STUDIES ON THE NATURE OF HEPATITIS B ANTIGEN

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ABSTRACT

Studies were undertaken to characterize the nature of hepatitis B antigens and their respective function in hepatitis B virus infection. Hepatitis B surface antigen (HB_sAg) was isolated from the plasma of asymptomatic chronic carriers by several methods including centrifugation, affinity chromatography and isoelectric focusing. Analysis of purified antigen revealed the presence of both lipid and protein together with significant levels of carbohydrate. A heterogeneity was demonstrated for HB_sAg and this was found to be related in part to the organization of the protein moiety of the antigen. Hepatitis B core antigen (HB_cAg) was isolated from the plasma of a proportion of carrier plasma. Activation of an associated DNA polymerase active on an endogenous template permitted the radiolabelling of HB_cAg. Owing to the close association of the reaction product, such preparations were found suitable for use in a radioimmune procedure for the detection of antibody to this antigen. The possible location of all or part of the viral genome is discussed in relation to type B viral antigens and their expression during the course of infection.

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INTRODUCTION

A Background

Viral hepatitis is defined as acute inflammation of the liver resulting from infection by one of two or more serologically distinct viral agents (Memorandum, 1970); the term excludes, by common usage, hepatitis resulting from infection by a number of well-characterised viruses including herpes simplex virus, cytomegalovirus, Epstein-Barr virus and yellow fever virus. Clinical studies of experimental infection in human volunteers has clearly established the existence of two epidemiologically and immunologically distinct forms of viral hepatitis; infectious hepatitis having a short incubation period and serum hepatitis with a long incubation period (MacCallum and Bradley, 1944; Havens et al., 1944; Neefe et al., 1945). The causative agents have been designated hepatitis A virus (HAV) and hepatitis B virus (HBV) respectively. In addition, there is recent evidence to suggest that a significant number of post-transfusion hepatitis may be due to agents other than HAV or HBV (Prince et al., 1974; Feinstone et al., 1975). In parallel with these studies, a major stimulus to research has been the identification and characterisation of a unique antigen specific to type B hepatitis.

In 1964 Blumberg and coworkers described an antigen (Australia or Au antigen) present in the serum of an Australian aborigine which was found to react with antibodies in sera obtained from

multiply transfused haemophiliacs, and suggested this to represent a possible serum protein polymorphism (Blumberg, 1964; Blumberg et al., 1965). Further work however has demonstrated a close association between circulating Au antigen and viral hepatitis type B (Prince, 1968; Memorandum, 1970). Detection of this reaction has now become a routine and essential procedure in the diagnosis and control of type B viral hepatitis.

Virus-like particles bearing these specific antigenic determinants can be isolated from the blood, liver tissue and body secretions of patients with type B viral hepatitis. Although the aetiological agent has so far not been successfully cultured in the laboratory, a whole body of knowledge has been obtained by various techniques such as electron microscopy and serology relating to the virology of hepatitis type B. Further studies on the nature of its associated antigens will continue to add to our understanding of the epidemiology and prophylaxis of this disease.

B Nomenclature of antigens

Hepatitis B antigen (HBAg) is a general term to describe antigenic material produced during the expression of the genome of HBV. Previously used terms for HBAg include Australia antigen, SH antigen, Au/FH, and hepatitis-associated antigen (HAA).

During the past few years, several studies have shown the HBAg particle surface antigen (HB_sAg) to be antigenically complex.

A 'group' specificity, a, is thought to be shared by all samples of HBAg. In addition, the particles generally carry two subspecific determinants which belong to two sets of generally mutually exclusive determinants, d/y and w/r (Le Bouvier, 1971; Bancroft et al., 1972). Thus at least four phenotypic combinations or 'subtypes' are possible - HB_sAg/adw, HB_sAg/adr, HB_sAg/ayw and HB_sAg/ayr. In addition, phenotypic variations or subgroups within the a determinant have been suggested (Soulier and Courouce-Pauty, 1973; Courouce-Pauty and Soulier, 1974). A second, apparently unrelated antigen system has been described, the determinants of which are not generally exposed in fresh serum (Almeida et al., 1971). This has been designated the hepatitis B core antigen (HB_cAg) by virtue of its enclosure by HB_sAg-reacting material. Should phenotypic variants of HB_cAg become identified, these can be indicated in a similar way to the HB_sAg phenotypes.

An additional antigen, designated "e", has been reported in some HBAg-positive sera and appears to be specific for HBV infection (Magnius et al., 1972). This determinant will be referred to as HB_eAg. Recent work has indicated that HB_eAg may also contain complex heterogeneous determinants (Williams and Le Bouvier, 1975).

Antibodies to these various determinants are designated anti-HB_s, anti-HB_s/adw, anti-HB_c, anti/HB_e, etc.

C. Association of hepatitis B antigens and hepatitis B infection

Experimental studies carried out by Krugman and colleagues at the Willowbrook State School have clearly demonstrated the existence of at least two forms of viral hepatitis (Krugman et al., 1967). Each one clinically and epidemiologically resembled those described previously in adult human volunteer experiments (reviewed by Barker et al., 1971; Krugman, 1974). Two serum pools (MS-1 and MS-2) obtained from one individual in the Willowbrook study on two separate occasions produced short- and long-incubation period hepatitis respectively in human transmission studies. Further experiments showed that there was no cross-immunity between hepatitis induced by MS-1 (infectious hepatitis) and the disease induced by MS-2 (serum hepatitis). Although parenteral inoculation is the major route of serum hepatitis transmission, these studies clearly showed MS-2 to be infectious when administered orally, confirming the longstanding clinical observation of secondary infection in the absence of apparent parenteral inoculation in individuals living in close contact with cases of serum hepatitis (Propert, 1938).

The identification of an antigen closely associated with the causative agent of serum hepatitis represented a considerable advance in the understanding of type B viral hepatitis. This antigen (Australia antigen) was found incidentally by Blumberg (1964) during an investigation of β-lipoprotein allotype precipitins. The new precipitin was identified in the serum of a haemophiliac who had received multiple transfusions as a result

of its affinity for an antigen present in the serum of an Australian aborigine. Although initially regarded as a recessive trait, a relationship was soon recognized between Australia antigen and leukaemia, Down's syndrome, lepromatous leprosy and hepatitis (Blumberg et al., 1967a, 1967b). The electron microscopy of MS-2 serum has shown this material to contain both the small 16 - 25 nm diameter spherical and tubular forms of HB_sAg in addition to the complex 42 nm double-shelled particle (Krugman et al., 1974a). These morphological forms closely resemble two of the structures seen in sera containing Australia antigen as originally described by Bayer et al. (1968) and a close serological relationship has been demonstrated (Giles et al., 1969). The antigenic determinants associated with these virus-like structures are now referred to as HB_sAg and HB_cAg (see Section 2 above).

HB_sAg is first detected in the serum of an infected individual on average 4 weeks prior to clinical or laboratory evidence of liver damage and may persist in most cases until the onset of symptoms and liver dysfunction (Shulman et al., 1970; Krugman and Giles, 1970). In the majority of naturally-occurring cases of serum hepatitis, HB_sAg is most likely to be detected during the first week of the acute phase of illness and may persist from a few days to several weeks. Persistence of antigenaemia is known to occur in a small percentage of cases: HB_sAg has been continually detected in the serum of one individual over a period as long as 20 years (Zuckerman and Taylor, 1969).

Anti-HB_s generally develops some weeks or months following recovery, albeit at a low titre detectable only by sensitive assay methods such as radioimmunoassay or passive haemagglutination. During antigenaemia, anti-HB_s can occasionally be detected by electron microscopy as circulating antigen-antibody complexes. Ion exchange chromatographic analysis of these complexes has shown precipitating antibody to HB_sAg in acute hepatitis to contain IgG, IgM and IgA (Madalinski et al., 1974). Peters and Johnson (1972) found no significant change in the level of immunoglobulin at the onset of the acute phase, but subsequent serial determinations revealed a fall in IgG. This decrease may reflect an immunosuppression process related to the evolution of chronic antigenaemia observed in some patients with HB_sAg-positive hepatitis. A high anti-HB_s titre is frequent if there is a history of repeated exposure to the antigen, often in the absence of clinical disease. The use of a sensitive radioimmunoassay technique has shown that over 80% of haemophiliacs in the United States of America possess circulating anti-HB_s as opposed to 15% or less in the general blood donor population (WHO, 1975).

In parallel with the development of a humoral response to HB_sAg, specific cell-mediated immunity has been demonstrated. Reed et al. (1974) showed delayed hypersensitivity to HB_sAg using the leucocyte migration test in 6 patients recovering from type B viral hepatitis. All 6 had detectable levels of anti-HB_s and 4 still had evidence of circulating HB_sAg. The positive cell-mediated response appears to be transient, beginning 2 to 3 months

after the onset of disease and accompanies the clearing of HB_sAg from the circulation (Ibrahim et al., 1975). However, anti-HB_s was not detected in the latter study for several weeks, probably due to the use of a less sensitive assay technique.

Almeida et al. (1971) clearly demonstrated the presence of antibody in convalescent sera to the inner HB_cAg component of the complex 42 nm double-shelled particle. The same sera did not react with the outer HB_sAg coat, and the HB_sAg immune complexes closely resembled those obtained from homogenates of infected liver. It was suggested that recovery from HBV infection is accompanied by a relatively short-lived anti-HB_s response whilst a normal immune response of greater longevity is produced against HB_cAg. This hypothesis is supported by the observation of core-like particles confined to the nuclei of infected hepatocytes (Nowoslawski et al., 1970; Almeida et al., 1970). The availability of an infected chimpanzee liver containing HB_sAg has resulted in the development of a complement fixation test to this antigen (Hoofnagle et al., 1973). In 15 cases of HB_sAg-associated acute hepatitis, anti-HB_c was found to appear in all the patients during or immediately after HB_sAg antigenaemia. A strong correlation with the persistence of HB_sAg suggests anti-HB_c to be produced in response to the active replication of the virus.

The relatively high frequency of anti-HB_s at low titre in the population indicates HBV infection may frequently be silent and probably transient. However, mild forms of acute hepatitis may provide an unusually good background for the development of

severe HBAg-associated chronic hepatitis (Redeker, 1975). In addition, extra-hepatic lesions may be associated with the presence of HBAg eg polyarteritis nodosa (Trepo et al., 1972; Trepo et al., 1974), hepatocellular carcinoma (Prince et al., 1970; Vogel et al., 1970), and some cases of glomerulonephritis in children (Brzosko et al., 1974).

Redeker (1975) found chronic hepatitis to develop in 10% of patients admitted to hospital with acute icteric type B hepatitis. Of these, one third developed chronic active hepatitis, displaying a spectrum of hepatic lesions and sporadic episodes of jaundice. The remaining two thirds showed signs of persistence of HB_sAg associated with a continuing elevation of transaminase levels, but otherwise in good health. In the latter group, resolution of chronic persistent hepatitis may occur over one to three years, but serum HB_sAg persists. Comparison of HB_sAg titres between the two groups showed a significantly higher titre of circulating HB_sAg in cases of chronic persistent hepatitis.

In general terms, the pathology of HBAg-associated chronic aggressive hepatitis closely resembles the clinical syndrome of active chronic hepatitis in which 10% of patients possess circulating HB_sAg (Reed et al., 1971). However, of the remaining HB_sAg-negative active chronic hepatitis cases, 61% were found to possess a significant delayed hypersensitivity response to HB_sAg suggesting that a past exposure to HBV may have been an important event in the development of chronic liver disease (Williams, 1975). This is further substantiated by the observations

of Nielsen et al. (1971) who found a progression from HB_sAg-positive acute to HB_sAg-negative active chronic hepatitis. Successful immunosuppressive therapy for the treatment of active chronic hepatitis has implicated autoimmunity as an important factor in the pathogenesis of this condition. A recent hypothesis has suggested the stimulation of sensitized T-cell lymphocytes to the surface of normal uninfected hepatocytes is one result of viral-induced changes at the plasma membrane of infected cells (Eddleston and Williams, 1974).

Immunofluorescence has been frequently used to study the distribution of HBV gene products in vivo. HB_cAg reactivity appears by this technique to be restricted to the nucleus or perinuclear region whereas HB_sAg is confined to the cytoplasm. This duality of reactivity, first observed by Nowoslawski et al. (1970) has additionally been observed in experimentally infected chimpanzees (Barker et al., 1973). During the early stages of acute hepatitis, both reactivities are present in liver tissue. HB_sAg is characteristically distributed in a diffuse fashion in the cytoplasm of hepatocytes throughout the liver (Edgington, 1974). Extensive proliferation of the smooth endoplasmic reticulum of these cells gives rise to the 'ground-glass' appearance under the light microscope. The restriction of HB_cAg to the nucleus is accompanied by enlargement of the nucleoli and extensive proliferation of the chromatin (Huang, 1971). Gudat et al. (1975) examined over 100 liver biopsies from patients with type B acute and chronic hepatitis. A spectrum of antigenic expression was observed by immunofluorescence, ranging from extensive HB_sAg

reactivity in cases of chronic persistent hepatitis accompanied by few HB_cAg-positive nuclei to focal areas of limited HB_cAg and HB_cAg in every nuclei seen in heavily immunosuppressed transplant patients. The pattern of fluorescence in cases of chronic aggressive hepatitis was intermediate with equal expression of each antigen in focal areas. These findings indicate the immune response to be of paramount importance in determining the course of the disease. The presence of HB_cAg, a recently described antigenic moiety distinct from the HB_s/HB_c systems, appears to predispose the patient to the development of the chronic disease. Magnius and Espmark (1972) described HB_sAg as being present in the sera of 18 of 23 persistent carriers of HB_sAg found in haemodialysis units, but it was not present in any of the chronic carriers examined in the donor population. Nordenfelt and Kjellen (1975) have shown a close correlation between HB_eAg and the presence of HB_cAg. In contrast, carriers of HB_sAg in the donor population show no histological or biochemical signs of liver disease (Reinicke et al., 1972). Hence the presence or absence of HB_eAg may be an indication of HBV infectivity and the subsequent course of the disease after infection (Magnius et al., 1975).

Studies of the HB_sAg subtypes associated with type B viral hepatitis showed that the *ay* subtype to predominate in outbreaks associated with haemodialysis units, whereas the predominant subtype in asymptomatic carriers is *ad*. It has been proposed, therefore, that an HB_s/ad antigenaemia promotes the formation of

detectable levels of precipitating antibodies against other specificities such as HB_sAg (Magnius and Espmark, 1972). However, in geographical areas where *ayw* is the predominant subtype, its occurrence is associated with all categories of acute and chronic hepatitis as well as asymptomatic carriage (Nadziyannis and La Bouvier, 1972). The opposite appears to be true for zones where *adw* predominates. However, in mixed-zone populations, *adw* is found at a high frequency among volunteer blood donors as well as patients with chronic aggressive hepatitis, whereas *ayw* is more frequently encountered in haemodialysis units in addition to drug-abusers and their contacts (Gordon et al., 1972; Nielsen and La Bouvier 1973). The complexity of HBV epidemiology was also reported by Pons-Romero et al. (1974) who found both subtypes *adw* and *ayw* to be associated with all forms of acute and chronic type B viral hepatitis although the geographical origin of the HB_sAg-positive individual, and possibly the route of infection, may influence the subtype findings in any one area.

B Ultrastructure of hepatitis B antigen

Examination by negative staining and electron microscopy of HB antigen-containing sera reveals at least 3 discrete virus-like forms, all of which are agglutinated by anti-HB_s. By far the most common is a roughly spherical particle of variable diameter in the range 16 nm to 25 nm (Almeida et al., 1969). Interpretation of surface structure is difficult by negative staining with phosphotungstic acid, probably because of the poor penetration by

this large hydrated ion (Haschmeyer, 1968). However, Bayer et al. (1968) were able to resolve surface sub-units approximately 1 nm in diameter using sodium silicotungstate as a negative stain. Examination of immune aggregates shows these small particles to possess antigenic determinants in common with long filamentous forms which are also a characteristic feature of HB antigen-containing sera. Although possessing a diameter close to 20 nm their length may vary widely from less than 50 nm to over 200 nm. Regular non-helical transverse striations approximately every 3 nm have been additionally described (Almeida et al., 1969). The presence of long filaments can make for difficult recovery from rate zonal gradients of a larger double-shelled particle described by Dane et al. (1970). Present in far fewer numbers than either of the more common filaments or numerous smaller spherical particles, their detection is enhanced by the use of immune electron microscopy techniques, which reveal immune aggregates containing all three forms. Occasionally, aggregates consisting entirely of double-shelled particles are seen free in serum, and Moodie et al. (1976) have proposed that an additional antigen-antibody system may be present on the surface of these particles.

Treatment of a preparation consisting almost entirely of small particles with ether was found by Barker et al. (1969) to reduce the diameter by approximately 4 nm, suggesting the presence of an outer lipid-rich layer of 2 nm. The reduction in size was accompanied by an increase in buoyant density from 1.24 g cm^{-3} in CsCl to 1.27 - 1.28 g cm^{-3}. Their finding of

only one predominant size class of small particle is somewhat at variance with the report of Dreesman et al. (1972) that small particles were distributed in two discrete size populations of 19 nm and 25 nm respectively. Subsequent work from the same laboratory has indicated the 25 nm particles to be non-reactive with HB_sAg antiserum after separation by rate zonal centrifugation. In addition, a third size class was isolated of intermediate diameter 20 - 22 nm. As yet, the significance of this size heterogeneity remains unclear, but should be carefully considered in comparing biochemical studies on different preparations of small particles.

Hirschman et al. (1971) examined the morphological change accompanying the treatment of HBAg preparations with a wide variety of reagents. The size of the small particle was found to vary according to the purification method adopted. Small particles recovered in the void volume after chromatography on Sephadex G-200 were found to possess a modal distribution of diameter sizes from 15 to 20 nm, whilst particles purified by acid treatment followed by equilibrium centrifugation in caesium chloride were found to possess on average a somewhat smaller diameter of 13 nm. The loss of filaments resulting from the last procedure was suggested to indicate their derivation from the small particle in a manner suggested for TMV helix assembly (Durham and Klug, 1971). An additional particle was observed when HBAg previously banded in caesium chloride was treated with phosphate buffer pH 7, at concentrations equal to or in excess of 0.125M. These forms were readily penetrated with stain; similar ring forms have been

reported after equilibrium centrifugation in sucrose gradients (Bayer et al., 1968), after treatment with sodium deoxycholate (Bukema et al., 1974), and following immunoadsorption (Bouwes et al., 1973). Neurath et al. (1975b) have also described hexagonal ring structures after treatment with thyroxine. Treatment with 0.1% chymotrypsin resulted in the appearance of many lamellae-like structures, some containing obvious nicks (Hirschman et al., 1973). The susceptibility to this enzyme is in accordance with the reported high content of hydrophobic amino-acid residues, especially trytophan. A similarity was seen in the effects of varying reagents on both the small spherical particles and filaments, an observation in broad agreement with separate findings that there are neither antigenic nor gross amino-acid composition differences between these two forms (Vyas et al., 1972b). Furthermore, the circulating small particles may arise as a result of filament breakdown at some stage during or following cellular release (Tranvik et al., 1973; Huang et al., 1974).

Krzosko et al. (1972) investigated further the ultrastructure of the small particles obtained from the sera of three patients with chronic hepatitis. In the presence of 0.1 - 0.5M β-mercaptoethanol, particles of 8 - 10 nm diameter were observed possessing a high buoyant density typical of nucleoprotein. These structures were found to be further degraded by exposure to ribonuclease, and it was concluded that the small particles contained a ribonuclease core surrounded by a lipoprotein layer 5 nm thick which was readily removed by the sulphydryl reagent.

This observation has yet to be confirmed, although 10 nm structures are seen as a result of treatment of the small particles with the nonionic detergent Nonidet P40 (Wallace and Gordon, 1975).

It has also been reported that under certain conditions of purification surface projections are visible on the surface of the small spherical particles (Neurath et al., 1973b) but this has not been confirmed nor has it been examined as to what is the nature of the projections.

An early study of the double-shelled particle indicated a constant overall diameter of 42 nm, with an inner immunologically distinct core component (HB_cAg) of diameter 25 - 27 nm (Almeida et al., 1971). These workers speculated that the inner component was similar to that described by Nowoslawski et al. (1970) in thin-sectioned liver tissue obtained from cases of type B hepatitis; the reported value of 20 nm diameter for the intranuclear particles observed by electron microscopy is compatible with a diameter of 26 nm obtained by negative staining. A similar particle with a larger diameter of 55 nm has recently been reported in the serum of a case of active chronic hepatitis (Suzuki et al., 1974). This form was not penetrated by negative stain, and was absent in sera from 11 cases of acute hepatitis or chronic HBAg carriers.

Jokelainen et al. (1970) confirmed the double membrane structure of the 42 nm particle by positive staining with potassium permanganate which preferentially stains lipid-containing membranes. The outer membrane was revealed to be composed of

membrane sub-units of similar size to identically-stained filaments and small spherical particles in the same preparation. The inner core component was not differentiated in this study. However, the core component was found to stain with 5% uranyl acetate, pH 5, a result suggesting the presence of nucleoprotein. No uptake of stain into the other HBAg morphological forms was noted.

Tranvik et al. (1973) found the core component as released by Tween 80 treatment to consist of an outer shell possessing capsomere-like structures approximately 4 nm in diameter. This observation is consistent with the study of Yamada et al. (1973) who demonstrated by optical rotation electron microscopy the inner core of the 42 nm particle to possess typical icosahedral symmetry.

Lipman et al. (1973) have described in addition isolated core component possessing 14 nm long projections originating from their surface. The method of isolation unusually consisted of a caesium chloride gradient overlaid with 8% Nonidet P40 in 12% sucrose followed by a layer of 8% sucrose. The possession of projections with knob-like termini gave the particle an overall diameter of approximately 55 nm. The resulting areola appearance may represent an intermediate layer of matrix protein or an alteration in the outside of the core itself. The use of ficoll-sucrose gradients as an alternative method of isolation gave rise to 27 nm core particles characteristic of those seen by other workers and did not possess surface projections.

As yet, there has been no indication that HB_sAg is associated with a unique particulate form. Circulating HB_sAg appears to be of lower molecular weight than IgM (Magnius, 1975) although there is some evidence that it may be present additionally on the surface of both the filamentous and 42 nm double-shelled particles together with HB_sAg (Neurath et al., 197[illegible]).

E Properties of hepatitis B antigens

1 Physical properties

The location of antigenic determinants on particles of various morphological forms has facilitated the isolation of this antigen from normal serum proteins to a standard of purity suitable for chemical studies and for its use as an immunogen in the laboratory. Successful isolation is dependent on knowledge of both the physical and chemical properties of the antigen. These are summarized in Table (1). Although inherent variation of at least some of these properties may exist according to the initial source of antigen, a purification procedure includes at least one of the following steps: (i) bulk removal of low molecular weight serum protein; (ii) separation from other large macro-molecules and cell debris by virtue of physical properties; (iii) specific isolation of antigen by formation of antigen-antibody complexes; (iv) separation of the differing morphological forms bearing similar antigenic determinants.

Exclusion chromatography of antigen-containing sera, using cross-linked dextran (Sephadex) or spherical agarose particles, results in the appearance of HB antigen in or close to the void volume, indicating the high molecular weight of this fraction. Skinhøj and Hansen (1971) were able to estimate the molecular weight of the small spherical particle to be approximately 2.5×10^6 after chromatography through a column of Sepharose 4B. This is in close agreement with values obtained by centrifugation methods (see below). Protein of lower molecular weight was eluted much later.

The replacement of the hydrodynamic force by an electrophoretic field has been recently reported to improve further the resolution obtained with Sephadex G200 (Luzzio, 1975). In particular, antigenic activity was found to be readily separated from macroglobulin as a result of their different electrophoretic mobilities.

Alternatively hepatitis B antigen may be partially separated from other serum proteins by virtue of its characteristic buoyant density. Antigenic activity is found at a density within the range defining the serum high density lipoproteins (HDL: 1.063 - 1.21 g cm^{-1}), the exact value varying between sera and according to the chemical employed in forming the density gradient. Centrifugation of serum in buffered caesium chloride results in the isolation of antigen at average density of 1.20 g cm^{-1}, although the presence of immune complexes may be indicated by a second band of antigenic activity at 1.25 g cm^{-3} (Gerin et al.,

1969). Large quantities up to 500 ml of serum may be readily centrifuged in the Oak Ridge B-XIX zonal centrifuge, good resolution being obtained at 30,000 rpm for 16 hours at 5°C (Gerin et al., 1971; Gerin et al., 1975). The separation can be further improved by adding solid salt to increase the density of the serum and placing the sample at the bottom of the gradient prior to centrifugation. A second run under similar conditions for 22 hours provides antigen suitable for radio-labelling. Both sucrose and potassium tartrate have been used in place of caesium chloride, in both instances antigenic activity being recovered at the lower density of 1.16 g cm^{-3} (Kim and Tillen, 1973; Gerin et al., 1969). Burrell (1975) reported some loss of antigenicity as a result of the use of gradients containing 28% w/v caesium chloride alone. This was considerably reduced by the use of discontinuous sucrose gradients (0 - 50% w/v) containing 14.3% w/v caesium chloride throughout. Antigenic activity was recovered at a density of 1.20 - 1.22 g cm^{-3}, depending on the antigen source.

Although the filamentous forms are recovered at a similar buoyant density to the small spherical particles, only a proportion of the 42 nm particles are recovered in the same fraction, the remainder being recovered at the higher density of 1.24 - 1.25 g cm^{-3} after equilibrium centrifugation in CsCl (Gerin, 1974). A similar value of 1.24 g cm^{-3} was reported by Barinsky and Bocharov (1974) whilst Chairez et al. (1974) recovered 42 nm particles at a slightly higher density of 1.26 - 1.27 g cm^{-3}, both in the presence of CsCl.

