested that non-covalent protein-protein interactions were not involved in maintaining the integrity of the antibody binding site and that the residual protein moiety did not contain accessible proteolytic cleavage sites. Some loss in activity occurred after treatment with 0.01 m-periodate or with mixed glycosidases and neuraminidase, indicating a role for carbohydrate in the full expression of antibody binding activity; reduction and alkylation destroyed 99% of the activity. Similar results with both the released material and untreated antigen suggested that these properties were due to the structure of the antibody binding site itself, rather than as a result of its involvement in the quaternary structure of the particles. In contrast, untreated antigen regained significant serological activity after reduction and dialysis to remove reducing agent, whereas the released material did not. This could occur if the mutual repulsion by the negative charge of bound SDS were preventing renaturation. Alternatively, if the released material were composed of more than one subunit joined by disulphide bonds and if antibody binding required these bonds intact, renaturation could be expected to proceed more efficiently where close apposition of these subunits had been maintained in the intact particle.

A mol. wt. estimate for the released material of 5000 to 15000 was obtained by gel filtration in Sephadex G-200 in the presence of 0.05% SDS, although this figure must remain an approximation due to the effects of the extent of binding of SDS, carbohydrate content, and incomplete unfolding of polypeptides containing intra-chain disulphide bonds.
(Fish et al. 1970). Correlation of this material with portions of any of the polypeptides detected in SDS-disrupted HBsAg particles in the absence of trypsin digestion (Shih & Gerin, 1975; Dreesman et al. 1975; I. Gordon, 1975, personal communication; Mackay & Burrell, 1976) would be of interest.

The above findings do not allow a description of the role of protein and carbohydrate in the binding of anti-HB, by the major antigenic determinant of HBsAg. Further studies, after purification of the released antibody-binding component described above, should lead to characterization of the chemical nature and configuration of the binding site by more conventional methods, and hence to information about the possible HBV gene product(s) involved in expression of serological activity. Finally, investigation of the immunogenicity of the released material should indicate if it has advantages over inactivated intact HBsAg particles in producing active immunity against hepatitis B.

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REFERENCES


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Examination of the Polypeptides of Hepatitis B Surface Antigen

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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. 60,000 to 70,000 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 x 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 HB.Ag 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 HB.Ag polypeptides has not been examined.

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

**METHODS**

**Purification and radiolabelling of HB.Ag.** HB.Ag 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 0.1% 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 HB.Ag 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 0.01% SDS, and centrifuged for 2.5 h at 42,000 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 sulphydryl 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 85×7 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 0.01 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 8.3, 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 HB,Ag with detergent and reducing agent

Solubilization of 125I-HB,Ag 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 36 S to 25 S. With increased time or temperature of incubation, relatively less 25 S 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 HB,Ag purified from one plasma source the 25 S component remained after treatment with SDS and reducing agent even at this temperature.

Irrespective of the source from which it was prepared the 25 S 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 10 S 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 HB,Ag into low mol. wt. material, but that
Fig. 1. Radioactivity profiles after centrifuging $^{125}$I-HB$_{Ag}$ (100 µl samples) through 5 to 20% linear sucrose gradients at 42000 rev/min for 2-5 h in a Spinco SW 50L rotor. Samples were treated with 1% SDS and 65 mM-DTT as follows: •••, room temperature, 15 min; ▲—▲, room temperature, 30 min; ■—■, 37°C, 30 min; ○—○, 100°C, 2 min. Untreated $^{125}$I-HB$_{Ag}$ spun in a similar gradient sedimented at 36S.

the 25S component, once formed, could not be adequately dissociated by further extensive disruptive procedures.

PAGE of HB$_{Ag}$ polypeptides detected by Coomassie blue staining

Preparations of HB$_{Ag}$ 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 HB$_{Ag}$ preparations after 2 cycles of equilibrium gradient sedimentation revealed nine or more polypeptides covering the mol. wt. range from 130000 to 14000 (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 HB$_{Ag}$ 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 70000 and 12 to 14000, and two minor polypeptides, 7 and 8, of mol wt. 17000 and 23000 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 30000 and 20 to 24000 respectively.
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 $^{125}$I-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 HB,Ag polypeptides were made using gel chromatography.

**Gel chromatography of HB,Ag polypeptides**

Samples of $^{125}$I-labelled HB,Ag were disrupted as for PAGE, and chromatographed in a 1.6 × 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 65,000 and 15 to 20,000 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 HB,Ag

When the rapidly migrating material isolated by disc-PAGE (12000 to 14000 daltons) was eluted from gel slices and chromatographed on Sepharose 6B, a single uniform peak of 15000 to 20000 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. 12000 to 100000. 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 HB,Ag polypeptides could occur during laboratory manipulations.

Analysis of material released from 25S component

\(^{125}\text{I}-\text{HB,Ag} \) 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. 83000 to 87000, but after boiling for 2 min with SDS and DTT before electrophoresis this large component dissociated to give a single polypeptide of mol. wt. 14000. Mild denaturation of HB,Ag 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. 82000 and 90000 described by Howard & Zuckerman (1974).

Proteins released during formation of the 25S component differed from the total \(^{125}\text{I}-\text{labelled} \) 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. 14000. These results suggest that the major 14000 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 HB,Ag 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 \(^{125}\text{I}-\text{HB,Ag} \) 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 HB,Ag reported by other workers using continuous buffer PAGE.

Thirdly, the proportion of radiolabelled material or the intensity of the stained protein band of 12000 to 14000 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 17000 to 24000 and 23000 to 30000, 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 $^{125}$I-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 HB,Ag 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 HB,Ag polypeptides after treatment with SDS and DTT. Reports that different HB,Ag 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 HB,Ag polypeptides. Two major polypeptides of mol. wt. 60,000 to 70,000 and 12,000 to 14,000 were consistently detected in purified HB,Ag. 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.

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REFERENCES


Polypeptides of HB,Ag


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