Lipman et al. (1973) were able to separate HB_cAg from the 42 nm particle by the prior layering of 10% Nonidet P40 over a composite density gradient containing both Ficoll and sucrose (14 - 56% combined w/w) in deuterium oxide. After centrifugation to equilibrium, core particles were recovered at a higher density of 1.28 - 1.30 g cm^{-3}, the exact value varying according to the effectiveness of HB_sAg removal. Gerin (1974) and colleagues isolated HB_cAg from CsCl gradients at a density of 1.31 g cm^{-3} after treating 42 nm particles with 1% Nonidet P40; the core particles appeared aggregated by small protein molecules which were considered either anti-HB_c antibody, or a 'matrix' protein situated between HB_cAg and HB_sAg and not removed by the nonionic detergent. Maritsugu et al. (1975) found HB_cAg, tracelabelled by endogenous DNA polymerase activity in the presence of 1% Nonidet P40, to be heterogeneous in buoyant density. A broad band of HB_cAg activity (1.28 - 1.32 g cm^{-3}) still contained detectable traces of HB_sAg whereas a heavier population (1.35 - 1.36 g cm^{-3}) contained no HB_sAg as detected by radioimmunoassay. This value is compatible with the hypothesis that such material represents nucleoprotein.

The morphological heterogeneity of intact HB_sAg-bearing particles may be revealed by further centrifugation under rate-zonal conditions. Bond and Hall (1972) used a B-14 zonal rotor containing a shallow caesium chloride gradient ranging in density from 1.04 to 1.20 g cm^{-3} dissolved in an ethylenediamine acetic acid buffer pH 7.4 containing 1.0 mM magnesium chloride. After centrifugation for 3.5 hours at 48,000 rpm relatively

homogeneous fractions were obtained although an almost continual range in the size of particles was reflected in significant overlapping between optical density peaks. As a result, complete separation of each particulate form could not be readily achieved by one rotational step alone; conditions should therefore be carefully chosen to permit maximum resolution of one form only per run. These conditions can be accurately predicted by means of computer analysis (Leach, 1971). Similar separations may be obtained using continuous sucrose gradients (Garin et al., 1971; Vyas et al., 1972b). In each of these cases a significant reduction in recoverable antigenic titre was noted, which was found to be prevented by the addition of 0.5% human serum albumin to the sucrose gradient before sedimentation of the antigen. It is possible that in the absence of stabilizing protein, a slight conformational change leads to a loss of antigenicity (Garin, 1972). Alternatively in the absence of protein aggregation may occur.

Aggregation of antigen particles may afford some explanation of the high sedimentation coefficient initially reported. In an early estimate, Garin et al. (1969) sedimented small antigen particles through a linear 5 to 20% w/w sucrose gradient using a swing-out rotor and calculated the sedimentation constant to be 110S. However in subsequent experiments a computer analysis of the separation obtained in a zonal rotor produced a lower estimate of 54S (Garin et al., 1971). More recent analyses with the model E ultracentrifuge suggest the value to be in the range of 30 - 40S. Böhme et al. (1972) performed several determinations in an An-D

12 nm standard cell using antigen previously purified by gel filtration and equilibrium centrifugation and obtained an average value of 30.8S. Schober et al. (1971) obtained a similar value of 34.1S. A slightly higher value of 40.2S was shown by Kim and Tillon (1973) by the extrapolation of results obtained by the same authors using serum as an antigen source. In a comparative study, HBAg particles of subtype ad and ay were removed from sera by ammonium sulphate precipitation and purified by pepsin treatment and gel filtration (Bourbonnais et al., 1975); the S-value was found to differ with subtype (33.0 and 40.1S respectively). Reduction and alkylation does not appear to markedly affect the sedimentation constant, a value of 31S having been reported following this treatment (Vyas et al., 1972a).

Barinsky and Bocharov (1974) isolated 42 nm particles by equilibrium centrifugation at a density of 1.24 g cm^{-3}, and determined their S-value by co-sedimentation with radio-iodinated small particles through a shallow CsCl gradient. Assuming an S-value of 34.1S for the small particle (Schober et al., 1971), a direct comparison of respective migration by the method of Martin and Ames (1961) gave a value of 58.5S for the 42 nm particle. However, a much greater value of 110S has been reported for the inner core component alone (Kaplan et al., 1973).

The diffusion constant of the small particle was estimated by Le Bouvier and McCollum (1970) to be about 2×10^{-7} cm^2 sec^{-1} by measuring the equivalent position in immunodiffusion studies; a subsequent detailed analysis in the analytical ultracentrifuge

gave a value of 2.278×10^{-7} cm^2 sec^{-1} (Kim and Tillas, 1973). Substitution of this value together with a sedimentation coefficient of 40.2S into the formula:

$$\text{Molecular weight} = \frac{RTs}{D(1 - \bar{v}\rho)}$$

where R = gas constant, S = sedimentation velocity, T = absolute temperature, $\bar{v}$ = partial specific volume (see Table 1) and ρ = density of the solvent, gives the molecular weight of the small particle to be 2.4×10^6. Bohme et al. (1972) found by similar methods the diffusion constant to be 6.3×10^{-7} cm^2 sec^{-1}. Using their observed S-value of 30.8, the molecular weight of the 20 nm particle was calculated to be correspondingly lower at 8.6×10^5, largely due to the higher estimate for the diffusion constant. Their derivation of $\bar{v}$ from an observed buoyant density of 1.14 g cm^{-3} in CsCl would also decrease the estimate of the small particle molecular weight. Dreesman et al. (1972b) found by similar means purified small particles of 20 - 22 nm diameter to possess a molecular weight of 9.6×10^6. By virtue of their differences in S-value, Bourhonnais et al. (1975) calculated the molecular weights of HBAg particles, subtype ad, as 2.5×10^6 and subtype ay as 3.0×10^6 respectively. However, these results were not correlated with particle size nor was the number quoted of samples examined.

The considerably lower S value of 11.9 determined for HB_sAg indicates its size to be considerably smaller than HB_sAg (Magnius,

1975). This distinction is in agreement with the absence of lipid associated with HB_sAg as judged from its estimated buoyant density of 1.20 g cm^{-3} in CsCl. However, there is some evidence to suggest HB_sAg is additionally found together with HB_sAg on the surface of both the 42 nm particles and filamentous forms of HBAg (Neurath et al., 1975c).

The purity of HB_sAg containing preparations may be considerably enhanced by the inclusion of a purification step utilising the surface charge properties of the antigen-containing particles. Alter and Blumberg (1966) reported that antigen eluted from DEAE-cellulose after IgG and together with or slightly in front of serum β-lipoprotein, IgA, IgM and albumin when developed with a 0.1 to 0.2M linear phosphate pH 7.0 buffer gradient. Similarly, Schober et al. (1971) found optimal elution of antigen using a 0.12M phosphate buffer pH 7.5 when examining a number of antigen-containing sera. Electron microscopy of the eluted particles demonstrated their morphology to remain intact after this treatment. Bukemo et al. (1972a) also employed DEAE-cellulose as a final step in a purification procedure involving several cycles of centrifugation and treatment with pronase. Intact 22 nm particles were eluted by 0.2M NaCl in a 0.01M tris buffer pH 7.5. Alternatively, the antigen may be precipitated from whole serum by the addition of ammonium sulphate or polyethylene glycol (PEG). Anan'ev et al. (1972) recovered HB_sAg by the addition of ammonium sulphate at 0.23 to 0.37 levels of saturation. After gel filtration through Sepharose 6B, the product was found to contain less than 1% serum proteins. Neurath et al. (1973a)

examined by electron microscopy both the pellet and the supernatant obtained after the addition of 9.5% PEG 6000 at neutral pH. Both ad and ay subtypes wer separated into two populations of particles after standing at 4°C overnight, each possessing distinct size distributions. The pellets were found to contain a significantly increased proportion of both 42 nm particles and filamentous forms. That a minor proportion of the 20 nm diameter forms are also precipitated may reflect a heterogeneity in the surface chemical composition of this smaller form.

Such heterogeneity was reported by Dreesman et al. (1972b) who recovered 2 peaks of activity, pI 4.0 and pI 4.4, by isoelectric focusing of radio-iodinated HBAg exhaustively purified by ultracentrifugation following acid treatment. The appearance of a second molecular species was attributed to the release of HB_sAg from 42 nm particles as a result of exposure to low pH. Further work by Chaires et al. (1974) showed that HBAg particles not subjected to acid treatment could be resolved into a number of size classes by rate-zonal centrifugation in a CsCl gradient. Isoelectric focusing of the fractions after iodination showed that a population of 25 nm spherical forms, previously found to be non-reactive in a complement-fixation test for HB_sAg, possessed a pI value of 4.15. SDS-acrylamide gel electrophoresis of their particles resolved two major polypeptide components similar in size, but not identical with, the two major polypeptides obtained on analysis of both 20 - 22 nm particles, pI 3.95, and filaments, pI 4.10. In addition, rate-zonal centrifugation separated a population of smaller,

15 - 19 nm spherical particles bearing HB_sAg determinants and a very acidic pI value of 3.65. Of the six component polypeptides obtained from this fraction, five were identical in size to five of ten components obtained from the larger 20 - 22 nm particles. The sixth polypeptide, molecular weight 32,000, appeared to be unique to its morphological form. Purification using polyethylene [illegible]ol enabled preparations rich in 42 nm particles to be characterised in a similar manner. A single peak of radioactivity was recovered at pH 3.82, indicating a similarity of surface charge between 42 nm particles and the other HB_sAg-containing particles.

With the advent of suitably prepared adsorbants (reviewed by Lowe and Dean, 1974) specific antiserum to HBAg may be readily immobilised on an inert support whilst still retaining its affinity for the antigen. All reports so far have described antibodies linked to Sepharose-4B activated with cyanogen bromide, although bound antigen has been eluted with a variety of reagents. Tripatzis & Horst (1971) successfully demonstrated HB_sAg in the urine of hepatitis patients using affinity chromatography; bound antigen was eluted by decreasing the pH to 1.8. Grabow and Prosesty (1973) used 5M sodium iodide to break antigen-antibody complexes on a column of immobilised baboon serum; this yielded greater than 80% recovery of HB_sAg at a purity superior to that obtained by equilibrium centrifugation in caesium chloride. Reduction of the molarity of sodium iodide resulted in only partial elution of bound antigen, suggesting some heterogeneity in affinity of the antigen for its antibody. The

use of a hyperimmune baboon serum for the immunoadsorbant was found to be particularly useful as antibody was preferentially bound to the activated Sepharose-4B without the prior separation of the serum gamma-globulins.

The morphology and titre of HB_sAg purified in this manner remained unchanged in the presence of high concentrations of sodium iodide. Shiraishi et al. (1974) found that 1M acetic acid containing 1M sodium chloride (pH 2.5) eluted less contaminating serum protein although the recovery of HB_sAg was not as great as that obtained with 5M sodium iodide.

Noureth'at al., (1973c) combined PEG precipitation of HBAg with affinity chromatography using concanavalin A covalently bound to Sepharose-4B as an adsorbant. This had previously been found to agglutinate HBAg-containing particles by reversible cross-linking of the carbohydrate present in the antigen (Cawley, 1972). Immobilized antigen was eluted by the addition of 5% α-methyl-D-mannoside in tris buffer at pH 7.4.

An alternative approach is the adsorption of unwanted serum proteins by immunoadsorption. Nouwen et al. (1973) used IgG fractions of rabbit antisera specific for normal human serum components. The antigen, previously treated with pepsin and chromatographed through Sepharose-4B, was mixed with the rabbit antisera fractions. Following incubation, rabbit IgG was removed by passage over a cellulose immunoadsorbent containing immobilized sheep anti-rabbit IgG serum.

However, Neurath et al. (1974) found that purified HB_sAg particles which had not been previously treated with pepsin were irreversibly bound to immunoadsorbents containing immobilised antisera to pre-albumin, albumin, apolipoproteins C and D, and the γ chain of immunoglobulin G.

2 Chemical Properties

The small spherical particle (as the predominant morphological form in HBAg containing sera) has been the most extensively characterised in chemical terms. Several laboratories have developed techniques to purify either the 42 nm particle or filamentous forms, but exhaustive analyses are still awaited.

i Protein

Current estimates of the percentage weight of protein in small HBAg particles show considerable discrepancies. Bond (1972) has stated that less than 20% of the particle weight can be attributed to protein, based on particle counts, nitrogen content, and an observed total molecular weight of 3.5×10^6 daltons. On the other hand, estimates of 70% protein have been reported using colorimetric assays for protein and lipid, by Jonniaux et al. (1971) and by Takahashi (1975). By analogy with high density lipoproteins (density 1.006 - 1.23 g cm^{-3}), the buoyant density of HBAg of 1.20 g cm^{-3} in caesium chloride suggests a probable protein content of 40 - 60%. It is possible that these discrepancies may be due in part to variations in composition in HBAg from different sources, and to selective

alterations in the composition of HBAg particles purified by different methods. By assuming a protein content of 60 - 70% and an estimated total molecular weight range of 2.4 - 3.5 x 10^6 daltons, the total molecular weight of the protein moiety can be calculated to lie in the range 1 - 2.4 x 10^6 daltons.

The amino acid composition of small HBAg particles has been examined by several laboratories. A tryptophan content as high as 25% was suggested by Garlick and May (1973), based on a comparison of the UV extinction profiles of purified HBAg and tryptophan. Rao and Vyas (1974a) have estimated the tryptophan content at 13.9% by spectrophotometric titration with N-bromosuccinimide. A substantial tryptophan content would account for the values reported for the extinction coefficient of HBAg; $E^{1\%}_{280}$ has been estimated at 37.26 (Vyas et al, 1972b; Dreesman et al., 1972; Gerin, 1973), although other figures in the range of 25 - 30 have been obtained (Ling and Overby, 1972; Takahashi, 1975). It can be noted that the protein moiety is also rich in proline and leucine and in hydrophobic amino acids in general, which would facilitate its close relationship with lipid.

Optical rotatory dispersion and circular dichroism studies have shown that purified HBAg possesses optical asymmetry compatible with 70 - 80% of the protein being present as α-helix (Sukeno et al., 1972b). Treatment with 8M urea, 1% SDS, reduction of -SH groups or carbamidomethylation were all without effect on the spectra, indicating that the gross secondary structure had not been influenced by these chemical modifications.

Initial studies of HBAg polypeptides by SDS-acrylamide gel electrophoresis and Coomassie Blue staining indicated that 2 major polypeptide species were present, with molecular weights 24 - 26,000 and 28 - 32,000 (Garin et al., 1971; Vyas et al., 1972b; Garin 1973). Additional higher molecular weight polypeptides were reported by the two former authors, which were variable in amount and lost on further purification of the preparations before electrophoresis; these were assumed to be contaminating serum proteins which may have a stabilizing role in preserving antigenic activity.

In further studies, additional polypeptides of larger and smaller molecular weights have been detected, both by Coomassie Blue staining and location of radioactive polypeptide peaks (Table 2). Reproducible differences have been reported between HBAg of ad and ay subtype, with ay material in several studies tending to show additional minor polypeptides (Garin, 1972; Chaires et al., 1973; Chaires et al., 1975). Carbohydrate has been detected, by periodic acid Schiff staining of acrylamide gels, in three polypeptides common to both subtypes (32,000, 27,000 and 22,000 mol wt; Chaires et al., 1973). Finally, Chaires et al. (1974) have used rate zonal centrifugation to separate 42 nm particles, filamentous forms, and spherical HBAg particles of differing sizes ranging from 25 to 15 nm, and found that particles of differing morphology showed small but significant differences in their constituent polypeptides.

ii Carbohydrate

The presence of carbohydrate in HBAg was suggested by the report of Cawley (1972) of the precipitation of ^{125}I labelled HBAg by concanavalin A. The reaction between HBAg and concanavalin A coupled to Sepharose 4B has subsequently been used for HBAg purification (Neurath et al., 1973). A positive anthrone reaction by purified HBAg was referred to by Bond (1972), also indicating the presence of carbohydrate. Russell et al. (1973), using the phenol-sulphuric acid method, demonstrated the presence of carbohydrate in different preparations of purified HBAg, and found a progressive loss of serological activity after mild periodate treatment.

Chairez et al. (1973) reported a carbohydrate content of 3.6 - 6.5% in purified HBAg. These workers detected glycoproteins of molecular weights 32,000, 27,000 and 22,000 by PAS staining of polyacrylamide gels, and also found an additional small molecular weight component containing carbohydrate at or near the migration front, which did not stain for protein. More recently Steiner et al. (1974) have separated this low molecular weight component into two non-sialic acid containing glycolipids one of which was characterised as a water-soluble glycosphingolipid similar to fucosylglycolipins or blood group glycolipids. The carbohydrate composition of these compounds, and their possible serological activity have not yet been characterised.

Neurath et al. (1975a) produced particles which had a greatly reduced in vivo life span when inoculated into rabbits

and produced a higher humoral antibody response than intact antigen. Serological activity remained unaffected. It therefore appears likely that 20 - 25 nm HBAg particles contain variable amounts of carbohydrate, present both as glycoprotein and glycolipid, and that sialic acid is present as the terminal residue of some polysaccharide moieties. No extensive carbohydrate analysis has been carried out although Garin (1974) reported the absence of amino sugars in both adw and ayw subtypes.

iii Lipid

Although the lipid content of purified hepatitis B antigen may account for up to 30% of its total weight (Takahashi, 1975) very few attempts have been made to determine the nature of the lipid components. In early experiments (Barker et al., 1969; Garin et al. 1969) pretreatment of partially purified HB_sAg antigen with ether or deoxycholate was found to result in an increase of buoyant density in CsCl equilibrium centrifugation experiments together with a substantial reduction in particle size, presumably as a result of lipid loss. There was no appreciable loss of serological activity. Kim and Bissell (1973) found purified HB_sAg antigen to be susceptible to attack by the proteolytic enzymes subtilisin and subtilopeptidase A after treatment with diethyl ether for 2 hours at 0°C. Both Barker et al. and Kim and Bissell interpreted their findings as demonstrating that lipid solvents could remove a lipid fraction from HB_sAg particles with no significant reduction of antigenic activity.

Kim and Bissell (1973) described the nature of the lipid moiety obtained after extraction of purified small particles with chloroform:methanol. One dimensional thin-layer chromatography using silica gel as the solid phase and a chloroform:methanol: water (65:25:4) solvent system, revealed a predominance of polar lipids. The major components were identified as phosphatidyl choline and sphingomyelin. A minor component identified as phosphatidyl ethanolamine was also present. A spot resembling lysophosphatidyl choline in behaviour was not detected by iodine vapour and was therefore assumed to be protein. Cholesterol and non-polar lipids migrated close to the solvent front. The use of chloroform:methanol was found to remove the lipid efficiently while diethyl ether only removed all the lipid if the antigen was treated first with 1% SDS.

Takahashi (1975) extracted the lipid moiety from purified HB_sAg using the extraction procedure outlined by Folch et al. (1957). The washed chloroform:methanol extract contained both neutral and phospholipids (19.3% and 78.7% respectively). Cholesterol and phosphatidyl choline were the predominant species (36.0% and 82% of each fraction respectively). Further analysis of free fatty acids recovered showed an 18 carbon atom chain to be the predominant alkyl group, with 20% containing more than one double bond. Thin-layer chromatography showed the phospholipid phosphatidyl serine to be noticeably absent.

Steiner et al. (1974) characterised the phospholipids obtained after chloroform:methanol (2:1 v/v) extraction of purified

antigen. Chromatography in one and two dimensions demonstrated phosphatidylcholine, sphingomyelin and lysophosphatidylcholine to be the major phospholipids present. The percentage composition based on lipid phosphorus was estimated at 65%, 30% and 5% respectively. There was no evidence of the presence of phosphatidyl serine, nor of phosphatidyl ethanolamine; this latter result differed from the previously published analysis of Kim and Bissell (1973). The presence of glycolipid had been previously suggested by the uptake of periodate-Schiff reagent in an area of polyacrylamide gels near the migration marker where there was no uptake of the protein stain Coomassie blue. Prior treatment of antigen with chloroform:methanol removed this band from the gels and analysis of the extract in chloroform:methanol:water (60:35:8 by volume) revealed the presence of two glycosphingolipids. Both were negative in tests for sialic acid and phosphorus and their mobility was unaffected by digestion with pronase. The sphingolipid nature of one of these was confirmed by the findings of a sphingosine base characterized as ceramide. The second suspected sphingolipid was present in too small amount for further chromatographic analysis. The authors commented that the water solubility and lack of sialic acid of the characterized sphingolipid closely resembled the properties of the fucosylglycolipids or blood group glycolipids; carbohydrate analysis of these compounds would be of interest.

iv Particle-associated nucleic acid polymerase

Much progress has recently been achieved in the search for a possible virion-associated nucleic acid polymerase. Hirschman

et al. (1971) collected four sera from patients with viral hepatitis and pelleted HB antigen by centrifugation at 40,000 x g in the absence of divalent cations. This preparation when incubated at 38°C was found to stimulate the incorporation of ^{3}H-TTP in the presence of dATP, dGTP and dCTP into an acid-insoluble product. A linear reaction rate was observed during the first three hours of incubation. One HBAg negative serum treated in a similar way contained no polymerase activity. The low level of endogenous activity found in pelleted HBAg was abolished by pretreatment with RNase whereas the labelled product was found to be sensitive to digestion by DNase. The reaction was greatly stimulated in the presence of the double stranded synthetic primer (dAT), but surprisingly, not by poly-rA.DT. Ethidium bromide was found to inhibit the reaction (Hirschman et al., 1971) which was insensitive to the presence of 4-N-dimethylriframpicin or 4-N-benzyldimethylriframpicin. The level of endogenous activity was found to be in direct proportion to the titre of antigen contained in the pellets. All the sera with DNA polymerase activity contained some 42 nm particles under the centrifugation conditions adopted. Garin (1972) examined highly purified preparations of small particles in an assay system which possessed the necessary conditions to detect similar enzymic activities in other virus systems, but found no evidence for the existence of either an RNA dependent or a DNA dependent polymerase in association with this particulate fraction.

Loeb and colleagues (1973) examined different partially purified preparations of HB antigen from 5 sera from patients with Down's syndrome. In one sample, a linear DNA polymerase reaction was found during a 90 minute incubation period in the presence of all four nucleoside triphosphates, magnesium ions and Triton-X100 non-ionic detergent. However, in the absence of electron microscopy or specific serological precipitation tests, this reaction was deemed to resemble closely a non-specific activity detected in a four-fold concentrate obtained from a HB negative serum. An extended study of 42 sera consisting of samples from age and sex-matched patients with and without HB antigen demonstrated no specific association of DNA polymerase activity with HB antigen or epidemiologically associated with hepatitis B infection.

A significant advance on this preliminary work was the report of Kaplan et al. (1973), who demonstrated a D.A polymerase activity associated with the core component of the 42 nm particle. Eight sera were selected for the experiments after prior screening of 60 chronic carrier sera by electron microscopy to select those which contained a large proportion of 42 nm particles. These were subsequently concentrated 20-fold by ultracentrifugation. In all eight preparations examined, a moderate rate of incorporation of ^{3}H-TTP into an acid-insoluble product was detected over a period of 6 hours of incubation at 37°C and in the absence of exogenous template. The reaction was stimulated by magnesium ions and was found to have a pH optimum of 7.7. The presence of nonidet-P40 enhanced the observed level of incorporation, presumably by removal of the outer or surface antigen coat of the 42 nm

particle. The reaction was reduced 20-fold in the presence of actinomycin D and by at least half in the presence of daunomycin. Rifampicin showed little effect, and Kaplan et al. (1973) tentatively concluded the template to be DNA. However, confirmation of this conclusion by incubation with DNase or RNase was not possible. The presence of either nuclease had little effect on the reaction supposedly because of the limited accessibility of the nucleic acid template.

Although the nature of the template remained unconfirmed, the enzyme product was found to be associated with material possessing a sedimentation coefficient of 110S. Immunoprecipitation studies showed the radiolabel to co-sediment with core-associated polymerase activity and to be precipitated by serum containing HB_c antibody but not by serum containing only HB_s antibody (Greenman and Robinson, 1974). The product was therefore closely associated with the core of the 42 nm particle. No release of newly synthesized DNA was noted. Brief centrifugation of antigen-containing material in 15 - 65% w/w sucrose gradients revealed the peak of DNA polymerase activity to precede the peak of core antigen by several fractions. This gave rise to the speculation that the enzyme activity was associated with only a fraction of the core antigen. Examination with the electron microscope of similar material previously treated with 2-mercaptoethanol and nonidet-P40 demonstrated typical 27 nm core antigen particles precipitable with anti-HB_c. Core antigen particles free of contaminating anti-HB_c were recovered from CsCl equilibrium centrifugation gradients at a density greater

than labelled core particles (approximately 1.34 and 1.36 g cm^{-3} respectively).

Kaplan et al. (1973) investigated the nature of the tritiated product further by disruption of radiolabelled core particles by heating for 15 minutes at 37°C in the presence of 1% sodium dodecyl sulphate and 1% mercaptoethanol. After phenol extraction of the digest, approximately 20% of the acid-precipitable label was recovered in the aqueous phase and it was subsequently found to possess a buoyant density typical of DNA, banding at 1.71 g cm^{-3}. Parallel experiments showed the labelled product to migrate at a reduced rate of 15% after disruption of the core particles. This value varied as little as 10% over a 0.002M to 0.160M range of salt concentrations indicating the labelled DNA product to be double-stranded (Robinson, 1974). This was confirmed by the complete resistance of the product to a single-stranded nuclease (S_1) at 37°C.

Finally the observation that synthesis was unchanged in the presence of DNase, antibody to HB_s or HB_c, nor stimulated by calf thymus DNA, lupus serum DNA or Sendai virus RNA (Kaplan et al., 1973; Greenman and Robinson, 1974) suggested that the polymerase enzyme, in addition to the template, was highly sequestered within the core.

v Nucleic acid

Examination by ultraviolet absorption spectroscopy of small spherical forms and filaments purified by ultracentrifugation in salt solutions produced an absorption spectrum typical of protein, the ratio of absorption at 260 to 280 nm being approximately 0.67 (Gerin et al., 1971; Gerlich and May, 1973). It is unlikely therefore that nucleic acid, if present, could account for greater than a few percent of the total chemical composition of antigen purified in this manner, unless there be less than one genome present per particle. As yet, there have been no spectra reported for purified preparations of the intact 42 nm particle.

The first positive finding of nucleic acid in preparations of HB_sAg was reported by Jomvish et al. (1971). Ten ml aliquots of antigen-containing sera were fractionated by starch block electrophoresis followed by ammonium sulphate precipitation. Chromatography of this material through Sephadex G-200 resulted in the elution of small particles in the void volume free of normal human serum proteins as monitored by immunodiffusion. The total chemical composition of this material was subsequently characterized by colorimetric assays as 70% protein, 25% lipid and 5% RNA. No DNA was detected by the diphenylamine reaction. Incubation of the preparation with pancreatic RNase for 20 minutes at 37°C neither altered its immunoreactivity nor released non-particulate RNA detectable by spectroscopy. Further analysis following butanol treatment and phenol extraction showed the RNA to possess a sedimentation coefficient of 9S in an environment

of low ionic strength, and to contain almost equimolar proportions of the bases adenosine and guanosine and similarly cytosine and uracil. In later experiments (Jonwiak and Kocielak, 1973) the recovery of RNA was found to be in direct proportion to the amount of antigen eluted after dissociation of antigen-antibody complexes with 6M guanidine hydrochloride, pH 3.0 from immunoabsorption columns of Sepharose-4B containing immobilized antibody to HBAg. Further evidence that RNA may be closely associated with HB_sAg has come from the finding of radioactive uridine incorporation into HB_sAg small particles (Jonwiak et al., 1975). HB_sAg particles were isolated by immunoprecipitation with specific chimpanzee anti-HB_s. All the acid-precipitable counts associated with the purified antigen were identified as being incorporated into RNA. Pretreatment of the serum with RNase did not significantly decrease the amount of radioactivity recovered and there was no evidence of chimpanzee serum protein precipitation in the immune complex.

Kim (1971) also found RNA closely associated with preparations derived from papain-treated acute viral hepatitis sera. Purified HBAg possessing an extinction coefficient of 9.42 at 260 nm for a 1% suspension, was subsequently disrupted with SDS and chloroform followed by digestion with subtilopeptidase A. Nucleic acid was recovered from the lower phase after phenol extration and further characterized as possessing an E value of 6. It was suggested that this material was double-stranded due to its resistance to RNase digestion and the increase in observed optical density at 260 nm on heating. A good correlation was

found between DNA content (2.1%) and buoyant density. Shikata and Hanson (1973) obtained a filament-rich preparation after polyethylene glycol precipitation and gel filtration which showed a uv absorption spectrum suggesting a nucleic acid content of approximately 5%.

There have recently been reports of the successful isolation of double-stranded DNA from both circulating 42 nm particles (Robinson, 1974) and particles closely resembling cores from the nuclei of infected hepatocytes (Hirschman et al., 1971). These structures are purported to provide both initiation sites and a suitable template facility for the core-associated polymerase. However, these contentions remain to be exhaustively investigated. Core particles isolated from serum by Robinson and colleagues were concentrated more than a 1,000-fold prior to the polymerase reaction before disruption of the core particles with SDS to expose extraparticulate DNA. Mounting of this material onto 0.25M ammonium acetate pH 7.0 (Clayton et al., 1970) followed by shadow casting revealed circular nucleic acid molecules of mean contour length 0.79 ± 0.09 μm. Spreading with 60% formamide at pH 8.0 to demonstrate single-stranded nucleic acid revealed no additional molecules, thereby suggesting the molecular form to be double-stranded; a length of 0.79 μm would therefore correspond to a molecular weight of approximately 1.4×10^6. No supercoiled structures were seen. The elimination of the polymerase reaction step prior to SDS treatment or exposure to 7M lithium thiocyanate gave similar results, suggesting the double-stranded circular form was not modified by, nor was a product of, the polymerase

reaction (Robinson, 1974). A number of additional linear forms varying between 0.5 and 12 μm in length were observed if the DNase incubation was omitted or nucleic acid extracted from whole 42 nm particles. These structures were thought to represent the extraparticulate DNA present in all human sera (Kamm and Smith, 1972). Overby et al. (1975) have recently found similar molecules in the 42 nm particle cores, prepared and treated in a similar manner from approximately 6 litres each of HB_sAg positive plasma of subtypes ay and ad. Examination by electron microscopy of the DNA preparations after spreading with formamide revealed a predominance of double-stranded open circles having a similar mean contour length of 0.78 ± 0.1 μm. An additional feature was the presence of circular DNA molecules possessing also linear segments of varying lengths up to approximately 0.8 microns in about 5% of the observed structures after the polymerase reaction had been allowed to proceed for 6 hours prior to nucleic acid extraction. Overby et al. suggested that these structures represented various stages of DNA synthesis which proceeds by the rolling circle model (Gilbert and Dressler, 1968), a negative closed circular strand serving as a template for elongation of the positive strand by successive addition of nucleotides to its 3'hydroxyl end. This model is compatible with observed single stranded regions at the junction of the closed circle and the growing linear portion. These replicating forms may have been missed by Robinson et al. as a result of the smaller starting volume of plasma used. Assuming the DNA molecules to be completely closed, a circular molecule of 1.6×10^6 daltons can be predicted to sediment at a value of 14S, closely resembling the experimental

value of 15S obtained by Robinson et al. Open circular forms as observed by both Robinson et al. (1974) and Overby et al. (1975) of similar dimensions would be expected to possess a reduced sedimentation coefficient of 10S. Unfortunately, no estimates of sedimentation velocity were reported by Overby et al. In a series of thermal denaturation studies, Robinson et al. (1974) demonstrated a relatively sharp transition to an S1 nuclease-susceptible state commencing at approximately 70°C becoming 50% denatured at 72°C (Tm). By a direct comparison to PM-2 virus DNA treated in a similar manner, the G + C content was estimated at 49%. This was in good agreement with the value of 48% determined by buoyant density measurement using SV40 virus DNA as a marker.

Hung et al. (1975) compared the sequence homology of the reaction product with human embryonic liver DNA. The kinetic hybridisation technique employed about 2,500 cpm of labelled DNA product from core material denatured in parallel with the unlabelled test DNA at pH 13. After neutralisation, both preparations were incubated together for 4 hours at 68°C prior to digestion of unhybridized single-stranded DNA with S1 nuclease. No significant homology was observed between the labelled polymerase product and DNA from human liver, Wi-38 cells, salmon sperm or calf thymus. However, of 21 HB_sAg antigen-positive plasmas examined, all were found to contain 0.1 to 1 $\mu g\ ml^{-1}$ of free DNA, which subsequently exhibited a highly significant degree of homology by molecular hybridisation with the labelled polymerase product. The free DNA, isolated by equilibrium

centrifugation of dialysed plasma at a density of approximately 1.7 g cm^{-3}, consisted of linear molecules up to 1 microns in length. Two of the 21 HB_s antigen negative control groups of plasma gave similar results.

It has been suggested that the core particles obtained by detergent treatment are similar to the 27 nm diameter naked particles seen in the nuclei of hepatitis B virus infected human livers (Nowoslawski et al., 1970). This hypothesis has been further strengthened by the finding of core antigen in the nuclei of chimpanzee liver cells successfully infected with type B viral hepatitis (Barker et al., 1973). Equilibrium centrifugation of a liver tissue homogenate revealed 27 nm particles present possessing a buoyant density of 1.32 g cm^{-3}. Furthermore, these particles were aggregated by sera known to contain anti-HB_sAg specificity (Barker et al., 1974). Particles of similar size and density were successfully isolated from a similar homogenate of human liver obtained by Hirschman et al (1974a) at necropsy from a patient with chronic hepatitis B infection. As with cores isolated from experimentally infected chimpanzees, only a low level of DNA polymerase activity was detectable. Together with their lighter densities, these studies suggest further maturation of these particles may occur prior to or during encapsulation and release as 42 nm particles.

Hirschman et al. (1974b) examined the ultraviolet absorption spectrum of these particles and found a peak of absorption at 264 nm together with a shoulder at 280 nm, indicating the presence

of both nucleic acid and protein. Following treatment with SDS and exhaustive extraction with phenol, material was released which possessed an ultraviolet absorption spectrum typical of nucleic acid. Treatment with DNase resulted in an immediate increase in absorption over the ultraviolet wavelength range whilst treatment with RNase produced no appreciable change. There was no reaction when neutralised formaldehyde was added to a concentration of 1.8%, and these results taken together were indicative that double-stranded DNA was present in the core particles isolated from the hepatocyte nuclei. Electron microscopy showed strands of heterogeneous lengths, the longest being just over half that seen on examination of DNA extracted from the 42 nm particles in serum. However, random breakage of DNA molecules during extraction could not be excluded. Thermal denaturation gave a Tm of 77°C, indicating a G + C content of about 56%. A preliminary comparison of hyperchromic spectra with the spectrum obtained after heating to 87°C suggested 60% of the DNA to be 52% composed of G + C base pairs, with the remaining 40% having a much higher content of 68%. However, much higher concentrations of extracted DNA are required in order to confirm this finding.

It would be of considerable interest to examine the sequence homology of this DNA with the nucleic acid found in the 42 nm particle core, and hence establish other than by serological means whether the two forms of particle are closely related structures. Such a finding would strengthen the proposal that these particles represent nucleocapsids of the hepatitis B virion containing unique viral nucleotide sequences.

F Immunochemistry of hepatitis B antigens

Early studies involving the treatment of purified HBAg with organic solvents and dissociating reagents revealed that HB_sAg immunoreactivity was remarkably stable in the presence of compounds promoting denaturation, in particular diethyl ether, urea, sodium dodecyl sulphate and various proteolytic enzymes. Anan'ev et al. (1972) showed there was no loss of HB_sAg reactivity following treatment with 50% chloroform or diethyl ether. However, there was a complete loss of reactivity after exposure to ethanol. A similar loss has also been reported after treatment with butanol (Jomvish et al., 1971).

Several studies have shown HB_sAg to be stable for many hours at an acidic pH (Anan'ev et al., 1972; Dreesman et al., 1972a). Kim et al. (1971) found that treatment of a pool of serum by fivefold dilution with 0.02N HCl pH 2.3 containing 0.02% pepsin provided antigen free of normal serum proteins. This preparation was suitable for the immunization of both guinea-pigs and rabbits. However, it was noted by Kim and Bissell (1973) that pretreatment with sodium dodecyl sulphate or diethyl ether increased the susceptibility of HB_sAg to proteolytic enzymes.

The reduction of disulphide bonds results in the complete loss of HB_sAg reactivity (Vyas et al., 1972a; Sukeno et al., 1972a), although considerable antigenic activity may be regained by the alkylation of free sulphydryl groups with iodoacetamide. After alkylation, intact particles with a sedimentation constant

of 31Å were reformed (Vyas et al., 1972a). Imai et al. (1974) were able to define by the use of immunodiffusion and haemagglutination-inhibition techniques reduction-sensitive and reduction-resistant components of HB_sAg. The group determinant a was destroyed by exposure to dithiothreitol at concentrations below 10mM. At higher concentration resistance to reduction was serologically detected in all HB_sAg preparations examined, regardless of the subtype determinants present.

The reactivity of HB_sAg is remarkably heat-stable. Anan'ev et al. (1972) found no loss of reactivity after heating purified antigen for 10 hours at 60°C, but heating for 5 minutes at 100°C completely abolished its affinity for antibody. Similarly, Millman et al. (1970) noted a total loss of antigenic activity following 60 minutes incubation at 85°C. In a detailed study, Bond et al. (1974) demonstrated that the a group-specific determinant was stable at 60°C for periods up to 21 hours, whereas the d and y subtype reactivities were markedly reduced after only 3 hours of incubation at the same temperature.

The stability of HB_sAg at high temperatures together with resistance to protease digestion strongly suggests the presence of carbohydrate. Burrell et al. (1973) found a 90% reduction in the serological activity of purified HB_sAg particles after treatment with 0.01M sodium periodate for 4 hours at 37°C. A significant amount of carbohydrate relative to the protein content was found in the same preparations by the phenol-sulphuric acid method (Dubois et al., 1956). Chaires et al. (1973) estimated

3.6 to 4.5% carbohydrate content in HB_sAg by the same method. The carbohydrate moiety was found as glycoprotein and glycolipid (Steiner et al., 1974).

There have been several recent attempts to raise specific antisera in animals using individual polypeptides separated by acrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Dreesman et al. (1975) isolated from purified HB_sAg/adw and HB_sAg/ayw three glycopeptides of molecular weights 19,000, 24,000 and 27,000 and two larger non-glycosylated polypeptides of molecular weights 35,000 and 40,000. The 19,000 glycopeptide from HB_sAg/ayw together with the 27,000 molecular weight glycopeptides from both sources failed to elicit an antibody response in guinea-pigs. The non-glycosylated polypeptides derived from both HB_sAg/adw and HB_sAg/ayw elicited antibodies which cross-reacted with intact HB_sAg particles in a radioimmunoprecipitation assay. Both polypeptides were therefore assumed to contain at least the a group-specific determinant. However, the 24,000 molecular weight glycopeptide from both sources produced antibodies which reacted only with the homologous antigen subtype. Further studies demonstrated a cell-mediated immune response to the 24,000 and 40,000 molecular weight components (Cabral et al., 1975). Peritoneal exudate cells from guinea-pigs inoculated with the 40,000 molecular weight polypeptide showed a significant response when challenged with intact homologous and intact heterologous HB_sAg particles. Exudate cells from animals immunized with the 24,000 molecular weight glycopeptide derived from HB_sAg/adw responded to intact homologous

antigen and its 24,000 and 40,000 molecular weight components. A poor response to HB_sAg/ayw was observed in these animals.

Shih and Gerin (1975) were also successful in raising antibodies to polypeptide components of HB_sAg. Antisera to seven polypeptides obtained by sodium dodecyl sulphate-acrylamide gel electrophoresis of HB_sAg/adw were found to react with ad and ay-coated red blood cells by passive haemagglutination assay, indicating that each of the seven polypeptides possessed at least one common group-specific determinant. Competition inhibition experiments with intact HB_sAg/ad as the competing antigen resulted in parallel slopes for the antisera. The displacement of the linear portion of the inhibition curve reflected a difference in binding affinity of these antisera for the intact HB_sAg particle. Further characterization using the passive haemagglutination assay for antibody subtype analysis has shown that each polypeptide stimulated subtype-specific as well as group-specific antibodies (Gerin, 1975).

Although the HB_sAg preparations in the studies of Dreesman et al. and Shih and Gerin contained no demonstrable normal human serum proteins, Cabral et al (1975) demonstrated a positive cell-mediated immune response in guinea-pigs immunized with normal human serum when challenged with the 24,000 molecular weight glycopeptide isolated by Dreesman et al. (1975). This finding suggests that the 24,000 molecular weight glycopeptide contains at least one antigenic determinant related to certain constituents of normal human serum.

Several workers have previously indicated that HB_sAg particles may contain traces of normal serum components. Millman et al. (1971) found purified HB_sAg particles produced precipitin lines in immunodiffusion tests with antisera to several human serum components after treatment with 1% Tween 80. Specific immunoreactivity was weakened or abolished by this treatment. Recently, Neurath et al. (1974) demonstrated HB_sAg was specifically adsorbed to immunoadsorbent columns containing sheep anti-human plasma immunoglobulins covalently linked to Sepharose-4B. Prior treatment of purified HB_sAg with proteases and nonionic detergents, in the presence and absence of diethyl ether failed to prevent HB_sAg adsorption, indicating that antigenic determinants related to host proteins were integral components of HB_sAg particles. Reduction and alkylation of the preparation abolished HB_sAg reactivity but did not prevent its adsorption, indicating that the HBAg-associated antigenic determinants related to plasma proteins were distinct from the group- and subtype-specific determinants of HB_sAg. Burrell (1975) also reported additional antigenic determinants to be present in close association with HB_sAg particles. Low affinity immunoprecipitation reactions with antisera to a range of normal human serum components were demonstrated. These determinants were not released by exposure to acid, Tween 80 or ether, but were removed by exposure of HB_sAg to trypsin or bromelain under conditions that otherwise preserved the structure of the small particles.

There is very little information on the immunochemistry of HB_cAg determinants. Huang (1975) found that the immunoreactivity of HB_cAg in formalin-fixed tissue sections obtained from liver biopsy specimens resisted protease digestion. Furthermore, treatment of fresh tissue with a variety of reagents including diastase, hyaluronidase, neuraminidase, lipase, lysozyme and Tween 80 did not reduce the affinity of nuclear HB_cAg for fluorescein-labelled anti-HB_c.

MATERIALS AND METHODS

A General reagents

All laboratory reagents were obtained from B.D.H. Ltd., Poole, England and were of 'Analar' grade wherever available. Phosphate buffered saline (0.14M NaCl, 0.011M Na_2HPO_4, 0.0017M NaH_2PO_4) was prepared from tablets (Oxoid Ltd., London) according to the manufacturer's instructions. 'Tris' buffers were formulated from 'Trizma' reagents (tris-(hydroxymethyl)amino methane and hydrochloride: Sigma Chemical Co., St. Louis, USA).

Protein standards for molecular weight estimations were obtained from Merck Chemicals (Darmstadt, West Germany). Nucleases, enzyme inhibitors, nucleotides and chromatography standards for lipid analysis were all purchased from the Sigma Chemical Co.

All isotopes were obtained carrier-free from The Radiochemical Centre, Amersham, England.

Primary and secondary scintillants for β^- particle counting were purchased from Koch-Light Ltd., Colnbrook, England and dissolved in 'Analar' grade toluene.

Acrylamide and N'N'-methylene-bis-acrylamide were used directly as supplied by Eastman-Kodak Chemicals, Rochester, USA.

The sources of other reagents and fine biochemicals for specified purposes are mentioned in the text where appropriate.

B Hepatitis B antigen

Plasma was obtained from the routine screening of blood donors for hepatitis B antigen by discontinuous counter-immunoelectrophoresis and radioimmunoassay. The principal antigen subspecificities, d and y, were determined (phenotypes ad and ay) by solid-phase radioimmunoassay using monospecific guinea-pig antisera. Specific anti-HB_s was not found by either of these methods in any of the samples. Sera were stored at -20°C in 100 ml volumes until required. Those found on examination to possess HB_sAg-associated DNA polymerase activity were subsequently stored at +4°C.

C Serological methods

Four techniques, counter-immunoelectrophoresis, latex particle agglutination, reverse passive haemagglutination and radioimmunoassay were employed at various times for the detection of HB_sAg and its antibody in plasma samples and experimentally-obtained fractions.

Electrophoresis was carried out using a modification of the technique described by Wallis and Melnick (1971). Agarose gels, 0.5% in 0.02M barbitone buffer pH 8.5, were prepared to a thickness of 2 mm on 76 x 25 mm glass microscope slides. Samples were dispensed into 3 mm wells 11 mm apart and subjected to electrophoresis for 2 hours at a constant current of 3 mA per slide. A 0.1M barbitone buffer pH 8.5 in both the anode and cathode compartments provided a discontinuous buffer system. Considerable enhancement in the case of precipitin recognition was achieved by the staining of slides following electrophoresis in 0.2% Coomassie Brilliant Blue.

The detection of HB_sAg by latex particle agglutination in fractions obtained following separation experiments was found to be both rapid and sensitive. Latex particles coated with guinea-pig antibody to HB_sAg were used essentially as described by Leach and Buch (1971). Approximately 50 µl volumes of sample and reagent were mixed with a wooden spatula on a glass surface. After 10 minutes of gentle rocking, the mixtures were examined for the presence of particle aggregates as evidence of HB_sAg.

Reverse passive haemagglutination was carried out using turkey erythrocytes coated with horse anti-HB_s ('Hepatest': Wellcome Reagents Ltd., Beckenham, Kent) as described by Cayser et al. (1974). Geometric mean titres of samples were obtained by two-fold dilution of 25 µl volumes in the test diluent. Optimum results were obtained after standing overnight at room

temperature, and titres expressed as the reciprocal of that dilution giving rise to a 50% haemagglutination pattern.

Radioimmunoassay was routinely carried out using a direct one-step non-competitive technique as described by Ling and Overby (1972). Early use of this method involved incubation of 100 µl sample volumes for 16 hours in polystyrene tubes coated with guinea-pig anti-HB_s ('Austria I', Abbott Pharmaceuticals Inc., North Chicago, USA). Radioactively-labelled anti-HB_s from the same source was used as an indicator of HB_sAg bound to the solid phase. More recent assays were performed by a modified technique using a polystyrene bead coated with guinea-pig anti-HB_s ('Austria II'). Sample volumes of 200 µl were incubated for the shorter time of 2 hours at 45°C prior to the addition of labelled human anti-HB_s to the solid-phase. Confirmation of specificity was achieved by incubating separate aliquots of sample with equal volumes of human convalescent anti-HB_s and normal human serum respectively. A greater than 50% reduction in the number of bound counts after incubation with specific antibody was taken as confirmation of a positive sample.

A modification of this technique was used for HB_sAg subtype (d or y) determination (Ling et al., 1973).

The presence of anti-HB_s was determined either by counter-immunoelectrophoresis as described or by solid-phase radioimmunoassay. The latter method was similar in all respects to the corresponding test for HB_sAg, with HB_sAg-coated polystyrene

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Tests for serological identity were performed by immunodiffusion in 0.9% agarose buffered with 0.01M tris pH 8.9 containing 0.1% protamine sulphate and 1 mM ethylenediaminetetraacetic acid (EDTA).

D Electron microscopy

Samples were briefly centrifuged at 3000 g for 30 minutes to remove cell debris that might have been present prior to centrifugation at 45,000 g for 2 hours to precipitate HBAg particles. The resulting precipitate was then resuspended in 2 ml of PBS and HBAg particles precipitated for a second time by centrifuging at 45,000 g for 2 hours. The supernatant was

beads for the immobilization of antibody and radiolabelled HB_sAg as indicator antigen.

All solid-phase radioimmunoassays were evaluated by the comparison of unknowns with a mean value obtained from a number of controls reacted with a negative serum pool. Values greater than 2.1 times the negative control mean (approximately 7 standard deviations) were deemed as positive by this test. For experimental purposes, results are quoted as a ratio of counts obtained for each sample: negative control mean, per unit time.

Immunoprecipitation assays for HB_cAg and anti-HB_c are described in Results for each individual experiment.

discarded, and the tube containing the pellet was inverted in a beaker lined with dry filter paper to allow any remaining fluid to drain off. The pellet was resuspended in 0.1 ml of sterile distilled water, and a drop was mixed on a clean glass slide with an equal volume of 4% ammonium molybdate solution adjusted to pH 5.3 with potassium hydroxide. A drop of the stained suspension was transferred to carbon-formvar-coated copper grids (Smethurst No. 400: Smethurst Highlight Ltd., England) and excess fluid was removed by touching the grid with the torn edge of a piece of filter paper. The grids were immediately examined in an AEI 801 electron microscope (AEI, London, England). A 'positive control' consisting of a serum previously characterised as containing all three morphological forms of HBAg was included in each test run.

Immune electron microscopy was performed by the addition of an equal volume of hyperimmune antiserum to HB$_s$Ag to the clarified sample prior to ultracentrifugation. The optimal dilution in PBS was previously determined by experiment and immune complexes allowed to develop by incubation for 16 hours at 4°C.

E. Gel chromatography

1 Gel filtration

Cross-linked dextran gels for molecular sieving ('Sephadex' Pharmacia Fine Chemicals, Uppsala, Sweden) were swollen in PBS in a steam bath to exclude trapped air. After cooling, gels were

poured in columns to give a minimum diameter to height ratio of 20:1. Gels were equilibrated with at least one bed volume of the desired eluent buffer before use. A 0.5% solution of Blue Dextran (Pharmacia) was used to indicate void volume (V_o). Samples were applied under reduced pressure either through end-adapters in direct contact with the gel surface or by underlaying following the addition of solid sucrose to increase the density of the sample.

2 Affinity chromatography

Agarose gel beads (Sepharose 4B: Pharmacia) were activated with cyanogen bromide by the method of Cautrecasas (1970) or purchased in an activated freeze-dried form. Coupling of immune globulin was accomplished in 0.1M bicarbonate buffer pH 8.3 by gentle mixing for 2 hours at 37°C or 16 hours at 4°C. Any remaining unfilled sites on the activated Sepharose were blocked by a further 1 hour of incubation in 1M ethanolamine. Before use, the gel was thoroughly washed in alternating cycles of 0.1M sodium bicarbonate + 0.5M NaCl pH 8 and 0.1M sodium acetate + 0.1M NaCl pH 4 buffer.

The prepared immunoadsorbants were packed in 5 ml bed volumes into 10 ml plastic syringe barrels over a layer of glass wool. A maximum of 4 ml of HB$_s$Ag-positive plasma was run onto the immunoadsorbant and incubated for 2 hours at 35°C prior to washing with bicarbonate buffer. Washing was continued until the optical density of the eluate was less than 0.1 at 280 nm. Bound antigen was eluted with 1M acetic acid + 1M NaCl pH 2.5.

Concanavalin A was obtained already immobilised onto activated Sepharose 4B (Pharmacia Fine Chemicals). The gel was thoroughly washed with 0.01M tris pH 7.5, 0.14M NaCl, 1mM $MnCl_2$. HB_sAg-positive plasma was applied as for the immunoglobulin-immobilised columns save the column was washed directly the plasma was applied. Carbohydrate-containing material was eluted with 0.01M tris pH 7.5, 0.14M NaCl containing 5% α-methyl-D-mannoside (Neurath et al., 1973c).

9 Ultracentrifugation

In early experiments, hepatitis B antigen was precipitated prior to centrifugation from clarified serum by the addition of a 30% w/w stock solution of polyethylene glycol 6000 (Koch-Light Ltd., Colnbrook, England) to give a final concentration of 5%. After gentle mixing for 15 minutes followed by an overnight incubation at 4°C, the precipitate was collected by centrifugation at 500 g for 10 minutes at 4°C. The precipitate was redissolved in 0.05M tris-HCl buffer (pH 7.2) to one fifth of the original volume. Quantitative assessment by discontinuous counter-immunoelectrophoresis and reverse passive haemagglutination demonstrated at least 90% of the antigen present in the original serum was present in the precipitate.

Three to four ml aliquots of the redissolved precipitate were subsequently layered onto 20 ml volumes of caesium chloride (CsCl) solution at an initial density of 1.20 g cm^{-3} and buffered

with 0.05M tris-HCl pH 7.5. The antigen was banded at its buoyant density by centrifuging at 100,000 g for 18 hours at 4°C in an SW30 MSE swinging bucket rotor. The gradients were collected from the top in one ml volumes and each fraction screened for the presence of HB_sAg. Fractions containing a high titre of antigen were pooled, concentrated in a Minicon B15 ultrafiltration unit (Amicon Ltd.) and rebanded in CsCl for at least a further 18 hours at 100,000 g. Fractions containing the antigen were again pooled, concentrated, and exhaustively dialysed against either 0.05M phosphate buffer pH 7.5, or PBS prior to storage at -20°C.

In later experiments, the initial polyethylene glycol step was omitted and the following centrifugation procedure adopted. After clarification of the hepatitis B antigen containing serum at 3,000 rpm for 15 minutes, 11.5 ml samples (density approximately 1.005 g cm^{-3}) were applied to a discontinuous CsCl gradient containing 15 ml of 38.5% CsCl (1.4 g cm^{-3}) and 12 ml of 22.5% CsCl (1.2 g cm^{-3}), buffered with 0.01M tris-HCl pH 7.3. The completed gradients were centrifuged at 25,000 rpm (80,000 g average) for 16 hours at 10°C in a Beckman SW27 swinging bucket rotor. A total of thirty-seven 1 ml fractions were collected from the top of the gradient and the presence of antigen detected by reverse passive haemagglutination. The refractive index was also determined for every fifth fraction. The five fractions containing the highest titres of antigen were pooled and 0.635 g of solid CsCl added (final density approximately 1.3 g cm^{-3}). After solubilization, the pool was clarified at 1500 rpm for

2 minutes and 4 ml layered over 5 ml of 38.5% CsCl. The discontinuous density gradient was completed by the addition of 4 ml of 22.5% CsCl (1.2 g cm^{-3}) and 5 ml of 11.25% CsCl (1.1 g cm^{-3}). The antigen was then allowed to float in the gradient to its buoyant density during centrifugation at 25,000 rpm (80,000 g average) for 16 hours at 10°C in a Beckman SW27.1 rotor. After centrifugation, the gradients were fractionated into twenty four 700 µl volumes and the presence of antigen detected as before. Refractive indices were determined for every third fraction. The three fractions containing the highest titres of HB_sAg were pooled and dialysed either against PBS or against a 1% solution of Urografin (sodium- and meglumine-amidotrizoate, 10:66; Schering Chemicals Ltd.).

Rate-zonal gradients were made by dilution of a 60% Urografin solution in 0.01M tris pH 7.3, 0.1M NaCl, 1mM EDTA. Five ml discontinuous 5 - 20% gradients were made in 5% incremental steps and allowed to diffuse for 1 hour at room temperature prior to the overlaying of 200 µl of antigen in 1% Urografin and centrifugation for 80 minutes at 60,000 rpm (260,000 g) at 10°C in a Beckman SW65 Ti rotor. Hepatitis B antigen was precipitated from fractions of interest by diluting 1:20 in water and centrifuging for 2 hours at 65,000 rpm (310,000 g) at 10°C in the SW65 rotor.

Equilibrium centrifugation of HB_cAg-containing samples was performed in 20 - 60% w/w preformed linear sucrose solutions. Five ml aliquots of 60%, 40% and 20% sucrose in 0.01 tris-HCl pH 7.4 were layered consecutively in a 17 ml centrifuge tube and

allowed to diffuse for at least 1 hour at 4°C prior to use. A 1 ml sample was placed on top of the gradient and the latter centrifuged at 25,000 rpm (80,000 g) for 16 hours at 4°C in a Beckman SW27.1 rotor. Fractions were collected in one ml amounts from the meniscus. In some experiments, sucrose-ficoll gradients were used as an alternative method. Following the method of Lipman et al. (1973), a 43% w/w sucrose, 13% w/w ficoll stock solution was prepared in D_2O (density 1.106 g cm^{-3}) containing 0.01M Na_3PO_4. Incremental dilutions were made 1:2, 1:3 and 1:4 respectively in D_2O containing phosphate and 4 ml gradients prepared using mixtures of an equal volume of dilution and stock solution. After storage overnight at 4°C, one ml of sample was placed on top of the gradient and the latter centrifuged at 57,000 rpm (235,000 g) for 4 hours at 4°C in a Beckman SW65 rotor. Fractions were collected in 500 µl amounts from the meniscus and the linearity of the gradient checked by refractometry.

C Electrophoretic methods

1 Isoelectric focusing

Protein mixtures were separated according to their isoelectric points in preformed sucrose gradients containing carrier ampholytes ('Ampholine' LKB Produkter, Bromma, Sweden). Samples containing between 1 and 10 mg of protein were added to the mixing chamber of a gradient former designed to deliver a 40 to 0% w/v sucrose gradient into a specially constructed glass column (Vesterberg and Svensson, 1966). The anode was protected with a lock

solution of 1.4% orthophosphoric acid (pKa 2.1) and 60% w/v sucrose. The preformed sucrose gradient was overlaid with 2% ethanolamine (pKa 9.5) as the cathode lock solution.

The carrier ampholytes were generally added to a final concentration of 1%. In some experiments, this was increased to 2% if solubility problems were encountered. The rapidly migrating carrier ampholytes established a pH gradient in under 8 hours at a maximum power output of 3 watts. Proteins were allowed to migrate to their respective isoelectric points for at least a further 24 hours before collecting the gradient by downward displacement with water.

Flat-bed isoelectric focusing was carried out in thin-layer plates of polyacrylamide as the stabilisation medium (Awdeh et al., 1968). Commercially prepared acrylamide layers impregnated with Ampholine (Ampholine-PAG plates, LKB Produkter) were used as described by Davies (1975).

2 Polyacrylamide analytical gel electrophoresis

The constituent polypeptides of HB_sAg were characterised by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), separation being effected at alkaline pH. Gels were prepared from a stock solution containing 20% w/v monomer and 1.4% methylene bisacrylamide as cross-linking reagent. This was diluted to the required concentration and buffered with 0.375M tris-HCl pH 8.9. Gels in addition contained 0.5M urea and 0.1% SDS. Polymerisation was brought about in 5 mm internal diameter

precision-bore glass tubes by the addition of N,N,N',N'-tetramethyl ethylenediamine and ammonium persulphate at final concentrations of 0.03 and 0.035% respectively. After allowing polymerization to proceed for several hours at room temperature, final polymerization was achieved by storage for 16 hours at 4°C. Polymerizing ions were removed by a preliminary electrophoretic step with 0.375M tris-HCl buffer containing 0.5M urea and 0.1% SDS. Resolution was considerably improved by the additional soaking of the gels in the same buffer containing additionally 0.1% dithiothreitol. Immediately before use, the gels were returned to clean running tubes and trimmed to the required running length of 8 to 10 cm.

Samples for analysis were disrupted in 1% SDS, 0.5M urea and 0.1% dithiothreitol at 80°C for 15 minutes or 100°C for 2 minutes. A one-fifth volume of 0.47M tris-phosphate buffer pH 6.7 was added and the density increased by adding a two-fifths volume of 80% w/v sucrose. Samples containing a maximum of 250 µg of protein were electrophoresed at 2.5 mA/gel in [illegible] tris-38mM glycine, pH 8.6, containing 0.1% SDS, 0.05M urea and 0.02% dithiothreitol. Phenol red was added to the samples as a tracking dye.

After electrophoresis, gels were extruded into 5% trichloroacetic acid and separated polypeptides fixed for at least 16 hours. Protein was detected by staining with 0.5% Coomassie Brilliant Blue and destained in 7% acetic acid. Carbohydrate was visualised by periodate-Schiff staining as outlined by Zacharius et al. (1969).

Stained gels were scanned at 620 nm (protein-stained) or 540 nm (carbohydrate-stained) in a Joyce-Loebl Chromoscan 200 densitometer. Trace-labelled components were detected by the freezing of the gels in the presence of glycerol prior to cutting the gels into 1 mm slices. Radioiodine was detected by the placing of slices directly into a Gamma-Guard well-type scintillation counter.

H Chemical analyses

1 Total protein content

The gross amount of protein present was estimated by a modification of the technique originally published by Lowry et al. (1951). To 100 µl of sample was added 1 ml of 0.01% copper sulphate, 0.02% potassium tartrate in 0.1M NaOH containing 2% sodium carbonate. After 10 minutes at room temperature, 50 µl of Folin-reagent diluted 1:1.4 in distilled water was added by vortex mixing and the development of a blue colour allowed to proceed for 30 minutes prior to the measurement of absorbance at 700 nm. Protein concentrations were estimated with reference to a curve obtained with crystallised bovine serum albumin (Sigma Chemicals Ltd.) dissolved to predetermined concentrations.

The concentration of HBAg in purified preparations was estimated where appropriate using an extinction coefficient of 37.26 for a 0.1% solution at 280 nm (Vyas et al., 1972). Corrections for light scattering were made where necessary by

taking a further reading at 320 nm.

2 Amino acid analysis

Samples for analysis were hydrolyzed in 6N hydrochloric acid at a final concentration of 0.1 - 1.0 mg of protein. A crystal of phenol was added to each mixture to prevent the hydrolysis of tyrosine together with 50 µl of β-mercaptoethanol for the reduction of disulphide bonds. The atmosphere above the mixtures, contained in heat-resistant glass ampoules, was replaced with nitrogen and heated for 1 to 3 days at 110°C in a hot air oven. After cooling the ampoules were broken and the hydrolysate concentrated by freeze-drying in vacuo in the presence of solid sodium hydroxide.

Dilutions of lyophilised material were made in 0.01N hydrochloric acid prior to injection into a Jeol 6-AH automatic amino acid analyser (Jeol Ltd., Tokyo, Japan). Constituent amino acids were separated on two columns of Jeol resin LC-R-2. Neutral and acidic amino acids were separated on a long column (0.8 cm x 50 cm) and basic amino acids on a short column (0.8 cm x 15 cm) using a predetermined sequence of sodium citrate buffers (pH 3.25, 4.25, 5.28) as eluents. Eluted amino acids were detected by spectrophotometry at 570 and 440 nm after reaction with ninhydrin. Quantitative estimates for each amino acid species were determined by comparison of integrals with those obtained using a standardized commercial mixture of α-amino acids (Calbiochem Ltd.). Norleucine (50 - 100 nmoles) was occasionally added as an internal marker. Variable lengths of time for the

hydrolysis of samples enabled corrections to be made for both the partial destruction of threonine and serine and the incomplete hydrolysis of isoleucine and valine.

Owing to the complete destruction of tryptophan during acid hydrolysis, the total content of this amino acid in purified MB_gAg preparations was determined separately by two different methods.

In the first method, samples were examined by spectrophotometry at 280 nm both before and after exposure to N-bromosuccinide following the method of Spande and Witkop (1967). Samples were diluted in 8M urea adjusted to pH 6.0 with acetic acid to give a final absorbance of 0.8 to 1.0. An initial ultra-violet spectrum was recorded prior to the addition of 10 µl aliquots of 10mM N-bromosuccinimide at 15 minute intervals to both the sample and the reagent blank. Bromosuccinimide was added until there was no further decrease in absorbance at 280 nm. A spectrum was again plotted and the percentage tryptophan content calculated using the formula of Spande and Witkop, viz:

$$\%_{\text{tryptophan}} = \frac{\Delta \text{ O.D.}_{280\text{nm}} \times 1.31 \times \text{m wt of tryptophan}}{\text{weight of sample/ml} \times \text{molar extinction coefficient of tryptophan}} \times 100$$

Alternatively, tryptophan content was estimated by non-destructive spectrophotometry by the method of Bredderman (1974). A known weight of sample was added to 6M guanidine-hydrochloride in 0.02M phosphate buffer pH 6.8 in order to expose and normalise

all available tryptophan residues. Optical density measurements were obtained at 275, 280 and 288 nm prior to the addition of 50 µl concentrated NaOH. The resulting shift in tyrosine absorption at alkaline pH was monitored at 295 nm over a 15 minute interval and extrapolated to zero time in order to minimise variations due to turbidity (Edelhoch, 1967). The tyrosine content was then estimated using a ΔE_{295} of 2400 M^{-1} and the result used to calculate the absorbance due to tryptophan at 275, 280 and 288 nm measured at neutral pH. These values were then inserted into the following formula modified from Breddermann (1974):

$$\%_{\text{tryptophan}} = \frac{(1.861\ A_{280} - 0.716\ A_{288} - 1.146\ A_{275}) \times \text{dilution}}{\text{wt of sample (µg/ml)} \times 10} \times \frac{\text{mw tryptophan}}{\text{mw}}$$

The presence of free sulphydryl groups was estimated with the use of Ellman's reagent (5',5-dithiobis-2-nitrobenzoic acid; Ellman, 1959). Approximately 2 mg of HB_sAg in 0.05M tris-phosphate buffer, pH 7.5 was added to 5M guanidine hydrochloride containing 10mM of Ellman's reagent. The optical density of the solution at 412 nm was measured 15 minutes later against a reagent blank and the sulphydryl content estimated assuming a molar extinction coefficient of 13,600 at this wavelength.

3 Carbohydrate content

The total carbohydrate content of HB_sAg was estimated by the phenol-sulphuric acid method (Dubois et al., 1956). The phenol solution (80% by weight in water) was freshly prepared on the

day of use. A 20 µl volume followed by 1 ml of concentrated sulphuric acid produced a suitable coloration within 30 minutes of addition to 400 µl of sample. The optical density of each solution was measured at 480 nm (pentoses) and 490 nm (hexoses) with a similarly-treated reagent blank in the reference beam. The amount of carbohydrate present was estimated using a calibration curve obtained by analysis of standard glucose solutions.

4 Extraction of lipid

Lipid was extracted from HB_sAg by a modification of the procedure described by Bligh and Dyer (1959). Approximately 2 mg of purified HB_sAg in 0.5 ml of PBS was extracted by the addition of 1.5 ml of methanol-chloroform (2:1 v/v) in a conical glass centrifuge tube. The mixture was shaken intermittently for 1 hour at room temperature and the resulting protein precipitate collected by centrifugation. After removal of the supernate, the residue was resuspended in 0.4 ml of water and extracted with a second column of methanol-chloroform. The residue was again collected by centrifugation, and the supernatant decanted. One ml of water was added to the combined supernates and separated into two phases by the addition of 1 ml of chloroform. The lower chloroform phase was recovered and dried in a stream of nitrogen. No protein was detectable in this extract. The residue was redissolved immediately prior to analysis in 40 µl of methanol-chloroform.

The extracted lipids were analyzed by two-dimensional thin layer chromatography using activated silica gel as the stationary phase. Twenty microlitre volumes of each sample were spotted onto 20 x 20 cm glass plates coated with a 0.25 mm layer of silica gel containing a fluorescent indicator (Kieselguhr F_{254} TLC pre-coated plates, Merck Chemicals). Sample spots were air-dried and developed in chloroform-methanol-ammonia-water (66:27:3:0.9), dried, turned through 90^{o} and then developed for a second time in chloroform-methanol-acetone (16:2:3). After drying at 60^{o} for at least 15 minutes, separated components were visualized by examination under an ultra-violet light source for quenching of fluorescence and by exposure to iodine vapour. Permanent records were made by tracing the spots observed onto transparent film, and individual spots identified by reference to chromatograms developed using known standards.

In some experiments, spots visualized by fluorescence quenching were scraped from the plate and the lipid component extracted into 1 ml of PBS containing 0.1% Triton X-100. The extracts were then tested for antigenic activity by solid-phase radioimmunoassay.

I Radiolabelling procedures

Tracelabelling of HB_sAg with ^{125}Iodine, a gamma-emitting isotope of iodine, was routinely accomplished by a modification of the chloramine-T procedure as described by Hunter and

Greenwood (1962). Antigenic material, previously separated from plasma proteins, was quantitated by optical density measurement at 280 nm and adjusted to the required concentration with 0.05M phosphate buffer, pH 7.5. A 10 µl aliquot was mixed in a small glass vial with 1 mCi of $Na^{125}I$ in 10 µl. Chloramine-T in 100 µl of phosphate buffer was added dropwise through a No. 25 gauge syringe needle over a period of 30 seconds, to be followed 60 seconds later with a slight excess of sodium metabisulphite to terminate the reaction. The whole procedure was carried out at room temperature and unbound iodine immediately removed either by dialysis or by elution through a 25 x 0.9 cm column of Sephadex G-25 previously equilibrated with PBS. In later experiments, marginally better separation of HB_sAg from the reactants was achieved by elution through a similarly-sized column of Sephadex G-200 following dialysis overnight at 4°C.

Iodination of surface protein was carried out using the method of Stanley and Whelan (1971). The antigen preparation was standardized as for the chloramine-T procedure. The reaction mixture consisted of 100 µg of lactoperoxidase (Sigma Chemical Co.) in 100 µl, 1 mCi of $Na^{125}I$ in 10 µl and 100 µl of hydrogen peroxide previously diluted 1:3 in 0.05M phosphate buffer pH 7.5. The reaction was allowed to proceed for 15 minutes at room temperature before terminating the reaction by the addition of 160 µg of cysteine-hydrochloride. Free iodine and other reactants were removed as before.

Free amino groups present on HB_sAg particles were reacted with an iodinated hydroxyphenyl propionic acid ester as an alternative means of radiolabelling. The iodinated ester was prepared as described by Bolton and Hunter (1973) and stored as a dried residue at 4°C until required. Conjugation of the ester to HB_sAg was achieved by adding a 10 µl volume containing antigen to the residue together with 10 µl of 0.1M borate buffer, pH 8.5 at 0°C. After 15 minutes, 0.2 ml of PBS was added and conjugated ester separated from unbound ester by immediate chromatography of the reaction mixture on a 22 x 1.6 cm column of Sephadex G-200 equilibrated with PBS.

J Assay of DNA-dependent DNA polymerase activity

Whole plasma and serum was centrifuged in order to concentrate HB antigen to the bottom of a centrifuge tube. Large volumes of plasma were centrifuged in the SW27.1 rotor at 25,000 rpm (80,000 g) for 16 hours at 4°C, although in later experiments the time was reduced to 4 hours. Small volumes of sera obtained from clinical cases of hepatitis were centrifuged in the Beckman AR40.1 rotor at 30,000 rpm (28,000 g) for 16 hours. Pellets were resuspended in 1/20 of the original sample volume and 25 µl volumes added to a reaction mixture containing 16 µmol of tris-hydrochloride, pH 7.5, 4 µmol of $MgCl_2$, 12 µmol of NH_4Cl and 0.05 µmol each of dATP, dCTP and dGTP (Kaplan et al., 1973). Tritiated-TTP was also included at a final activity of 1.4 µCi. Enzyme activity was activated by the addition of monidet P40 and β-mercaptoethanol at

final concentrations of 1% and 0.3% respectively. The concentration of mercaptoethanol was increased tenfold in the preparation of labelled $^{125}I_c$Ag (Moritsugu et al., 1975). The total volume of the reaction mixtures was 145 µl.

Following incubation at 37°C two 50 µl aliquots from each reaction mixture were spotted onto Whatman 3MM 2.5 cm diameter paper discs, air dried, and immersed in 5% trichloroacetic acid for 16 hours. Discs were rinsed in 5% trichloroacetic acid for 1 hour prior to dehydration in absolute alcohol and air drying for 20 minutes at 60°C. The dried discs were counted by immersion in 10 ml of scintillation fluid (4 g 2,5-diphenyloxazole and 0.1 g 1,4-di-(2-(5-phenyloxazolyl))-benzene) and placed into a Carumatic 200 2-channel liquid scintillation counter. Vials were each counted for ten minutes to obtain a calculated cpm with a standard deviation not greater than 1%. Under these conditions, counting efficiency for tritium was approximately 45 - 50% and the (efficiency)^2:background ratio greater than 100.

R E S U L T S

A Hepatitis B surface antigen

1 Subtypes of HB_sAg

Each plasma available in a sufficient quantity for further study was characterised as containing HB_sAg of subtype ad or ay using a modified solid-phase radioimmunoassay procedure as previously outlined. A total of 52 donations were available from Regional Blood Transfusion Centres in the UK. Of these 30 were characterized as containing HB_sAg/ad (58%); the remainder were all confirmed as containing HB_sAg/ay (42%). There were no equivocal results using this procedure. In parallel, a reference panel obtained from the Standards Laboratory of the Public Health Laboratory Service was found to contain 74% HB_sAg/ad and 26% HB_sAg/ay. A further series of 9 donations was obtained from Athens, Greece. All of these were found to contain HB_sAg/ay.

A collection of serial samples were available from clinical cases of acute type B viral hepatitis for further study. A limited number were similarly subtyped and individual results are quoted below in conjunction with the finding of HB_cAg activity.

2 Morphology of virus-like particles in HB_sAg-positive plasma and serum

Examination of HB_sAg containing plasma and serum by negative staining and electron microscopy revealed a variety of pleomorphic

virus-like particles (figure 1). By far the most common was a roughly spherical particle varying in diameter from 16 to over 30 nm. In some samples, repeated measurements indicated a bimodal distribution within this range centred around 25 nm and 30 nm (figure 2). Interpretation of surface structure proved difficult owing to poor penetration of the negative stain, although occasional surface structures 4 to 6 nm in diameter could be resolved. Filamentous forms possessing a similar range of diameters were a constant feature, although present in far fewer numbers. No surface striations were discernable along their length which varied from 40 to over 200 nm.

Less than 1% of the morphological forms observed possessed a diameter greater than 40 nm. Of these the majority were double-shelled in appearance with an outer diameter of 42 to 45 nm, and closely resembled the particles described by Dane et al. (1971) in possessing an inner core component of approximately 27 nm in diameter. Densitometric scanning of micrograph negatives confirmed the complex structure of this particle and suggested the existence of a 1.5 - 2 nm thick electron-dense layer immediately in contact with the inner core component. A number of similarly-sized particles were not penetrated by negative stain to reveal any internal structure. These were particularly prominent in preparations containing HB_cAg-associated DNA polymerase activity (figure 3B). The latter preparations also contained an increased number of filamentous forms and occasionally a number of unpenetrated spherical particles 36 nm in diameter were also observed.

All these morphological forms were aggregated by either human convalescent serum or horse hyperimmune anti-HB_sAg.

3 Isoelectric focusing of serum containing HB_sAg

The technique of isoelectric focusing may be used for analytical or preparative separation from heterogeneous mixtures of individual ampholytes, particularly proteins. Figure 3 illustrates the resolution this technique may offer, which is accompanied by an almost complete recovery of total protein after separation. A 100 µl volume of serum previously clarified by centrifugation was focused in a pH 3 to 10 gradient in order to examine the relationship of HB_sAg to other plasma proteins in conditions of low ionic strength (figure 4). After 3 days of isoelectric separation, solid-phase radioimmunoassay for HB_sAg activity revealed a close association of the antigen with the major serum components possessing isoelectric points within the pH range 4.0 - 7.0. Antigenic activity was not detected in fractions containing separated gamma globulins, the latter possessing isoelectric points greater than 7.

Fractionated material containing HB_sAg were examined by electron microscopy. Small spherical forms of HB_sAg were seen in each of the fractions examined, demonstrating that the presence of a number of serum components may be closely associated with HB_sAg particles. Moreover, the amount of antigenic activity recovered appeared proportional to the amount of protein present, assuming an approximate linear relationship over the range of radioimmunoassay results obtained (Overby et al., 1973).

4 Effect of various reagents on the immunoreactivity of HB_sAg in plasma

As part of a wider study (see Section D below) the effect of various reagents on the immunoreactivity of HB_sAg in whole plasma was examined. Incubation with nonionic detergents would be expected to remove any non-specifically bound serum protein and lipid from HB_sAg. The preservation of antigenic titre demonstrated that no additional determinants were unmasked, and exposed determinants remained unaffected by such treatment. Exposure to anionic detergents produced no change at 1% final concentration (sodium dodecyl sulphate) or slightly reduced the antigenic titre (sodium lauryl sarcosinate). In contrast, the positively charged detergent, cetyl trimethylammonium bromide, appeared to markedly reduce the affinity of HB_sAg for its antibody, indicating a negatively-charged moiety to be important in HB_sAg immunoreactivity (Table 3).

Various dissociating reagents were also examined at concentrations sufficient to rupture surface hydrogen-bonds without leading to extensive unfolding of the HB_sAg particle. Of these, only formamide produced any significant effect, probably as a result of an interaction with HB_sAg protein by a mechanism unique to this weakly protic amide solvent.

The reducing agents β-mercaptoethanol and dithiothreitol were found to have markedly different effects. Enhancement of titre was apparent following removal of β-mercaptoethanol whereas a similar removal of dithiothreitol did not allow a recovery of

the initial antigenic titre. Unlike β-mercaptoethanol, dithiothreitol is less sensitive to oxidation and may result in the irreversible reduction of disulphide bridges.

The effect of some of these reagents on purified HB_sAg is examined further in Section D.

B Separation of HB_sAg

1 Gel chromatography of HB_sAg-containing plasma

Sephadex G200 is a relatively simple and economical stationary phase for the separation of macromolecules from lower molecular weight material. Samples of plasma were clarified by preliminary centrifugation at 15,000 g for 30 minutes at 4° and applied directly to a 100 x 5 cm diameter column previously equilibrated with 0.05M tris-hydrochloric acid buffer pH 7.4. Applied volumes of up to 75 ml resulted in elution of 5% of the total applied protein in the void volume which also contained HB_sAg activity. Although there was considerable dilution of HB_sAg, there was little or no loss of antigenic titre. Figure 5 shows the elution profile of a typical separation on Sephadex G200. Peak I possessed all of the recovered HB_sAg activity. IgG and albumin were the prominent components of peaks III and IV respectively whereas the elongated molecule of fibrinogen resulted in its elution immediately behind the void volume in peak II.

The constituents of peak I were analysed by isoelectric focusing. The result following 2 days of electrophoretic separation in a pH 3 - 6 gradient is shown in figure 6 . The removal of the majority of normal plasma proteins by the previous step of gel chromatography allowed the focusing of HB_sAg into two bands corresponding closely to peaks I and II of absorbance at 280 nm. The broad peak of protein in peak V (pI 5.2) corresponds exactly with the behaviour of human serum albumin in isoelectric focusing (Carlsson and Perlmann, 1969). Peak IV may also represent a polymer of human serum albumin. Peaks III and VI were unidentified.

Normal serum proteins were not detected in either peak of HB_sAg by immunodiffusion against horse antiserum to whole human protein. The isoelectric focusing of HB_sAg/ad resulted in the recovery of the d determinant in both peaks of HB_sAg activity, demonstrating that each peak had at least one determinant in common. Immune electron microscopy confirmed the presence of HB_sAg as the small spherical form (see figure 7). Measurements obtained from enlarged micrographs show that the particles in peak II possess an average diameter of 23 - 25 nm. Examination of particles recovered from peak I proved more difficult due to the poor definition of these micrographs, although some measurements were possible to indicate an average diameter of 24 nm. A number of experiments with HB_sAg/ad showed antigenic activity to be consistently recovered at 3.65 and 4.33, indicating a heterogeneity of isoelectric point for the small spherical form of HB_sAg. One sample of HB_sAg/ay was included in this series of

experiments, antigenic activity being recovered at pH 3.95 and 6.90 respectively. This difference almost certainly reflects the antigenic composition of the particle surface.

2 Precipitation of HB_sAg with polyethylene glycol

The effect of addition of polyethylene glycol 6000 on the concentration of HB_sAg from normal plasma proteins was examined as a preliminary to ultracentrifugation techniques. In initial experiments with HB_sAg/ad, the addition of polyethylene glycol resulted in the precipitation of over 90% of the antigen present in the original plasma. However, the percentage recovered in the precipitate varied over a wide range of values for individual sera, with recoveries as low as 20% in some cases. In these instances, recoveries were considerably improved by the lowering of the pH by the addition of 2N HCl to the plasma to pH 4, nearing the isoelectric point of HB_sAg. The results of an experiment performed on one plasma containing HB_sAg/ay is shown in Table 4. In addition, the recovery was marginally improved by increasing the final concentration of polyethylene glycol to 8%. However, the use of polyethylene glycol at acid pH was not regarded as desirable owing to the possible effects of acid pH on particle structure (see Discussion) and therefore polyethylene glycol was not employed in later studies.

3 Separation of HB_sAg from plasma proteins by ultracentrifugation

The limited quantity of HB_sAg obtainable by isoelectric focusing precluded the use of this technique for purifying large quantities of antigen for chemical analysis. As an alternative

procedure, HB_sAg was prepared from either the original plasma or polyethylene glycol-treated plasma (see Materials and Methods) by a combination of isopycnic and rate-zonal centrifugation procedures. Figure 8a shows the result of layering plasma onto a preformed density gradient consisting of CsCl and centrifuging to equilibrium. Antigenic activity was recovered in a concentrated band with an average density of 1.195 g cm^{-3}. This value is greater than for low and very low density lipoproteins but less than for other plasma proteins, being within the range defining a fraction of the serum high density lipoproteins (HDL: 1.063 - 1.21 g cm^{-3}). Rebanding of HB_sAg by flotation in a similar gradient resulted again in a homogenous peak of antigenic activity at the same buoyant density value and removed from the main peak of optical density (figure 8b). Further purification was performed by rate-zonal centrifugation. Although sucrose solutions are often employed in forming anti-convective gradients for rate-zonal separations, its use results in a considerable loss of antigenic activity. Table 5 shows the recovery of HB_sAg was less than 10% of the original following rate-zonal centrifugation in sucrose gradients whereas the applied sample obtained by two successive bandings in CsCl represented a recovery of nearly 75% of the HB_sAg present in the oringinal plasma. This was not the result of any significant breakdown of particle structure as a comparison with an identical gradient containing HB_sAg positive plasma run in parallel revealed no significant change in sedimentation properties (figure 9). The sedimentation coefficient was calculated as 52S from the peak of antigenic activity using the method of McEven (1967). Of interest was the finding that

recovery from the HB_sAg-containing plasma gradient was 31% whereas the recovery of HB_sAg previously subjected to isopycnic centrifugation was much lower at 11% of the total antigenic activity applied to the gradient. The use of urografin in the formulation of the stabilising gradient considerably improved the recovery of HB_sAg after rate-zonal centrifugation. In common with sucrose-containing gradients, HB_sAg was recovered in a single, symmetrical peak of antigenic activity. However, urografin was found to absorb strongly at 280 nm and to interfere with the Lowry protein determination assay, and it was therefore necessary to remove HB_sAg from the urografin by differential centrifugation prior to its chemical quantitation. Urografin did not interfere with the reverse passive haemagglutination test in any way, and the resulting preparations were free of normal plasma proteins as assessed by immunodiffusion. Examination by electron microscopy showed the final preparation to contain predominantly the small spherical morphological form of HB_sAg with diameters in the range 22 - 26 nm (figure 10). A proportion of the particles were penetrated by the negative stain to give a ring-like appearance. A number of short filamentous forms were also seen. Rebanding in CsCl isopycnic gradients of HB_sAg recovered from rate-zonal gradients showed no significant change in buoyant density.

4 Affinity chromatography

The recent development in the use of spherical agarose gels as support phases in the immobilisation of macromolecules has proved useful in the extraction of antigens from fluids as a

result of binding onto immobilised antibodies during passage through a suitably-prepared column. As an alternative approach to the purification of HB_sAg from antigen-containing plasma, a number of immunoabsorption columns were prepared using guinea-pig hyperimmune sera to HB_sAg purified by a centrifugation procedure similar to that outlined in (3) above. Similar results were obtained either by mixing whole serum or guinea-pig IgG separated by ion-exchange chromatography with freshly-activated Sepharose 4B. After absorption of HB_sAg as outlined in Materials and Methods, HB_sAg was optimally recovered using 1M acetic acid + 1M NaCl pH 2.5 to rupture antigen-antibody bonds. High concentrations of various salts at neutral pH proved unsuitable in eluting bound HB_sAg. The column could be reused several times without a significant drop in HB_sAg recovery. Table 5 shows, however, this single step was inferior to ultracentrifugation procedures with respect to both the total amount of HB_sAg activity and the degree of purification obtained. Antigenic activity was not precipitated from the eluate by differential centrifugation at 60,000 rpm for 2 hours, suggesting the rupture of the antigen-antibody bonds had also resulted in the breakdown of HB_sAg particle structure. Owing to the small amount of HB_sAg recovered from the eluate, this was not verified by electron microscopy. HB_sAg obtained by this procedure was analysed for amino-acid content for comparison with antigen prepared by alternative methods: the results are outlined in Section D of Results.

Affinity chromatography was also performed using commercially-prepared concanavalin A - Sepharose 4B, this ligand possessing the property of binding to carbohydrate-containing structures. It had previously been observed that the addition of concanavalin A to HB_sAg isolated by isoelectric focusing resulted in precipitation of antigenic activity, an effect that was reversed by the addition of α-methyl-D-mannoside. HB_sAg activity in HB_sAg-positive serum was found to be retained on a column of immobilised concanavalin A. The requirement for calcium ions for binding to this ligand necessitated the recalcification of plasma prior to chromatography. This activity was subsequently eluted by the addition of 5% α-methyl-D-mannoside (figure 11), along with other glycosylated serum proteins. This experiment illustrates the potential usefulness of this non-specific method for HB_sAg isolation and reveals that HB_sAg contains a carbohydrate moiety. The degree of purification obtained was comparable to the isopycnic centrifugation of plasma once in a CsCl gradient (Table 5).

5 Criteria of purity

HB_sAg purified by isoelectric focusing or ultracentrifugation did not contain normal plasma proteins as assessed by immunodiffusion against animal hyperimmune serum to normal human serum. Although the possibility that an anti-human serum response may result on injection of purified HB_sAg into laboratory animals, Ling and Overby (1972) estimated contaminating material to account for less than 5% of the total protein recovered. The use of pepsin during HB_sAg purification (Kim et al., 1971; Leach, 1975) proved unsatisfactory. Identical amounts of HB_sAg banded twice

in isopycnic CsCl gradients were centrifuged and the pellets resuspended either in 0.02% in 0.02N HCl or TNE buffer for 1 hour at 37°C. After dilution and repelleting, the titre of the pepsin-treated HB_sAg was 1:128 as compared with $1:8 \times 10^6$ for the control, representing a reduction in titre of 99.99%.

The biophysical separation of HB_sAg from serum proteins was monitored by flat-bed isoelectric focusing (figure 12). Centrifugation of HB_sAg/ay in a urografin gradient resulted in a single band in a 3 - 10 pH gradient, corresponding to an isoelectric point of 5.1. This is higher than the value obtained by isoelectric focusing in preformed sucrose gradients (pI 4.90 for the major HB_sAg peak), although pH measurements on the surface of the acrylamide layer was subject to a large degree of experimental error. A second band was not detected, probably because of the small quantity of sample applied to the gel.

C Analysis of HB_sAg

1 Radiolabelling of HB_sAg

Antigenic material separated from plasma proteins was tracelabelled for analytical studies with an isotope of iodine (^{125}I, half-life 60 days). Oxidation of carrier-free $Na^{125}I$ was regularly carried out in the presence of the mild oxidizing agent chloramine-T. Preliminary experiments demonstrated optimal incorporation of radioactivity at pH 7.5 in 0.05M phosphate

buffer. Under these conditions free iodide ions are incorporated almost exclusively into the tyrosine residues of protein to produce mono- and di-iodotyrosine (Hunter, 1973). Variation of the amount of oxidising agent per unit weight of protein permitted the preparation of iodinated HB_sAg to different specific activities. Elution of the reaction product in a column containing Sephadex G200 resulted in a peak of radiolabel in the void volume which corresponded to the elution of antigenic activity (figure 13). In the presence of 10% trichloroacetic acid, greater than 97% of the radiolabel was precipitated, confirming the specific iodination of HB_sAg protein. The addition of 30 ug of chloramine-T per ug of HB_sAg in the presence of 1 mCi $Na^{125}I$ gave a specific activityof 0.25 uCi/ug. Storage at $4^{o}C$ led to a gradual release of free iodine: after 2 months, only 60 - 65% of the total activity was acid-precipitable. This necessitated the removal of free iodine by frequent dialysis.

A similar elution profile through Sephadex G200 was obtained for HB_sAg iodinated by the lactoperoxidase technique. The specific activity of preparations iodinated by this method was approximately 30% less than iodination of a similar amount by the chloramine-T method.

Conjugation of purified HB_sAg with an iodinated acylating agent as outlined in Materials and Methods resulted similarly in recovery of radiolabelled HB_sAg in the void volume of a column containing Sephadex G200. Approximately 92% of the radiolabel was precipitated by trichloroacetic acid. The specific activity

of the preparation was approximately 0.16 μCi/μg.

2 Properties of radiolabelled HB_sAg

Incubation of HB_sAg radiolabelled by the chloramine-T method with rabbit hyperimmune anti-HB_s showed that 50% of ^{125}I-HB_sAg precipitated at a final antiserum dilution of 1:1500 (figure 14). The same antiserum was found to have a titre of 1:16 by counter-immunoelectrophoresis. Approximately 90% of the acid-precipitable radiolabel was precipitated in conditions of antibody excess. ^{125}I-HB_sAg prepared in this manner may therefore be suitable for use as a reagent for radioimmunoassay procedures.

Isopycnic centrifugation of HB_sAg after iodination in the presence of an oxidising agent revealed an increase in the buoyant density of the antigen according to the specific activity of the preparation. At a specific activity of 0.25 μCi/μg. ^{125}I-HB_sAg was recovered at a buoyant density of 1.25 g cm^{-3} following centrifugation in caesium chloride. A similar increase was observed for HB_sAg conjugated to the acylating agent hydroxyphenyl propionic acid ester.

Isoelectric focusing of ^{125}I-HB_sAg prepared by the chloramine-T procedure resolved the antigen into two peaks of radiolabel (figure 15). The isoelectric points were determined as 4.7 and 4.9 respectively, although there was some variation between preparations of HB_sAg obtained from different plasma samples. These values were consistently higher than those determined for

unlabelled, partially purified HB_sAg which was also resolved into two peaks by isoelectric focusing (see Section B above). In one preparation, the second, more basic peak was recovered from the alkaline pH range (figure 16). The isoelectric point of this material was 9.6 - 9.8 and closely resembled the value obtained by the isoelectric focusing of a sample rich in the filamentous forms of HB_sAg previously separated by rate-zonal centrifugation (figure 17). Small spherical forms of HB_sAg obtained from the same gradient possessed an isoelectric point of approximately 3.6. Purified HB_sAg conjugated to an acylating agent was found to possess a single isoelectric point of 3.65 (figure 18).

3 Amino-acid composition of HB_sAg

The amino-acid composition of purified HB_sAg was analyzed following hydrolysis in concentrated hydrochloric acid. Table 6 compares the recoveries of amino acids from the acid hydrolysates of antigen prepared by several different procedures. To assess the compositional relatedness of two different protein samples, a 'difference index' (DI) may be calculated by obtaining the difference in the percentage of umoles recovered for each amino acid and dividing the sum of their absolute values by half (Metzger et al., 1968). Two proteins with a similar composition have a DI of zero, whereas proteins with no amino acid in common give a DI of 100. A comparison of the two major subtypes of HB_sAg in this study demonstrated that HB_sAg/ad and HB_sAg/ay possessed a largely identical protein moiety, purified either by isopycnic and rate-zonal procedures (DI 3.1) or only by isopycnic

centrifugation (DI 6.8). The introduction of the rate-zonal centrifugation step in sucrose gradients led to a slight change of gross amino acid composition for the protein recovered from the band of antigenic activity (DI 11 - 12).

A comparison of HB_sAg/ad extensively purified by centrifugation with the same subtype eluted from an immunosorbent containing immobilized anti-HB_s/ad (DI 7.4) indicates that centrifugation procedures do not significantly alter the gross protein composition of HB_sAg. Similarly, a comparison of the filamentous and small spherical forms of HB_sAg revealed very little difference in the gross composition of the protein moiety of these two morphological forms (DI 6.8: Table 6). However, both contained higher proportions of the amino acids threonine, serine and proline than recovered for other HB_sAg preparations.

Three serum proteins, human albumin, fibrinogen and a Factor VIII preparation, were also analysed. The relatively lower proportion of basic amino acids found in HB_sAg by comparison to human albumin and fibrinogen is in accord with the lower isoelectric point of the antigen.

Owing to the destruction of the amino acid tryptophan during acid hydrolysis purified preparations were titrated with N-bromosuccinimide, a reagent which selectively cleaves tryptophanyl and tyrosyl bonds (Spande and Witkop, 1967). Because of the very high molar extinction coefficient of tryptophan at 280 nm, the effect of this titration at acid pH was followed by

spectrophotometry in the ultra-violet range of wavelengths. Figure 19 shows the u/v spectra of both subtypes before and after exposure to N-bromosuccinimide. The tryptophan content was subsequently calculated as 10.4% and 7.4% for HB_sAg/ad and HB_sAg/ay respectively. However, considerable variation was obtained at concentrations above 100 ng cm^{-3}. A second method enabled accurate measurements to be made of the contribution of tyrosine to the absorbance of HB_sAg at 280 nm (see Materials and Methods). The tryptophan content was subsequently calculated as 13.65% and 13.16% for HB_sAg/ad and HB_sAg/ay respectively. These values are in good agreement with the independently obtained values for the extinction coefficients of both subtypes (Table 7) and the u/v spectra of both the small spherical and filamentous forms of the antigen (figure 20).

Determination of free sulphydryl groups by their reaction with Ellman's reagent in the presence of guanidine hydrochloride showed that HB_sAg/ay contained approximately 0.12% of its total protein as free, reactive -SH groups. By comparison with the previously determined values for cystine and methione, this result suggests at least 90% of cystine residues exist as cysteine.

The amino acid composition may be used for the calculation of the partial specific volume of a protein. This was calculated as 0.742 and 0.736 for HB_sAg/ad and HB_sAg/ay respectively.

4 Polypeptide composition of HB_sAg

Polypeptide components of HB_sAg were separated by acrylamide gel electrophoresis after dissociation and reduction of the antigen. The resolution obtained was considerably enhanced by the use of the discontinuous buffer system. Figure 21 illustrates the densitometric scanning of acrylamide gels stained either for the presence of protein (upper traces in the figure) or carbohydrate (lower traces) following electrophoresis. Both HB_sAg/ad and HB_sAg/ay gave similar electrophoretic profiles. Protein staining revealed two major components of 90,000 and 82,000 molecular weight together with three smaller, minor components of molecular weights 78,000, 51,000 and 30,000 respectively. In addition, a number of minor components considerably in excess of 150,000 molecular weight were detected. Examination of the gels stained for the presence of carbohydrate revealed both of the major polypeptide species were glycosylated, although a much larger component of high molecular weight was intensely stained by this method. None of the smaller components appeared to be glycopeptides.

Following the radioiodination of HB_sAg by the chloramine-T method, the polypeptide composition was similarly analysed (Figure 22). The majority of radioiodine was found to be associated with polypeptides of molecular weights which closely resembled those determined for the major species as observed after protein staining. The minor, 30,000 molecular weight component was also detected in ^{125}I-HB_sAg. Higher molecular weight material was not clearly discernable into distinct polypeptide components.

The lactoperoxidase technique for the iodination of HB_sAg was employed for the radiolabelling of the HB_sAg particle surface. Co-electrophoresis demonstrated that when this procedure was used very little iodination of the major polypeptides occured. The 30,000 molecular weight component was labelled to the same extent as that labelled when chloramine-T was used. However, a large number of counts remained at the top of the gel following the electrophoresis of ^{125}I-HB_sAg iodinated by the lactoperoxidase technique. These results suggest that the 90,000 and 82,000 molecular weight polypeptides are integral components of HB_sAg.

SDS-acrylamide gel electrophoresis of ^{125}I-HB_sAg resolved into two peaks by isoelectric focusing (figure 23) illustrates that the two major polypeptides are segregated into each population by this technique. The more acidic peak (pI = 4.7) possessed the bulk of radioiodine as the slower moving 90,000 molecular weight polypeptide whereas the more basic (pI = 4.9) contained the lower 82,000 molecular weight component (figure 15). Both peaks contained the smaller, 30,000 molecular weight polypeptide, as shown by iodination.

5 Carbohydrate content of HB_sAg

The capacity of the agglutinin concanavalin A to bind to HB_sAg has already been demonstrated in Section C. The ability of the sugar α-methyl-D-mannoside to reverse this affinity is further evidence that HB_sAg contains an integral carbohydrate moiety. The total carbohydrate content was estimated by reaction

with phenol in the presence of concentrated sulphuric acid. The resulting coloration was similar in its absorption spectrum as for that obtained for a reference solution containing glucose, the wavelength of maximum absorption being 492 nm. No significant contribution to the absorption spectrum was seen at 400 nm, this wavelength being the peak of absorption for a reference solution of glucosamine. These results suggest the carbohydrate moiety to consist largely of hexoses, there being no significant amounts of pentoses (maximum absorption 480 nm) or amino sugars. Assuming a lipid content of approximately 30%, the total carbohydrate content of HB_sAg was estimated at 8% by reference to a standard curve obtained for glucose at increasing concentrations.

6 Lipid composition of HB_sAg

Neutral lipids, fatty acids, sphingomyelin and phospholipids were all detected by the thin layer chromatography of organic solvent extracts. No attempt was made to assess the relative proportions of each class in the lipid moiety of HB_sAg. The solvent systems adopted for the development of the silica gel chromatograms achieved a full resolution of extracted phospholipids. Phosphatidyl ethanolamine and phosphatidyl choline were both identified, but phosphatidyl serine was absent. This finding suggests that the carboxyl group of this phospholipid does not account for the acidic nature of HB_sAg as indicated by the isoelectric focusing of the antigen.

Antigenic activity was not detected by solid-phase radioimmunoassay in any of the extracts prepared from the separated lipid species.

7 Immunochemistry of HB_sAg

Several methods were examined for the preparation of antigenically-active sub-units of HB_sAg. Preliminary experiments demonstrated that a number of detergents and dissociating reagents did not reduce significantly the antigenic titre of HB_sAg-containing plasma (Table 5). One exception was the finding that the cationic detergent cetyltrimethylammonium markedly reduced HB_sAg activity. A further experiment showed this detergent to reduce both the group and subtype reactivities of purified HB_sAg/ad (Table 6), the subtype determinant being affected at a lower detergent concentration.

Separation of the protein moiety of HB_sAg was attempted by treatment of ^{125}I-HB_sAg with the nonionic detergent Nonidet P40 and β-mercaptoethanol, both at 1%, in the presence of 4M urea. Isoelectric focusing in a preformed urea gradient resulted in a single band of radiolabel with a shoulder towards the anode (figure 24). The isoelectric point of this material was determined as 5.9. Acrylamide gel electrophoresis revealed essentially the same polypeptide components to be present as for the original, untreated ^{125}I-HB_sAg. However, rate-zonal centrifugation of this treated material showed exposure to Nonidet P40, β-mercaptoethanol and urea reduced the S-value of ^{125}I-HB_sAg to 11.8. At least 15% of the radiolabel recovered

from this experiment was removed from suspension by incubating 100 µl in a polystyrene tube coated with anti-HB_sAg.

Several alternative methods were compared for the cleavage of HB_sAg into antigenically active components. Table 9 compares the recovery of antigenic activity following exposure to a number of conventional dissociating reagents. Sodium dodecyl sulphate (SDS) in the presence of urea and mercaptoethanol was found to abolish almost all of the antigenic activity. Although only 12% of HB_sAg was recovered after treatment with lithium diiodosalicylate (figure 25), this figure represented a considerable improvement on the results obtained following disruption with SDS or guanidine-hydrochloride in the presence of β-mercaptoethanol. Examination by electron microscopy revealed the absence of the small morphological form of the antigen following this treatment (figure 26). Gel chromatography of similarly treated ^{125}I-HB_sAg through a column of Sephadex G200 resulted in the elution of antigenic activity close to the retention volume of the column, and represented a shoulder of low molecular weight material recovered in the eluate (figure 27).

The finding of an unusually high tryptophan content in the protein moiety led to a consideration of the immunochemical importance of this residue. Incubation of HB_sAg with N-bromosuccinimide was found to result in a considerable reduction of antigenic activity as assessed by solid-phase radioimmunoassay (Table 8). The preservation of tryptophanyl bonds therefore appears to be of importance in maintaining HB_sAg reactivity.

B Separation of HB_cAg

1 Ultracentrifugation of HB_cAg-associated polymerase activity

Plasma samples containing moderate to high levels of HB_cAg-associated polymerase activity were examined further in an effort to identify the particulate structure possessing this enzyme activity. Isopycnic centrifugation in preformed linear gradients of sucrose revealed the major portion of recovered enzyme activity at a density of 1.28 g cm^{-3} with a minor peak at 1.25 g cm^{-3} (figure 28). Although HB_sAg was detected in both of these fractions, the main peak of HB_sAg activity was recovered at a lighter density of 1.18 g cm^{-3}. Attempts to perform similar experiments in gradients of caesium chloride were unsuccessful due to the inhibition of polymerase activity after exposure to caesium ions. A duality in the peaks of recovered polymerase activity was also observed using isopycnic gradients of ficoll mixed together with a reduced concentration of sucrose in deuterium oxide in order to reduce the osmotic pressure due to high concentrations of sucrose. Similar results were obtained (figure 28) in that two peaks of enzyme activity were recovered in HB_sAg positive fractions removed from the peak of HB_sAg reactivity.

In order to prepare large quantities of specifically-labelled HB_sAg, a plasma pool was prepared containing samples previously identified as containing high levels of DNA polymerase activity that was precipitated by anti-HB_s (Table 10). Large volumes of this pool were centrifuged to concentrate the

HB_cAg-containing particles, and the resuspended pellet centrifuged through a 20% sucrose solution to form a translucent band above a 45% sucrose cushion. Preparations containing a high level of enzyme activity were consistently isolated from this band. A repeat of this step produced preparations rich in the double-shelled spherical form of HB_sAg. A large number of HB_sAg filamentous forms were also present (figure 29). At this stage, HB_cAg was separated from HB_sAg in the presence of 1% Nonidet P40 and 1% β-mercaptoethanol and the HB_cAg tracelabelled by means of the endogenous DNA polymerase activity. Isopycnic centrifugation in CsCl revealed the tracelabel to be associated with material possessing a density of 1.35 g cm^{-3} (figure 30). The peak of trichloroacetic acid-precipitable material was symmetrical and contained no minor peaks at lower densities. After isopycnic centrifugation, in excess of 98% of the total activity recovered was precipitated by a chimpanzee anti-HB_c serum at a dilution of 1:50 in a radioimmunoprecipitation procedure (figure 31). HB_sAg was not detected by solid-phase radioimmunoassay, and the material was therefore suitable for further studies (see Section F below).

The sedimentation behaviour of HB_cAg separated in this manner was compared with that of the intact double-shelled particle. Rate-zonal centrifugation in preformed linear sucrose gradients resulted in the recovery of HB_cAg in a broad band prior to removal of the outer, HB_sAg coat. Following the DNA polymerase reaction, the close association of the reaction product with HB_cAg enabled its detection as a narrow, slowly-sedimenting band with an

estimated S-value of 420 (figure 32)

2 Separation of HB_cAg by isoelectric focusing

The use of isoelectric focusing as an alternative procedure for the separation of HB_cAg from HB_sAg and other plasma proteins was examined. A plasma sample containing a high level of HB_cAg-associated DNA polymerase activity was subjected to centrifugation. The pellet was resuspended, incubated with the enzyme reactants as outlined in Materials and Methods, and the whole applied to an isoelectric focusing column as for the examination of HB_sAg. ^3H-HB_cAg was recovered in close association with the major peak of optical density at pH 4.2 - 4.3. The close resemblance in the behaviour of HB_cAg to that previously determined for HB_sAg suggested incomplete removal of the outer coat during the enzyme reaction. However HB_sAg was absent from the peak containing ^3H-HB_cAg as assessed by solid-phase radioimmunoassay: the surface antigen was found at a lower pH of 3.6 - 4.0 (figure 33).

E Properties of HB$_s$Ag-associated DNA polymerase activity

1 Determination of optimal enzyme reaction conditions

A proportion of concentrated HBAg preparations obtained by the centrifugation of HB$_s$Ag-containing plasma samples were found to catalyze the incorporation of tritiated thymidine triphosphate (^{3}H-TTP) into a trichloroacetic acid-insoluble product on incubation with four deoxyribonucleotide precursors in the presence of the nonionic detergent Nonidet P40. The rate of incorporation was approximately linear during 6 hours of incubation at 37°C. The presence of the nonionic detergent was necessary for the full expression of enzyme activity (figure 34).

Some of the reaction requirements for the polymerase activity are illustrated in Table 11. All four deoxynucleotide triphosphates were required for optimal activity, indicating that the reaction product is DNA. The addition of the complementary ribonucleotide triphosphates to the reaction mixture at increasing concentrations progressively inhibited the incorporation of ^{3}H-TTP (figure 35). The increased reduction of incorporation in the presence of UTP probably reflects the lower concentration of TTP with respect to the other deoxyribonucleotides present in the reaction mixture.

Variation in the concentration of magnesium ions over a range of 5 to 40 µM did not significantly alter the rate of ^{3}H-TTP incorporation. A slight enhancement was seen at 20 µM (figure 36) and this concentration was therefore used in all future reactions. The effect of adding manganese ions to the reaction mixture over

a similar range of concentrations was also examined. There was a progressive decrease in the incorporation of ^{3}H-TTP, the lowest concentration used (5 μM) producing approximately 69% of acid-precipitable product as compared to the equivalent concentration of magnesium ions (figure 36).

Serial two-fold dilution of concentrated HBAg prior to the assay of polymerase activity resulted in a logarithmic decline in the observed level of ^{3}H-TTP incorporation (figure 37). This result suggested the possibility that the enzyme, template and primer were not present in equimolar quantities in concentrated HBAg preparations. Electron microscopy of one of these preparations revealed a high content of the filamentous forms of HB_sAg (figure 38). The possibility that the enzyme activity may therefore be stimulated by other morphological forms of HBAg free of HB_cAg was investigated by the addition of HB_sAg small spheres and filaments to the reaction mixture. A marginal increase in the level of ^{3}H-TTP incorporation followed the addition of either morphological form (Table 12). This stimulation was highest for the 1:50 dilution of both preparations. No polymerase activity was detected when HB_sAg was incubated in the absence of HB_cAg.

2 The nature of the template

In order to determine the nature of the nucleic acid template ie, whether RNA or DNA, reaction mixtures were incubated in the presence of RNase or DNase. The presence of either nuclease failed to decrease the level of ^{3}H-TTP incorporation, indicating the template to be inaccessible to the action of either of these

enzyme (Table 13).

In the presence of the mutagenic dye ethidium bromide, ^{3}H-TTP incorporation was reduced by approximately 50% at a concentration of 100 µg/ml (figure 39). The intercalating mode of action reported for ethidium bromide (Crawford and Waring, 1967) suggests that HB$_s$Ag contains a double-stranded nucleic acid template, probably supercoiled in structure.

Further indirect evidence that the template for the reaction is indeed DNA was provided by the finding that actinomycin D, a potent inhibitor of DNA-dependent nucleic acid synthesis, significantly inhibited ^{3}H-TTP incorporation (figure 40).

3 Nature of the product

The requirements of the HB$_s$Ag-associated enzyme activity indicated the reaction product was DNA (Table 11). This was confirmed by extraction of the nucleic acid produced during the reaction with sodium dodecyl sulphate and pronase. Incubation of this material with DNase significantly reduced the recovery of radiolabel as a result of precipitation with trichloroacetic acid whereas RNase had little effect (Table 14). The product of the HB$_s$Ag-associated DNA polymerase was therefore tentatively identified as DNA.

F Incidence of HB_eAg and anti-HB_e

1 Incidence of HB_eAg in asymptomatic chronic carriers

The close association between HB_eAg and a specific DNA polymerase provides an assay of enzyme activity for the detection of this antigen. During a period of several months, confirmed HB_sAg-positive plasma samples supplied by two Regional Blood Transfusion Centres in the United Kingdom were concentrated 20-fold prior to examination for a particle-associated DNA polymerase activity. The specificity of the reaction was assessed by incubation with an equal volume of rabbit anti-HB_e. A reduction of 20% or more in the level of polymerase present in the supernatant with respect to a negative control reaction was taken as indicating the presence of HB_eAg-associated DNA polymerase activity (Table 10). Amongst 52 samples obtained from the first centre, 5 (9.6%) were found to possess the enzyme, whereas 8 of a total of 18 (47%) from the second centre contained high levels of enzyme activity. There was no apparent correlation between HB_sAg titre and the level of ^{3}H-TTP incorporation. No significant levels of incorporation were found in a group of HB_sAg-negative plasma samples.

2 Incidence of HB_eAg-associated DNA polymerase activity in acute hepatitis sera

Initially, a total of 20 sera were examined for the presence of enzyme activity. Hepatitis B antigens were initially concentrated by ultracentrifugation in order to minimise the detection of non-specific, soluble enzyme activity. From this group, 13

(65%) were characterised as containing a significant level of DNA polymerase (greater than 200 cpm of demonstrable ^{3}H-TTP incorporation). However, the range of values was considerably lower as compared to specimens from asymptomatic chronic carriers (figure 41). A separate group of sera obtained from 10 cases of HB_sAg-negative acute hepatitis closely resembled the 9 HB_sAg-negative donor sera: the level of ^{3}H-TTP incorporation exceeded 200 cpm.

This study was subsequently extended to include sera collected at weekly intervals over a period of 1 to 6 weeks from 16 cases of HB_sAg-positive acute hepatitis, the first sample being taken at the time of onset of jaundice. The results from each series of assays is illustrated in figure 42 together with the titre of HB_sAg present as determined by solid-phase radioimmunoassay. During the course of these experiments, the mean value of ^{3}H-TTP incorporation obtained in a series of negative control sera was below 100 cpm. Higher values for ^{3}H-TTP incorporation were frequently confined to the first 2 weeks of observation, although the peak of enzyme activity may have preceded the appearance of HB_sAg. In one case, however, enzyme activity was elevated towards the end of the period of observation and it was accompanied by a sharp decline in detectable HB_sAg (patient no. 2: figure 42). A collective examination of the results from all 16 cases revealed a negative correlation between HB_sAg titre and the level of DNA polymerase activity present in each serum (figure 43).

One preparation of purified HB_cAg obtained from the nuclei of infected hepatocytes was available. No significant amount of DNA polymerase activity was detected in this material.

3 Incidence of anti-HB_c

Tritium-labelled HB_cAg separated from HB_sAg by isopycnic centrifugation in CsCl was incubated with serial dilutions of a chimpanzee hyperimmune antiserum to HB_cAg. Following 5 days of incubation at 4°C maximum precipitation of ^3H-HB_cAg occurred over a 1:10 - 1:100 dilution range (figure 31). Approximately 50% of the total tracelabel was present in the precipitate at a dilution of 1:100,000, demonstrating the potentially high sensitivity of this procedure for the detection of anti-HB_c. The capacity of several different human sera to precipitate ^3H-HB_cAg is shown in figure 44. A serum sample known to contain anti-HB_c as detected by immune electron microscopy was found to possess only a low affinity for the labelled antigen at low dilutions. One sample routinely used in the laboratory as a reference anti-HB_s reagent was found to additionally possess some anti-HB_c activity, but this affinity decreased at high dilutions below that detected for the human anti-HB_c reagent at equivalent concentrations.

A significant amount of precipitation was observed at two dilutions of a reference anti-HB_s reagent examined. However, the presence of distinct populations of anti-HB_s and anti-HB_c γ-globulins was not distinguished.

In a routine procedure, the capacity of acute and early convalescent sera to precipitate ^{3}H-HB_cAg were assessed after dilutions of 1:5 and 1:50 for each sample. The results from 7 of 14 acute hepatitis cases together with the findings of HB_cAg and HB_sAg activity are illustrated in figure 42. At each dilution examined, a significant level of anti-HB_c activity was present from the second week of observation onwards, the extent of precipitation being greater at a 1:50 dilution in most cases. The comparatively high titres of anti-HB_c activity in these sera resembles the high degree of precipitation obtained with a dilution of chimpanzee anti-HB_c included as a positive control, illustrating a reaction of serological identity between HB_cAg produced in man and the HB_cAg recovered in the liver of infected chimpanzees.

In several instances, an increase in anti-HB_c activity was accompanied by a sharp, though brief, rise in titre of HB_sAg (patients 3, 4 and 5). Anti-HB_s was detected by solid-phase radioimmunoassay only as a transient response, the level of corresponding HB_sAg being low (patients 3, 6 and 7). One exception was the pattern observed for patient no. 2 who presented a brief anti-HB_s response at week 4 in the presence of a high titre of homologous antigen. This was immediately followed by a sharp rise in detectable ^{3}H-TTP incorporation. However, it was not possible to determine whether this was indicative of belated HB_cAg production or the result of extensive liver damage accompanying a high titre of anti-HB_s

D I S C U S S I O N

A Hepatitis B surface antigen

Early studies in volunteers indicated that the concentration of infectious particles in serum obtained from patients in the acute phase of hepatitis B infection was approximately 10^6 cm^{-1} (Mazur et al., 1975). The concentration of HBAg particles counted by electron microscopy in a similar acute phase serum, however, is usually greater than 10^{10} cm^{-3} (Shulman, 1970) and may be as high as 10^{13} particles cm^{-3}. These observations support the contention that the morphological forms identified with HBAg are predominantly excess viral coat protein. The examination of HBAg-containing plasma by electron microscopy demonstrated the small spherical form of HB_sAg to be the predominant particle in samples chosen for the isolation and analysis of HB_sAg. The considerable variation in the size of these particles together with their subsequently determined composition provided further indications that particles possessing HB_sAg determinants only represent excess viral protein.

Early experiments by flotation centrifugation and the staining of precipitin lines revealed HB_sAg to contain both lipid and protein (Millman et al., 1970). The recovery of HB_sAg at a density of 1.19 g cm^{-2} in isopycnic CsCl gradients confirms the lipoprotein nature of this antigen, and is in close agreement with previously reported values (Gerin et al., 1969; Gerin et al., 1975). The separation of HB_sAg from plasma proteins

does not grossly alter the chemical composition of the antigen as assessed by buoyant density measurements. In addition, purification of the small spherical forms did not significantly change the sedimentation behaviour of HB_sAg in linear gradients of sucrose. In both cases the peak of antigenic activity corresponded to an S-value of 52, and closely resembled the value of 54S as determined by Gerin et al. (1971). In order to compute the average molecular weight of HB_sAg, this value may be substituted into the Svedberg equation (see p 23) together with the value of the partial specific volume ($\bar{v}$ = 0.736) empirically estimated from the proportions and partial specific volumes of its components. Assuming a diffusion coefficient of 2.278×10^{-7} $cm^2\ sec^{-1}$ (Kim and Tillee, 1973), the molecular weight of HB_sAg preparations containing predominantly the small spherical form is 1.96×10^6 by this procedure. However, this estimate is subject to considerable inaccuracy as an error of 1% in the value employed for $\bar{v}$ will produce a 3% variation in the calculated molecular weight. Although measurements of particle sizes indicated a possible bimodal distribution in small particle diameter, these populations were not resolved sufficiently by centrifugation procedures to allow separate estimates of their respective sedimentation coefficients.

Kim and Tillee (1971) reported that purified HB_sAg, derived from the serum of individual patients with acute hepatitis B infection, migrated in an electrophoretic field either in the α_2-β-globulin region or in the β-globulin region with some trailing. This confirmed their earlier findings of electrophoretic

heterogeneity of serum samples obtained from such patients. A heterogeneity of HB_sAg surface properties was also revealed in these studies by the isoelectric focusing of purified preparations (figures 6 and 15). The small spherical form was consistently isolated as two discrete bands in the region of acidic pH. In sharp contrast, a filament preparation was characterised as possessing a basic isoelectric point. Although the basis of this heterogeneity remained unclear, its preservation following tracelabelling by the chloramine-T method allowed the analysis of the two heterogeneous bands for their constituent polypeptides (figure 23). Each band was found to contain one of the major iodinated polypeptides, together with a smaller, 30,000 molecular weight component. Both of these major peaks of activity represented integral components of the small particle structure as revealed by comparison with similarly prepared antigen iodinated by the lactoperoxidase method (figure 22). With a molecular weight of 87,000 lactoperoxidase specifically labels those protein components whose tyrosyl residues lie on or close below the surface of the antigen particle (Stanley and Maslam, 1971). Trace-labelling by means of conjugation to an iodinated acylating agent via free amino groups led to the recovery of only one radioactive band at pH 3.65. Although the technique of isoelectric focusing may be used with great effect in resolving different morphological forms, an apparent heterogeneity may be of minor importance as populations of particles may only differ in amide content. Interestingly enough, the filamentous particle in HB_sAg preparations contained similar proportions of amino acids after acid hydrolysis to the hydrolysates

of small particles isolated from the same plasma sample (Table 6b). Alternatively, particle populations may differ only in the extent of partially filled sites available for other small molecules eg phosphate groups, or metal ions, neither of which may be directly involved in the structure of the antigenic determinants. It is worthy of note that similarly low values of determined isoelectric points have been obtained for QB phage and several plant viruses (Rice and Horst, 1972) as well as for Rous sarcoma virus (Hung et al., 1971).

The close association of HB_sAg with normal plasma components has been an acknowledged difficulty in developing purification techniques for separation of the antigen from plasma prior to biochemical and serological characterization. This association with other proteins in unfractionated plasma was confirmed by isoelectric focusing (figure 4), and supports previous findings of HB_sAg by radioimmunoassay in certain blood product fractions (Zuckerman et al., 1971). The antigen, morphologically consisting almost entirely of the small spherical particles, was found to be associated with plasma proteins over a pH range of 4.0 - 7.0. Of interest is the observation that antigenic activity was not detected in association with separated γ-globulins, which is in accord with the long epidemiological and clinical experience that γ-globulin is free of the risk of transmitting hepatitis and with the failure to detect HB_sAg, a marker associated with infectivity, by electron microscopy after Cohn fractionation of human plasma known to contain the antigen (Zuckerman et al., 1971). Isoelectric focusing of HB_sAg after

passage through a column of Sephadex G200 resulted in several HB_sAg-negative peaks at isoelectric points that closely resembled the monomeric and polymeric forms of human albumin. HB_sAg may therefore be eluted in the void volume of the gel together with one or more proteins which would otherwise be retained: the reversible nature of this association is demonstrated by the separation of antigenic activity from these contaminants during isoelectric focusing. Burrell (1975) identified several plasma proteins including albumin which may remain tightly bound to HB_sAg following purification. These additional components were not removed by brief exposure to low pH or by treatment with the detergent Tween 80, but were released by protease digestion.

A useful separation procedure for monitoring purity is electrophoresis in a 7.5% polyacrylamide gel in the absence of detergent, which allows clear separation of smaller molecular weight contaminating protein (Gerin, 1972; Gerlich and May, 1973). Gerin et al. (1971) reported that serum albumin was the major contaminant found by such analysis after two cycles of equilibrium centrifugation in caesium chloride. A further rate zonal centrifugation step was found to remove this contaminant, thereby eliminating all traces of serum proteins detectable by this technique. The same preparation was subsequently used to immunise guinea-pigs (Purcell et al., 1970), with the result that although a specific high titred antiserum was produced, titration in a full block complement fixation titration against dilutions of normal serum revealed a zone of reactivity at high

dilutions of normal serum. Non-specific antibody was readily removed by passage through a column containing normal serum protein components coupled to activated Sepharose-2B. The recently developed technique of flat-bed acrylamide gel isoelectric focusing is also suitable for the monitoring of purity. HB_sAg subjected to several cycles of purification by centrifugation was found to produce a single asymetrical peak following a final rate-zonal step in Urografin (figure 12). The length of the gel did not allow resolution of HB_sAg heterogeneity as was seen by isoelectric focusing in sucrose gradients.

Removal of residual traces of normal serum proteins was achieved by Dreesman et al. (1972a) by treating purified HBAg with 0.05M phthalate buffer pH 2.4; the purity of material prepared largely by centrifugation may also be enhanced by pepsin treatment (Leach, 1975). Such procedures assist in removing bound contaminants (Burrell, 1975), but in chemical studies also increase the possibility of denaturation of particle structure or release of essential components which may play an integral role in HBAg structure. Exposure of HB_sAg to pepsin at low pH was found to result in a severe loss of antigenic activity (Results, p 85).

In addition to lipid and protein, colorimetric assay of HB_sAg revealed carbohydrate to account for approximately 8% of its total chemical composition. The irreversible binding of antigenic activity to immobilised concanavalin A further indicated the presence of carbohydrate on the surface of HB_sAg (figure 11).

The reaction between concanavalin A and carbohydrate-containing molecules is specific for α-D-glucosyl and sterically related residues (Goldstein et al., 1965). Neurath et al. (1975a) found neuraminidase treatment released sialic acid from HB_sAg with a concomitant increase in isoelectric point from pH 4.35 (subtype ad) or 4.9 (subtype ay) to pH 5.45. Clearance studies in rabbits revealed desialylation of HB_sAg accelerated the elimination of HB_sAg from the blood by 10 to 20 fold. This finding may be analogous to that found for certain human plasma glycoproteins eg ceruloplasmin, although the clearance of others eg transferrin, remains relatively unaffected by the presence or absence of sialic acid (Ashwell and Morell, 1974). In addition, desialylation of HB_sAg enhanced the humoral antibody response and stimulated lymphocyte transformation induced by intact HB_sAg. There is little information available regarding the turnover rate of circulating HBAg in man. Boulier (1975) reported the half-life of purified HB_sAg injected into carriers at 3 to 6 hours, the total renewal time being 3 days. This rapid renewal rate may explain the failure to treat the chronic carrier state with anti-HB_s. As a cell-mediated immune response appears to play an essential role in recovery from type B hepatitis (Dudley et al., 1972), the presence or absence of terminal sialic acid may be of significance in the interaction between the infected hepatocyte, circulating HBAg, and host defence mechanisms. In addition, the extent of sialylation may determine the degree of infectivity of HBV in a manner similar to the neuraminic acid content of vesicular stomatitis virus.

A comparative analysis of HB_sAg/ad and HB_sAg/ay indicated there was little difference in the amino acid composition and the number of polypeptide components of either subtype (figure 21 and Table 6a). Both of the major polypeptide species appeared to be present as glycoproteins. The estimated molecular weight of each component (90,000 and 82,000 respectively) was found to be considerably larger than either of the two major polypeptides isolated in other laboratories. This almost certainly reflects the variety of analytical techniques employed and the diversity of HB_sAg obtained from different sources. In addition, wide discrepancies may arise due to the presence of varying amounts of tightly bound host proteins in different HB_sAg proteins or to the fact that particles of similar general physical properties but of heterogeneous composition can be produced during HBV infection. The presence of varying amounts of carbohydrate covalently bound to protein molecules may also result in some variation in observed molecular weights. It has also been suggested that the variability in HB_sAg polypeptide profiles may be due to incomplete post-translational cleavage of large precursor polypeptides (Gerin, 1974; Neurath et al., 1974) or to incomplete dissolution of strong non-covalent protein bonds (Vyas, 1974). The discrepancies in the reported molecular weights of the component polypeptides of the small spherical particle do not allow at present an estimate of the size of the HBV genome necessary to code for structural protein. If the various polypeptides are each composed of unique viral amino acid sequences, the results quoted above and by others (Gerin, 1972; Chairez et al., 1973, 1974) would indicate a total unique viral protein content in

excess of 300,000 daltons; this would require a significantly larger genome than the proposed 1.6×10^6 daltons of double stranded DNA, to code for structural protein alone, and could only be achieved if co-operation occurred between different pieces of a segmented genome, or if considerable additional genetic information were provided eg from helper viruses. If, on the other hand, many of these polypeptides represent host material or contain sequences in common with other polypeptides, considerably less genetic information would be necessary.

The possibility of active immunisation against hepatitis B using HB_sAg-containing material has been suggested by limited volunteer studies in man (Krugman and Giles, 1973) and recent work with experimental infection of chimpanzees (Markenson et al., 1973), in which immunisation with heat-inactivated HB_sAg-positive serum or purified small particles has successfully modified or prevented disease on subsequent challenge with live material. A possible alternative approach is the molecular characterization of the major HB_sAg haptenic site, allowing its chemical synthesis and possible use as an immunogen after coupling to a suitable carrier. The use of such a vaccine would avoid the potential risk of infectivity associated with clinical material, and its production would not be dependent on continuing supplies of HB_sAg-positive plasma. The feasibility of such an approach has been demonstrated by the successful chemical synthesis of antigenic determinants of TMV protein and egg-white lysozome, and the use of this material to stimulate antibody production. Unfortunately, little significant progress has been made towards the first step

in this approach, namely the characterisation of HB_sAg haptenic sites.

Mild treatment of HB_sAg-containing plasma with a variety of nonionic detergents resulted in no significant decrease in antigenic activity (Table 3). Nonidet P40 used in combination with mercaptoethanol in the presence of urea at high concentration was subsequently found to alter both the surface properties and the size of the small HB_sAg particle (figures 24a and 24b). The reduction of disulphide bonds appears essential for the disruption of hydrogen and/or hydrophobic bonds which play a major role in the maintenance of HB_sAg morphological integrity (Dreesman et al., 1973). Following isoelectric focusing, an asymetrical peak was subsequently characterised as an HB_sAg subunit of approximately 150 - 200,000 in molecular weight (11.8%). The presence of β-mercaptoethanol may have contributed to the observed reduction in the affinity of this antigen for anti-HB_s as chemical analysis indicated a large proportion of the cystine residues present in the protein moiety of HB_sAg existed as cysteine. A similar finding has been reported by Sukeno et al. (1972a) who found approximately 80% of antigenic activity was restored after reoxidation. The reason for the anomalous effect of β-mercaptoethanol in HB_sAg activity in plasma is unclear. The possibility of immune complex dissociation is unlikely as no activity was recovered at a density of 1.25 g cm^{-3} where antigen-antibody complexes are thought to be recovered after isopycnic centrifugation (Garin et al., 1969). Alternatively, the reagent may allow the antigen to adopt a more favourable configuration

following reoxidation in the presence of large quantities of plasma proteins. Dithiothreitol is a more effective reducing agent and is not so prone to reoxidation.

Several laboratories have recently reported the use of SDS in the presence of a reducing agent and urea as being an effective method for the production of immunogenic polypeptide components from HB_sAg. Shih and Gerin (1975) recovered a total of 6 polypeptides from HB_sAg/ad and 7 from HB_sAg/ay after electrophoresis of solubilized small particles in polyacrylamide gels. Following elution and reoxidation, individual polypeptides were used for the immunization of guinea-pigs. Each antiserum reacted with both ad and ay coated red blood cells by passive haemagglutination assay which indicated that all of the constituent polypeptides contained the group a HB_sAg as part of their structure. The finding of similar results after immunization with both glycosylated and non-glycosylated polypeptides suggested the presence of carbohydrate was not essential for this response. In a similar study, Dreesman et al (1975) found group-specific responses to only 3 of 5 polypeptides isolated from HB_sAg/ay and to 2 of 5 isolated from HB_sAg/ad. The low protein content of each inoculum used in this study (0.05 - 1.0 ug protein per inoculum) may account for the failure to elicit a response against some of the components, although some success was achieved in producing a type-specific response by immunization with either the 19,000 or 24,000 molecular weight components isolated from HB_sAg/ad.

In a preliminary experiment the use of SDS, β-mercaptoethanol and urea was compared to the use of guanidine-HCl and β-mercaptoethanol, the latter combination having been reported to offer a considerably better recovery of viral protein antigenicity (Fleissner, 1971). Although the use of guanidine-HCl resulted in a 4 fold improvement in the recovery of HB_sAg, the use of the reagent lithium diiodosalicylate in the absence of a reducing agent represented a considerable improvement. Similar in effect to SDS but more readily separated from dissociated components, lithium diiodosalicylate has proved remarkably effective in the solubilization of a major glycoprotein component bearing both MN antigens and phytoagglutinin receptors from the plasma membranes of human erythrocytes (Marchesi and Andrews, 1971). Following exposure to this compound, the morphological integrity of HB_sAg small particles was disrupted and antigenic reactivity recovered from a single low molecular weight fraction after elution through a column of Sephadex G200. This material may be similar to the small antigenic moieties isolated by Dreesman et al., (1973) and Rao and Vyas (1973) after acid treatment or sonication respectively. Intact tryptophanyl bonds appear to be necessary for the preservation of HB_sAg reactivity (Table 8). Rao and Vyas (1974) found 15 tryptophan residues in a total of 48 after analysis of a 6,000 molecular weight antigenically active HB_sAg fragment. However, in the latter study there was a drastic loss of antigenic titre during the preparation of this component (Vyas, 1974). The nature of the determinants of HB_sAg may resemble the haemolytic glycoproteins of measles virus in requiring the presence of lipids in maintaining extracted

glycoproteins in a biologically active conformation (Hall and Martin, 1975). In particular, the presence of the phospholipid phosphatidyl ethanolamine was found to considerably enhance the activity produced by reassembly of the glycoproteins. Phosphatidyl ethanolamine, detected in the lipid moiety of HB_sAg, and other lipid species may similarly play a passive role in maintaining group and subtype reactivity.

Cabral et al. (1975) have extended this approach by the examination of cell-mediated immune responses to certain polypeptides obtained by SDS treatment of HB_sAg particles. A macrophage-inhibition assay demonstrated a positive response to intact HB_sAg using peritoneal exudate cells obtained from guinea-pigs immunized with a 40,000 molecular weight component. In preliminary experiments, HB_sAg disrupted with lithium diiodosalicylate has been found to stimulate a cell-mediated response in guinea-pigs as revealed by both lymphocyte transformation and macrophage inhibition assays (Kremastinou, 1975).

Several ultrastructural and immunofluorescence studies of human liver tissue have indicated that HBV and its related products were derived from infected liver (Nowoslawski et al., 1970). More recently, Huang and Groh (1973) have demonstrated that homogenates of liver obtained at autopsy from chronic active hepatitis cases contained a large number of 42 nm particles, many of which were isolated within microsomal vesicles, together with numerous long filamentous forms. Examination of thin sections showed the filaments to be situated within the cisternae of the

smooth endoplasmic reticulum, which themselves showed extensive proliferation (Huang et al., 1974). The membranes of the endoplasmic reticulum surrounding the filaments have been found to bind labelled anti-HB_s, suggesting that the antigenic material in the filaments is manufactured or at least assembled at this site in the infected hepatocyte (Schaffner and Gerber, 1974). Fluorescent-labelled anti-HB_s has been shown to stain the liver cell cytoplasm in areas corresponding to the filament-containing foci observed with the electron microscope (Gerber et al., 1973). However, the small particles found in high numbers in the serum of the same patients have only infrequently been observed in both liver homogenates and thin sections of infected tissue (Huang and Groh, 1973; Huang et al., 1974). On the basis of these observations, it is possible that many of the circulating small particles may be derived from breakdown of filaments occurring in the serum or in hepatocytes. There is evidence that such breakdown can be induced to occur experimentally by treating filaments with pronase (Huang and Groh, 1973), Tween 80 (Trasvik et al., 1973), ether (Barker et al., 1969), or with low pH and high salt (Hirschman et al., 1973). Both morphological forms possess a similar amino acid composition (Table 6b) although the proportions of their respective polypeptide components may differ (Corin, 1972). The comparatively high isoelectric point of HB_sAg filaments suggests that the generation of the small spherical forms may be accompanied either by a reduction in amide content or by a rearrangement of HB_sAg structure to expose carboxyl groups.

A further possible relationship between these two morphological classes has been suggested by the studies of Hirschman (1974); purified HBAg in phosphate-buffered saline, incubated for 24 hours with wheat germ or yeast RNA, showed a statistically significant increase in the number of filaments. The high α-helix content (70 - 80%) reported by Sukeno et al. (1972b) suggests that HBAg may readily associate with nucleic acid in a similar fashion to the coat protein of phage fd which contains as much as 95% of its protein in an α-helix (Marvin and Wachtel, 1975). The figure for HB_sAg should be considered as an upper limit, however, as interactions other than α-helices could in theory contribute in part to the observed asymmetry; it should be noted that proline, which contributes approximately 10% of the total amino acids, does not take part in α-helix formation. Proline has been reported to be totally absent from at least one HB_sAg determinant (Rao and Vyas, 1974). Preparations rich in filaments occasionally possess a spectroscopic profile resembling that of nucleoprotein (Shinhój and Hansen, 1973). In this study, HB_sAg filaments possessed a similar local peak of absorption at 290 nm indicative of high tryptophan content as seen for the small spheres but the relative absorbances at 260 nm and 280 nm suggested the filaments additionally contained material which absorbs light at the same wavelengths as nucleic acid. It is therefore possible that the filamentous forms represent linear aggregates of material structurally similar to small particles, with their integrity maintained by a lipid matrix (Le Bouvier and McCollum, 1970) or by the presence of nucleic acid. In support of the contention that HB_sAg bearing filaments and/or

small particles contain low quantities of DNA, Jeusiak et al. (1975) have recently reported the incorporation of tritiated uridine into HB_sAg circulating in a male chimpanzee carrier. Furthermore, the radiolabel was precipitated by anti-HB_s and believed to be protected from external nuclease action by the surrounding lipoprotein coat of the HB_sAg particle. In this context, it was of considerable interest to find certain preparations of HB_sAg stimulated the incorporation of ^{3}H-TTP by the HB_sAg-associated DNA polymerase activity (Table 12). This effect is explicable in terms of HB_sAg particles containing a polynucleotide molecule or DNA which may function as a primer for this reaction.

B Hepatitis B core antigen

Since the description of the double-shelled 42 nm HBAg particle (Dane et al., 1970) and the unique antigenic specificity of its core component (Almeida et al., 1971), extensive studies have been initiated to establish if this particle contains part or all of the HBV genome. Speculation that the core may represent the HBV nucleocapsid is strengthened by the finding of similar particles in liver homogenates obtained from chronic hepatitis cases at autopsy. However, it remains to be demonstrated that particles obtained from liver homogenates, and the core of circulating 42 nm HBAg particles, are indeed identical. Both types of particles bear similar antigenic determinants, since antisera to particles purified from liver have been shown to

react with cores prepared from serum (Barker et al., 1974; Moritsugu et al., 1975). Although similar diameters have been reported for particles from both sources, (Table 1), cores prepared from plasma were isolated at a higher density (1.35 g cm^{-3}; figure 28) than has been reported for particles extracted from infected liver (1.30 g cm^{-3}; Hirschman et al., 1974a). Moritsugu et al, (1975), also examining cores prepared from plasma, found particles free of HB_sAg and globulins at a density of 1.36 g cm^{-3}, whereas particles associated with such material were found at a density of 1.28 - 1.32 g cm^{-3}. It is probable that particles from either source may vary in their nucleic acid or polymerase content (Gerin, 1974; Hirschman et al., 1974a) and the presence of an internal matrix protein between core and envelope has been suggested (Gerin, 1974). Although two peaks were observed for the recovery of enzyme activity from isopycnic gradients (figure 28), repetitive precipitation in discontinuous sucrose gradients followed by an increase in the concentration of Nonidet P40 resulted in a symmetrical band of ^3H-HB_cAg activity on rebanding in CsCl gradients. Almost 100% of this preparation was found to be reactive with anti-HB_c and there were no significant quantities of acid-precipitable material found at lighter densities. The density of ^3H-HB_cAg is indicative of a nucleoprotein structure. Electron microscopy of particles purified from liver has suggested a subunit structure for HB_cAg resembling the capsomeres of small icosahedral viruses (Barker et al., 1974).

The finding of DNA within the core particle from either infected hepatocytes or circulating double-shelled particles strongly indicates any new DNA synthesis to be DNA-directed. Direct confirmation of this, however, is difficult due to the inapparent inaccessibility of the template to both DNase and RNase (Table 13). Although synthesis was depressed in the presence of actinomycin D (figure 40), this is not conclusive evidence that the enzyme requires a DNA template. Kaplan et al. (1973) found that DNA synthesis was insensitive to rifampin, a potent inhibitor of RNA directed DNA polymerase. However, this compound has been reported to be hardly active in mammalian nucleic acid polymerase reactions (Gurgo et al., 1971; Ting et al., 1972). Perhaps more conclusive is the finding that DNA synthesis is substantially inhibited in the presence of ethidium bromide (figure 39). Furthermore, the intercalating mode of inhibition for this compound suggests the template to be double stranded.

Whether the enzyme is host or virus-coded may only be readily determined after its extraction from HB_cAg in an active state. Clearly it should be differentiated from either the small, nuclear or larger, mainly cytoplasmic mammalian polymerases. It is noteworthy that its requirements for 20 uM to 40 uM magnesium ions is two to four times as great as the cation requirement of either of the two mammalian polymerases. In this property, together with its sensitivity to ethidium bromide, the HB_cAg-associated polymerase resembles mitochondrial DNA polymerases; stimulation by high salt concentrations has also been reported

for phage T5 polymerase (Orr et al., 1965).

By analogy with known mechanisms of DNA replication, the possible requirement for a hitherto unidentified primer for initiating DNA synthesis is likely. Serial dilution of polymerase-containing concentrates resulted in a decrease in DNA synthesis greater than could be accounted for by dilution alone. The possibility, therefore, that a primer function may be associated with a particle other than that containing the enzyme warranted further investigation. Although spectrophotometric examination of purified HB_sAg suggested the absence of significant levels of nucleic acid greater than 1 or 2% of the total antigens recovered, a small but significant stimulation of polymerase activity was noted which could not be due to the addition of further enzyme molecules to the reaction. Occasional reports that at least a proportion of the small spherical and/or filamentous forms contain nucleic acid should be reconsidered in this light, bearing in mind the lack of enzyme activity in DNA-containing core particles purified from infected liver.

A model of replication that does not require an exogenous primer for initiating the synthesis of one DNA strand has been suggested by Overby et al. (1975). The open double-stranded circles visualised by electron microscopy may arise by a 'nick' in one of the two strands, thereby allowing strand elongation to take place from the exposed 3'-hydroxyl terminal nucleotide. However, it is difficult to see how this mechanism could operate within the confines of the core. In addition, exogenous primers

would be required for the synthesis of complementary linear strands on the growing 'tail' (Dressler, 1975). Given that a DNA base pair is 2 nm in diameter (Bradley, 1971), a 27 nm core could only possess at any one time a maximum of 3 to 4 copies of the DS DNA template. If the molecule was replicated in this fashion, only a very restricted number of copies could therefore be manufactured within the core. Since the product remains firmly associated within the core and the size of the product of the reaction equals the size of the template, the heterogeneous linear DNA free in some plasmas, which contains significant homology with the DNA product of the reaction, must be manufactured on intracellular templates and not on those in circulating double-shelled particles (Overby et al., 1975). This free DNA could additionally be explained as the product of a defective mode of virus replication. Robinson et al. (1974) have indicated that the heterogeneity of observed lengths for intraparticulate DNA is similar to that seen in SV40 infection at high multiplicities when a large number of defective virions are produced (Tai et al., 1972). However, the possibility that the free DNA in plasma and that contained within double-shelled particles represent excised fragments of host material, remains to be excluded.

Summers et al. (1975) have confirmed that the DNA template was circular. However, fragmentation with the restriction enzyme endonuclease R. Hae III both before and after <u>in vitro</u> DNA replication suggested the existence of single-stranded gaps along 10 - 20% of the total template length. These single-

stranded regions may have contributed to the wide range of molecular lengths observed in the electron microscope by Robinson et al. as the length of single-stranded DNA molecules is strongly dependent on ionic conditions. In this study, the endogeneous polymerase reaction appeared to repair the single-stranded gap in the double-stranded circular DNA. Moreover, the ability of a polymerase obtained from avian myeloblastosis virus to synthesize a DNA product indicates the specificity observed in the endogeneous reaction appears to reside with the template DNA and not in the HB_cAg-associated DNA polymerase.

After the addition of ethylmaleimide to the extraction buffer to inhibit cell nucleases, Hirschman (1975) reported the recovery of linear DNA of 2.3×10^6 m wt from intranuclear core particles. This DNA is some 40% larger than the circular molecules isolated by Robinson et al., from circulating 42 nm particles. In addition, this larger DNA molecule contained a large tract rich in the bases guanosine and cytosine, the loss of which would result in a similar size and G-C content to that reported in the studies by Robinson and colleagues. As intranuclear particles have been found to be lacking in DNA polymerase activity, Hirschman has suggested this activity may be acquired upon passage of core particles through the hepatocyte cytoplasm, together with the loss of part of its DNA content and the acquisition of an outer coat of HB_sAg. However, this is difficult to envisage, given the sequestered nature of the enzyme activity and the apparent morphological integrity of the particles prior to their entry into the cytoplasm. Circularisation

of the template may be necessary for its packing into the confines of the core particle, as well as providing a favourable configuration for integration into the host genome as suggested by Hirschman.

C The nature of hepatitis B virus

Since the original observations of Almeida et al. (1971) several laboratories have confirmed the existence of at least two distinct antigen-antibody systems associated with type B viral hepatitis. Whereas HB_sAg can be detected free in the serum of patients acutely or chronically infected with the virus, HB_cAg is generally sequestered within a surrounding coat of HB_sAg. The resulting double-shelled particle described by Dane et al. (1970) contains HB_cAg as an inner electron-dense component that morphologically resembles the 27 nm diameter nucleocapsid-like particle found in the nuclei of infected hepatocytes. This material stains positively for HB_cAg in immunofluorescent examination of liver biopsies and is distinct from the HB_sAg reactivity found in the cytoplasm (Brzosko et al., 1973).

Barker et al (1973b) successfully transmitted type B hepatitis to chimpanzees. A human convalescent serum containing anti-HB_c but not anti-HB_s specifically stained the nuclei of infected hepatocytes whereas a hyperimmune anti-HB_s exclusively stained the cytoplasm of these cells. The isolation of HB_cAg from the liver of an infected, immunosuppressed chimpanzee (Markenson et al., 1975)

enabled Hoofnagle et al. (1973) to characterize the pattern of antibody reactions in 15 patients during the course of type B hepatitis. Anti-HB_c reactivity was detected during HB_sAg antigenaemia by a complement fixation procedure within 3 weeks from the onset of jaundice: the presence of these antibodies did not signal the beginning of recovery from clinical infection. The response to HB_sAg appeared much later during convalescence and it was apparently of a lasting nature. In the present study, a similar rise in anti-HB_c was detected by a radioimmunoassay procedure during the period of HB_sAg production, suggesting that antibodies are produced in response to active virus replication. Anti-HB_c was detected in the presence of both HB_sAg/ad and HB_sAg/ay, indicating at least one common HB_sAg determinant in HBV infection of both subtypes. The increase in anti-HB_c reactivity was generally accompanied by a reduction in the titre of circulating HB_sAg. A brief anti-HB_s response was occasionally detected by radioimmunoassay but the very low titre found precluded confirmation of the specificity of this reaction. HB_cAg production, as detected by DNA polymerase activity was highest during the first two weeks of observation. A sample was considered to be DNA polymerase-positive if the assay for enzyme activity resulted in approximately 200 cpm of incorporated ^{3}H-TTP and represented a clear rise above the serum background. A negative correlation between enzyme activity and HB_sAg was apparent, confirming that there is no correlation between HB_sAg titre and HB_cAg-associated polymerase activity (Kaplan et al., 1973; Krugman et al., 1974). In a study of 3 cases of post-transfusion hepatitis, Kaplan et al. (1974) similarly found maximum DNA polymerase activity 3 weeks

after the first appearance of HB_sAg, but the level rapidly declined before the onset of liver dysfunction. The use of the DNA polymerase activity as an indicator of HB_cAg may therefore present a clearer indication of the presence of the virus than the detection of HB_sAg alone. In this context, Krugman et al. (1974b) found that DNA polymerase activity was not present in two persons who were protected against hepatitis by immunization with heat-inactivated MS-2 serum and in one who was protected by hepatitis B immune serum globulin.

Although the titre of anti-HB_c eventually declines to a low level during convalescence, the development of the HB_sAg chronic carrier state is closely associated with the continuing presence of anti-HB_c at high titre. Hoofnagle et al. (1974) found all of 100 chronic carrier sera to contain anti-HB_c at a titre of 1:64 or higher by complement fixation regardless of the HB_sAg subtype present, whereas only 1% of volunteer blood donors had evidence of exposure to HB_cAg. The incidence of anti-HB_s was somewhat higher in the same group (4%). Tsuda et al. (1975) using an immune adherence method found a slightly higher prevalence rate of anti-HB_c reactivity than for anti-HB_s (17% and 14% respectively). These estimates will almost certainly be revised as the use of radioimmune techniques is extended.

Purified immune globulin containing anti-HB_s has also been tested for the presence of anti-HB_c. In one study, all 5 preparations examined were found to be negative by radioimmunoassay

(Purcell et al., 1974) whereas Greenman et al. (1975) found anti-HB_c to be present in two batches examined. Clearly more preparations will have to be examined before this discrepancy is resolved.

The radioimmunoassay described in the present study offers a highly sensitive procedure for the detection of anti-HB_c. A chimpanzee serum used at a dilution of 1:500,000 was found to precipitate 50% of added ^{3}H-HB_cAg in comparison to a titre of 1:256 - 1:1024 by complement fixation in a previous study (Barker et al., 1974). This degree of sensitivity is similar to that obtained by Moritsugu et al. (1975) also using purified HB_cAg from human plasma. The use of Staphylococcal protein A for the removal of immune complexes (Figenschau and Ulstrup, 1974) offers the added advantage that a second, precipitating antibody is not required. A method involving simultaneous activation of polymerase activity and incubation with antibody has been described by Greenman et al. (1975), but appears to be somewhat less sensitive. Despite the need for human plasma containing a high proportion of HB_cAg, a substantial number of HB_sAg chronic carriers possessed significant levels of specific polymerase activity. From an initial volume of 200 ml, it was possible to prepare sufficient ^{3}H-HB_cAg for the assay of anti-HB_c in 300 - 350 samples. The use of the detergent Nonidet P40 at increased concentrations was adequate for the removal of HB_sAg reactivity: this treatment has been found to be superior to Tween 80 for release of the core component from the 42 nm particles (Purcell et al., 1974).

Cell-mediated immunity is believed to play a key role in recovery from viral infections. The ability of both HB_sAg and HB_cAg to stimulate distinct cell-mediated responses in immunized guinea-pigs was reported by Gerety et al. (1974). Delayed cutaneous hypersensitivity appeared to provide a more sensitive indicator of exposure to HB_cAg, while anti-HB_s was apparently a more sensitive means of assessing exposure to HB_sAg. The authors suggest that this reflects a difference in biochemical composition between these antigens and/or their relative effectiveness in inducing responses in T- and B-cell lymphocyte populations. Although no comparable data are yet available for the nature of similar responses to HB_sAg in man, patients recovering from HBV infection have been shown to possess cell-mediated immunity to HB_sAg (Yeung Laiwah et al., 1973; Reed et al., 1974; Ibrahim et al., 1975). This response appears during the period HB_sAg antigenaemia and may persist for several months either in the presence or absence of anti-HB_s. However, in the development of chronic liver disease, cell-mediated immunity persists (Dudley et al., 1972). In addition, anti-HB_c has been detected regardless of whether or not the infection was clinically evident (Purcell et al., 1974). Although high titres of anti-HB_c have been found in the absence of anti-HB_s during the development of the chronic carrier state (Macfnagle et al., 1975) little is known about the production of antibodies in the course of chronic aggressive hepatitis. It has been proposed that the development of chronic liver damage is associated with either a failure to produce sufficient anti-HB_s or the production of low affinity antibody (Eddleston and Williams, 1974). Cellular responses to a human,

liver-specific lipoprotein present as a normal constituent of liver cell membranes have also been identified in 92% of untreated cases (Miller et al., 1972). Treatment with prednisone has been shown to control the perpetuation of the autoimmune reaction in these cases (Williams, 1975). Long-term administration of specific immunoglobulin containing anti-HB_e may offer an alternative means of treating HB_eAg-positive chronic aggressive hepatitis although the initial results were not encouraging (Reed et al., 1973b).

The recent recognition of a third, apparently unrelated, antigen-antibody system may prove to be of considerable value in the prognosis of progressive liver damage resulting from HBV infection. HB_eAg has been commonly found in haemodialysis patients in whom liver cell damage is probably less severe than in hepatitis patients (Magnius and Espmark, 1972). Furthermore, this antigen has been found to be closely associated with the presence of HB_eAg-associated DNA polymerase activity among HB_eAg-positive dialysis patients who are recognised as a 'high' risk for transmitting type B hepatitis (Nordenfelt and Kjellen, 1975). In a separate study, Sheikh et al. (1975) found HB_eAg with greater frequency in chronic aggressive hepatitis than in chronic persistent hepatitis, suggesting that the presence of this antigen is linked with continuing liver damage. The demonstration of a high concentration of 42 nm particles in sera positive for HB_eAg but not for anti-HB_e (Nielsen et al., 1974; Nordenfelt and Kjellen, 1975) indicates the antigen may additionally be contained within this particle (eg HB_cAg, DNA polymerase, matrix protein)

as well as being released in a soluble form from damaged cells. Indeed, Neurath et al. (1975c) have found a determinant closely related to HB_sAg on the surface of both the 42 nm particle and the filamentous form of HB_sAg present in the sera of patients with chronic or acute hepatitis. Similar findings were not obtained using sera from asymptomatic chronic carriers of HB_sAg. In five patients infected with MS-2 serum in the Willowbrook study, HB_eAg appeared simultaneously with HB_sAg and preceded detectable liver damage (Magnius et al., 1975). Although HB_eAg therefore appears to be closely associated with infectivity, the appearance of this antigen in soluble form suggests such an association may reflect a host response to HBV infection rather than be a specific viral gene product.

As has already been stated, it is unlikely that the low molecular weight of the DNA recovered by several workers from HB_cAg would contain sufficient information to code for a specific DNA polymerase activity in addition to the antigenically complex HB_sAg. This raises the interesting possibility of the HB_cAg-associated enzyme being acquired from the infected host cell. The absence of activity in HB_cAg isolated from the nuclei of infected cells further indicates that the enzyme may be added to the maturing HB_cAg particle in the host-cell cytoplasm: a host enzyme possessing a DNA polymerase activity in addition to a requirement for a circular DNA molecule is present within the mitochondria. Both the mitochondrial enzyme and that associated with HB_cAg are stimulated in the presence of salt, are functional in the presence of sulphydryl reagents, and are thought

to have an endonuclease activity. A common sensitivity to the mutagenic dye ethidium bromide seems to distinguish both of these activities from the larger cytoplasmic DNA polymerases. The finding of single-strand gaps in the DNA of HB_cAg (Summers et al., 1975) additionally suggests a similar template for both activities as mitochondrial DNA also contains single-strand gaps which arise as a result of asynchrony in the replication of the two strands of the circular DNA molecule (Kasamatsu and Vinograd, 1974). Summers et al. also found the specificity of the HB_cAg-associated polymerase reaction to reside entirely in the DNA template, indicating that this enzyme need not necessarily be virus-coded.

This hypothesis may be taken further and HB_eAg postulated as representing this host enzyme. Dilution experiments (figure 37) revealed a possible multicomponent effect to be operating, which may be explained by the presence of a bound polymerase within the circulating DNA particle in close association with its template, and a similar activity free in the serum that has access to the same template. HB_eAg has been shown to exist both free in the serum and bound to particulate hepatitis B antigens. The proposed size of HB_eAg is also consistent with this hypothesis. The appearance of antibodies to this antigen may then be explained as an autoimmune response to an antigen normally sequestered within the liver-cell mitochondria but released in large quantities during type B hepatitis as a result of extensive mitochondrial degradation. Indeed, virus-like particles have been detected within the mitochondria of lymphoid cells exposed to serum

believed to contain the virus (Jenson et al., 1970). The apparent lack of antibodies to this cellular organelle in HB_sAg-positive cases of acute (Farrow et al., 1970) and chronic hepatitis (Dudley et al., 1973) is also consistent with this view.

The role of the small spheres and filamentous forms of HB_sAg remains unsolved. Although small quantities of nucleic acid have been detected in HB_sAg preparations, it is unclear whether this represents viral or host material or indeed plays any part in initiating HBV infection. If these particles contain excess viral coat material, the large amount usually synthesized in infection may be due to overproduction of some viral gene products and rate-limiting production of others, due to differing rates of translation or transcription of different parts of the genome, or selective availability of genetic material. However, intracellular accumulation of 27 nm spheres resembling circulating cores can often be seen in infected cell nuclei, while small particles are rarely seen. Accordingly, transport from nucleus to cytoplasm, or maturation of core structures, may be steps which limit the production of double-shelled particles. Major defects in genome expression at one of these levels may account for the limited success in attempts to isolate HBV in tissue culture.

Although there are many encouraging reports to indicate that the 42 nm double-shelled HBAg particle may be closely related, if not identical to the putative hepatitis B virus, this suggestion must remain hypothetical until the infectivity of these particles

has been conclusively demonstrated. Should the viral nature of the DNA be clearly shown, the finding of complementary DNA of heterogeneous lengths free in plasma, and of DNA molecules larger than 1.4×10^6 in intranuclear core particles, strongly suggests that a complete genome larger than this size may be involved, and that the majority of double-shelled particles may contain only a proportion of the complete genome. Additional genetic material could be provided by non-identical nucleotide sequences of similar lengths, carried in different 42 nm particles or by occasional particles as yet unrecognised with a significantly larger content of nucleic acid; alternatively, host DNA, or genetic material from other viruses commonly infecting the host, may play an essential role in a manner perhaps similar to the Fraenkel-Conrat 'covirus' model for some plant viruses which require two or more particles. This concept, which was extrapolated by Zuckerman (1970) to human type B hepatitis, cannot yet be ruled out. Finally, free viral DNA in plasma released from infected cells, may provide necessary information for complete replication, thus helping to account for the preferred parenteral route of transmission in this disease.

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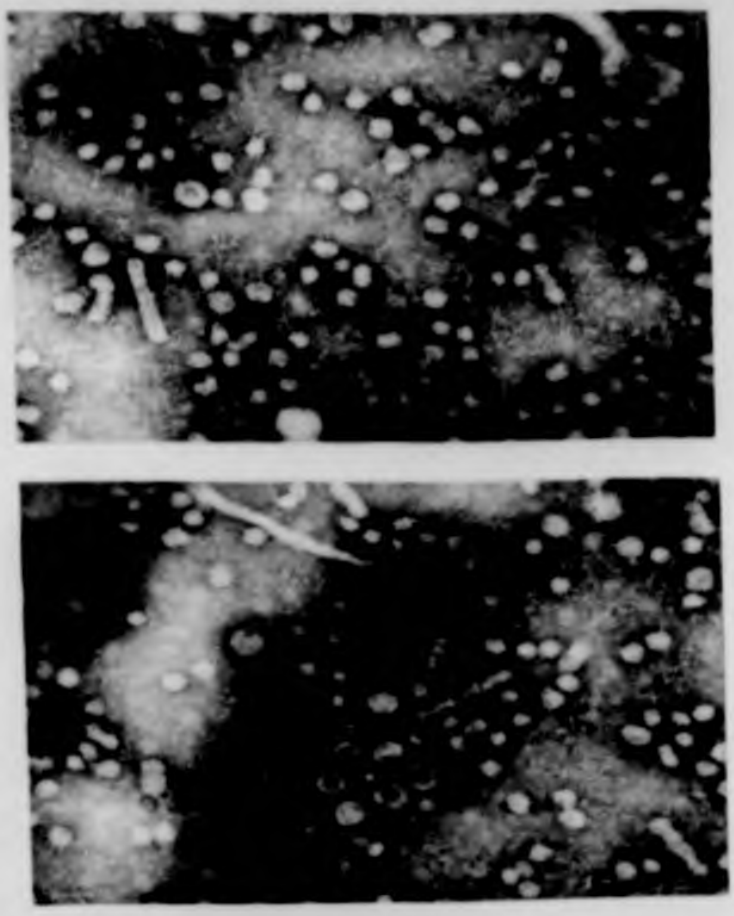


FIGURE 1

Electron microscopy of HB_sAg-containing plasma. By far the most prominent form was a small pleomorphic sphere (upper micrograph). Occasional aggregates consisting of double-shelled particles and filamentous forms of varying lengths were also seen (lower micrograph). Transverse striation occurred with a periodicity of 4 nm along the lengths of the filaments.

Magnification: x 126,000

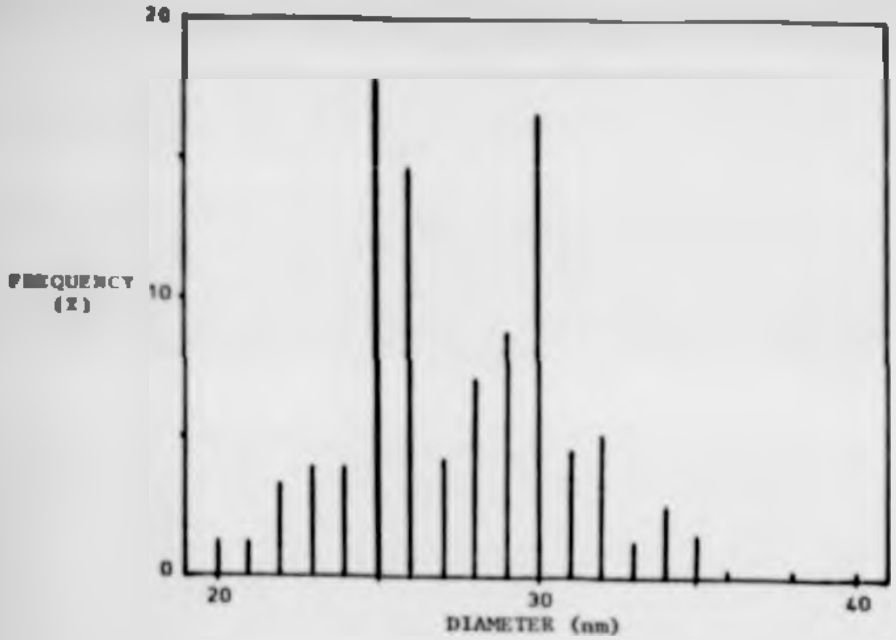


FIGURE 2

Size distribution of spherical particles in NH_4Ag-containing plasma. A total of 305 particles were measured by micrometer from an electron micrograph at a final magnification of 100,000.

FIGURE 3

Isoelectric focusing of proteins. The apparatus of Vesterberg and Svensson (1966) is designed to contain a 110 ml preformed sucrose gradient. The central location of the anode allows the escape of gas without the disturbance of the gradient. Both electrodes are protected by lock solutions.

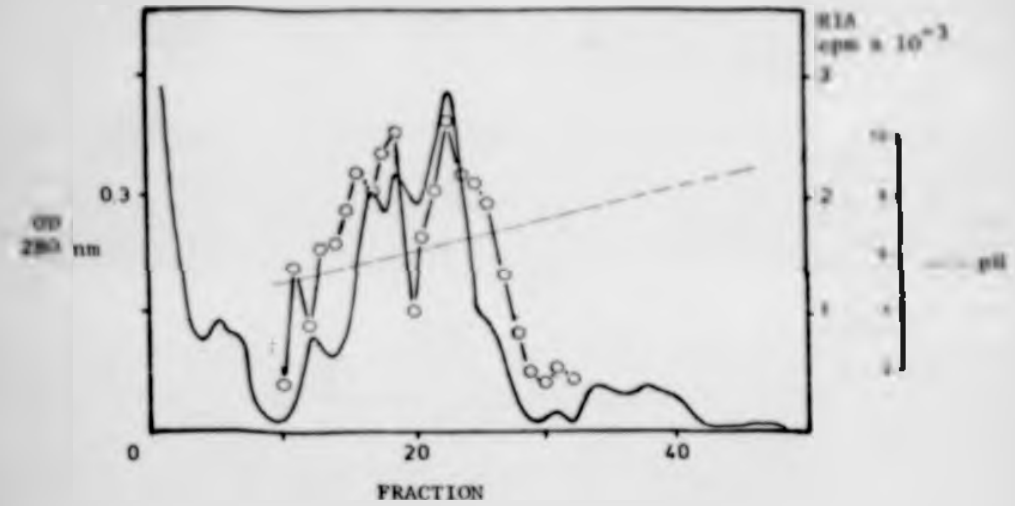
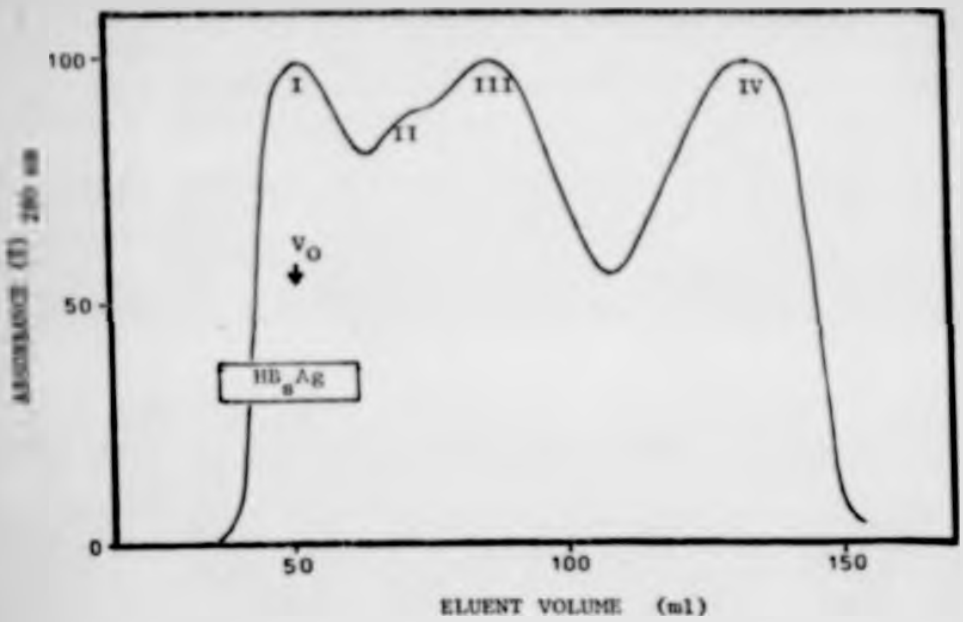


FIGURE 4

Isoelectric focusing of HB Ag-containing plasma. One hundred microlitres of HB_sAg/ad-positive plasma were focused in a preformed 0 - 40% w/v sucrose gradient containing carrier ampholytes to establish a pH 3 - 10 gradient. After 72 hours of electrophoretic separation with a maximum power output of 3 watts, separated proteins were fractionated by downward displacement. Only radioimmunoassay results positive for HB_sAg are shown.



F I G U R E 5

Gel chromatography of HB_sAg-containing plasma. A 80 x 2.5 cm diameter column of Sephadex G200 previously equilibrated with PBS was loaded with 25 ml of clarified HB_sAg-positive plasma. Antigenic activity was found to be eluted close to the void volume (v_o). IgG and albumin were identified by electrophoresis in peaks III and IV respectively. Fibrinogen was recovered from peak II.

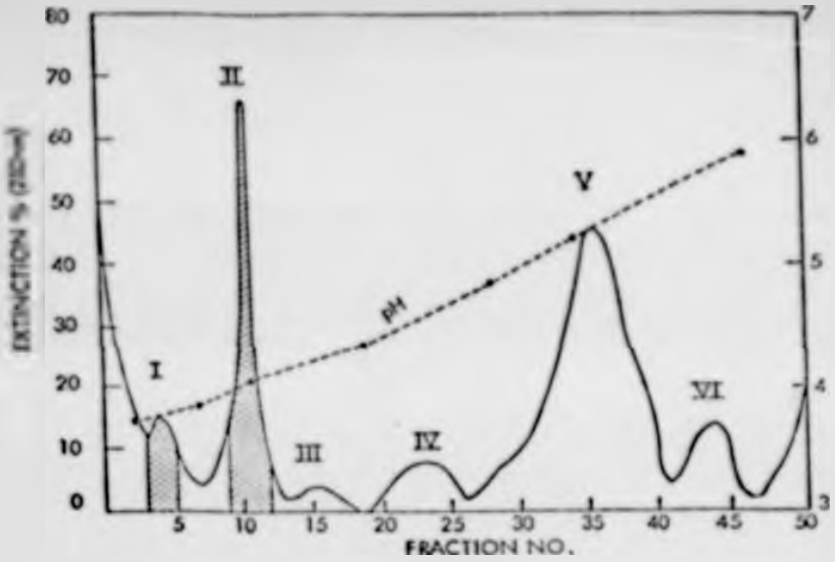


FIGURE 6

Isoelectric focusing of HB_sAg.
Extinction profile obtained 48 hours after applying partially purified HB_sAg prepared by gel filtration to a preformed 0 to 40% (w/v) sucrose gradient containing carrier ampholytes.
The shaded areas under peaks I and II were found to contain antigen.

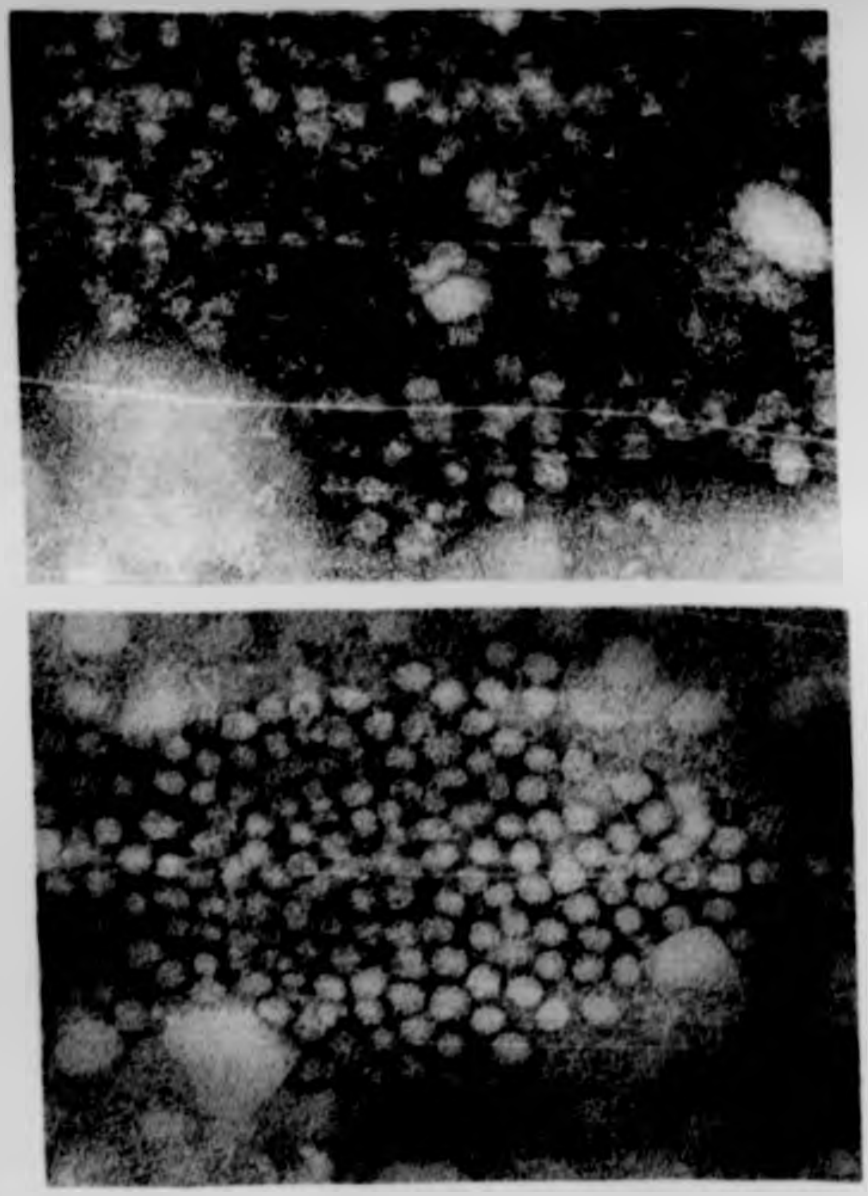


FIGURE 7

Electron microscopy of HB$_s$Ag separated by isoelectric focusing (see figure 6).
Top: Antigen recovered from peak I
Bottom: Antigen recovered from peak II.

Magnification: x 252,000

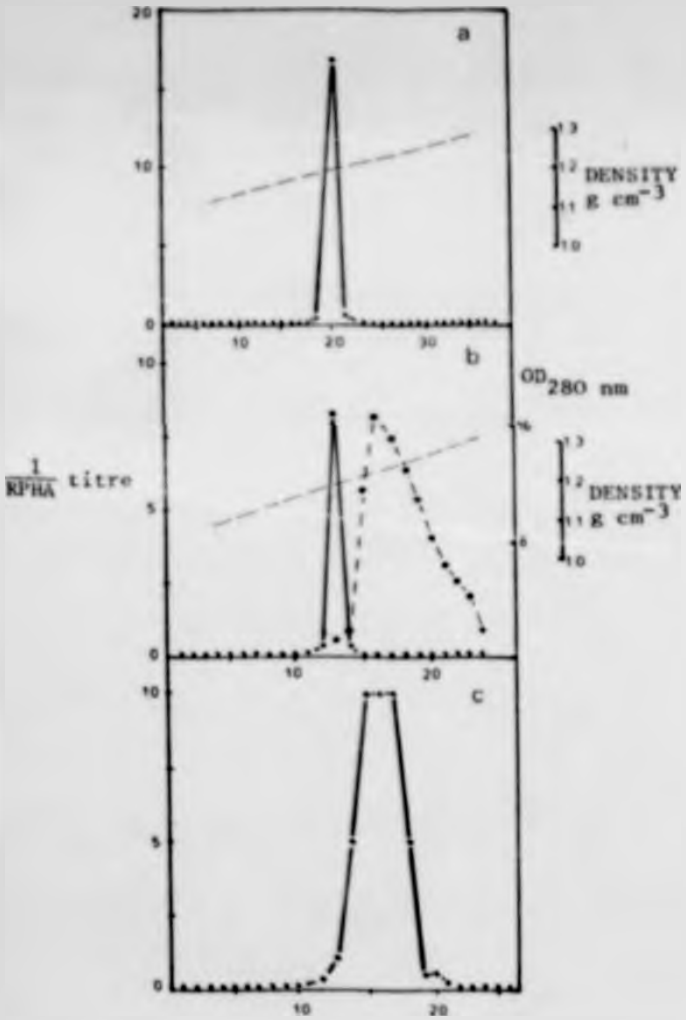
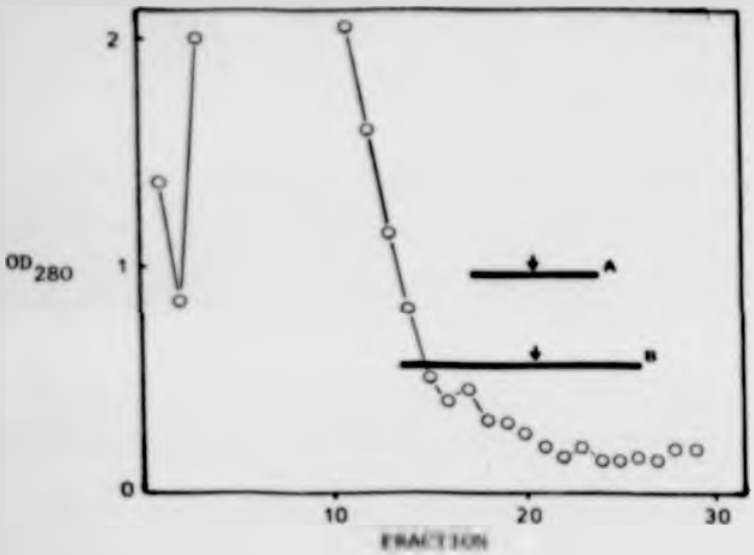


FIGURE 8

Ultracentrifugation of HB_sAg-containing plasma.
Two successive bandings of HB_sAg in isopycnic gradients of CsCl (a and b) resulted in the recovery of antigenic activity at a density of 1.195 g cm^{-3}. On rebanding, HB_sAg was floated away from the main peak of optical density (b). Subsequently, rate-zonal centrifugation in Urografin gradients showed HB_sAg to sediment as one broad peak (c). Centrifugation conditions were as described in the text.
HB_sAg activity, x 10^{-3} (a and b) or x 10^{-2} (c): closed circles.
Optical density, 280 nm, 1 cm: open circles
Density, g cm^{-3}: dashed line.



F I G U R E 9

Rate-zonal centrifugation of HB_sAg in sucrose gradients. Centrifugation of HB_sAg on a 10 to 30% (w/v) preformed sucrose gradient in 0.01M tris buffer pH 7.4 containing 0.1M NaCl and 0.001M EDTA was for 16 hours at 80,000g. The positions of fractions containing antigenic activity > 1:64 by reverse passive haemagglutination are shown by the horizontal bars (A) for purified HB_sAg and (B) for HB_sAg separated from plasma proteins. The optical density values of the fractions after the separation of HB_sAg-containing plasma are additionally shown. The peaks of antigenic activity corresponded to a calculated sedimentation coefficient of 22S for HB_sAg in both instances.

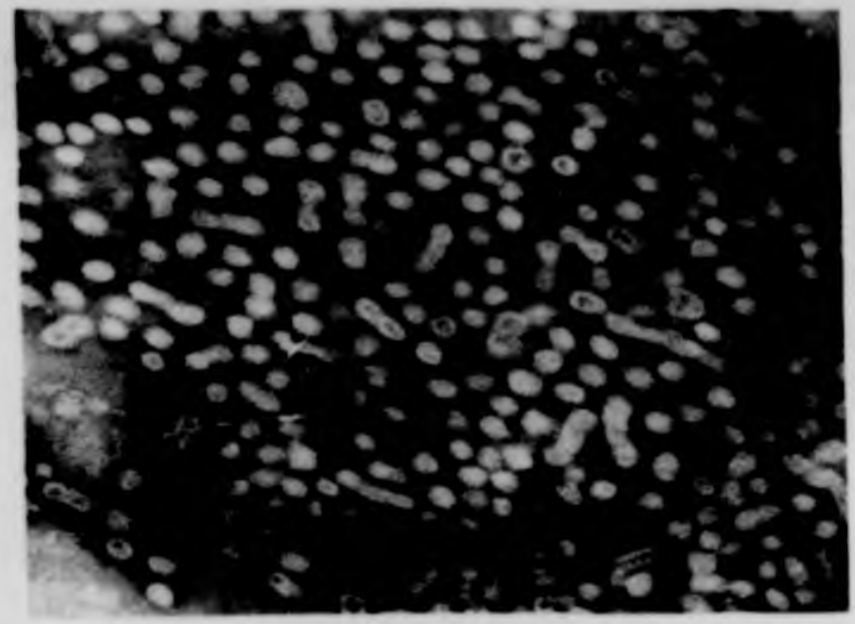


FIGURE 10

Electron microscopy of HB_sAg separated in Urografin gradients. Occasional ring-like structures were visible possessing an inner diameter of approximately 10 nm.

Magnification: x 189,000

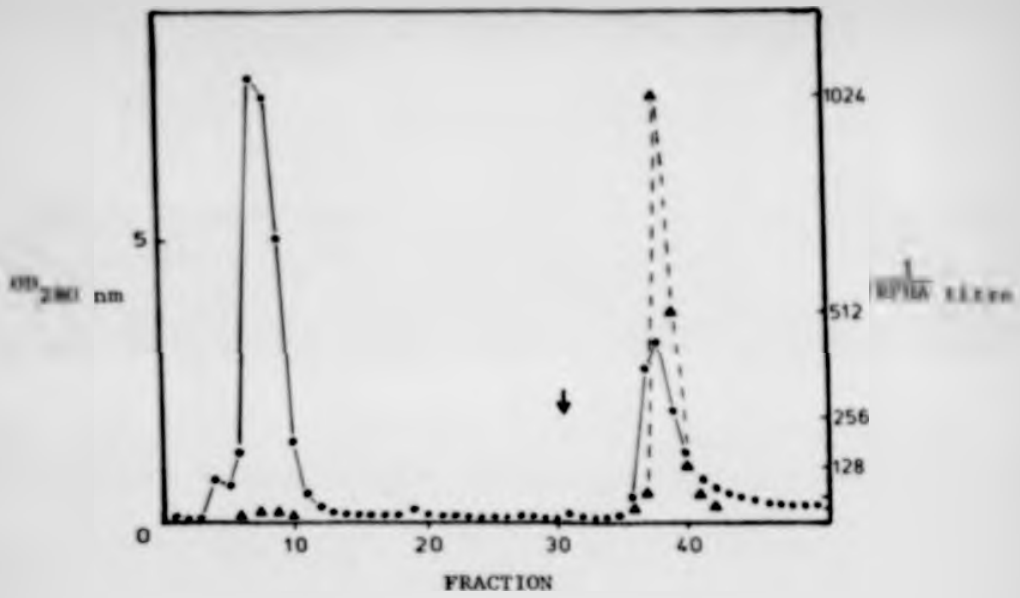
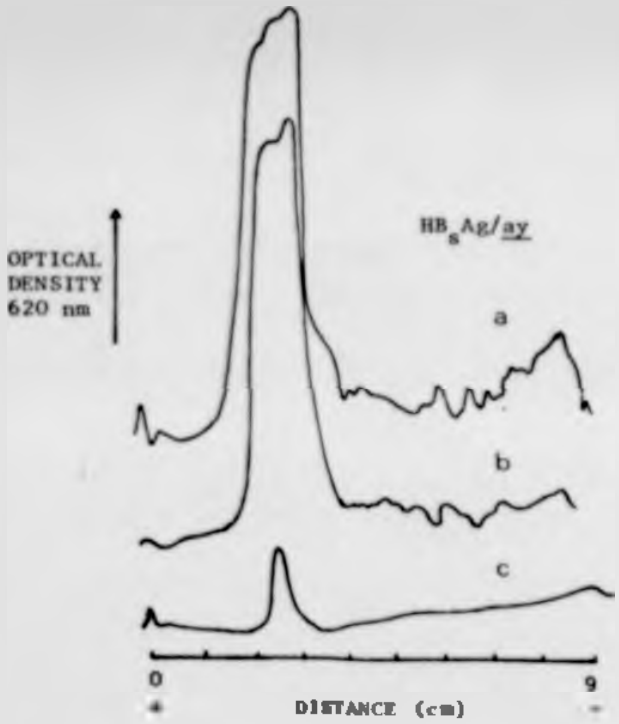


FIGURE 11

Affinity chromatography of HB_sAg on concanavalin A-Sepharose. One ml of recalcified HB_sAg-positive plasma was applied to a 17 x 0.9 cm column of immobilized concanavalin A and washed with tris buffer pH 7.5 containing Na^+, Ca^{2+} and Mn^{2+}. Adsorbed HB_sAg was eluted by the addition of tris buffer containing Na^+ and α-methyl-D-mannoside (arrow). Only those fractions positive by reverse passive haemagglutination are indicated (triangles). Antigenic activity was almost exclusively eluted with the second peak of optical density (closed circles).



F I G U R E 1 2

Flat-bed isoelectric focusing of HBsAg-containing preparations

a) after banding of HBsAg-positive plasma in an isopycnic gradient of CsCl

b) after rebanding in an isopycnic gradient

c) after isopycnic centrifugation and rate-zonal sedimentation through a preformed linear gradient of Urografin.

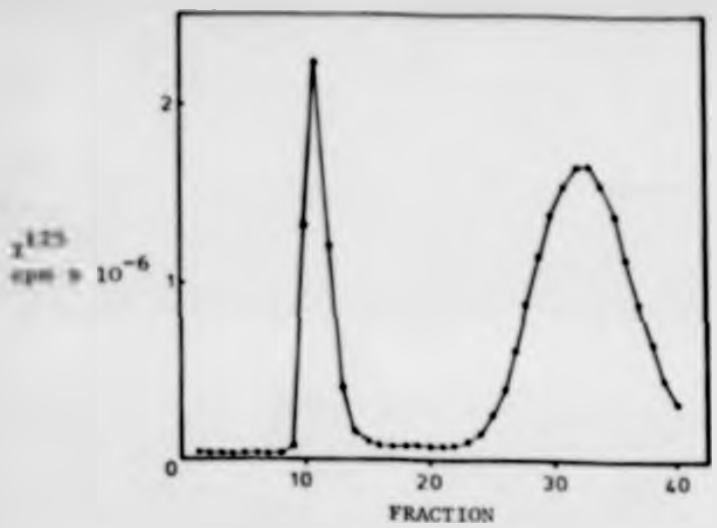


FIGURE 11

Gel chromatography of HB_sAg iodinated by the chloramine-T procedure. After brief dialysis, the iodination reaction mixture was chromatographed on a 22 x 1.5 cm column of Sephadex G200 equilibrated in PBS. Fraction no. 11 constituted the void volume of the column and fractions nos. 11 - 13 found to contain detectable antigenic activity by reverse passive haemagglutination.

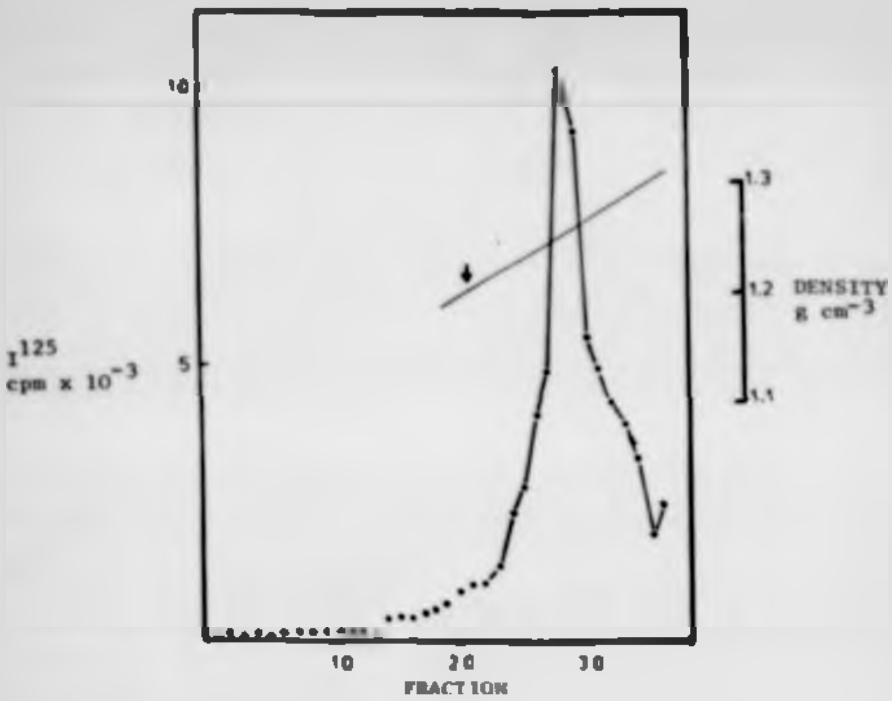


FIGURE 13a

Isopycnic centrifugation of HB_sAg tracelabelled by the chloramine-T method. I^{125}-HB_sAg was recovered at a density of 1.25 g cm^{-3} after centrifugation at 150,000 g for 3 days at 20°C. The arrow represents the position of unlabelled HB_sAg centrifuged under the same conditions.

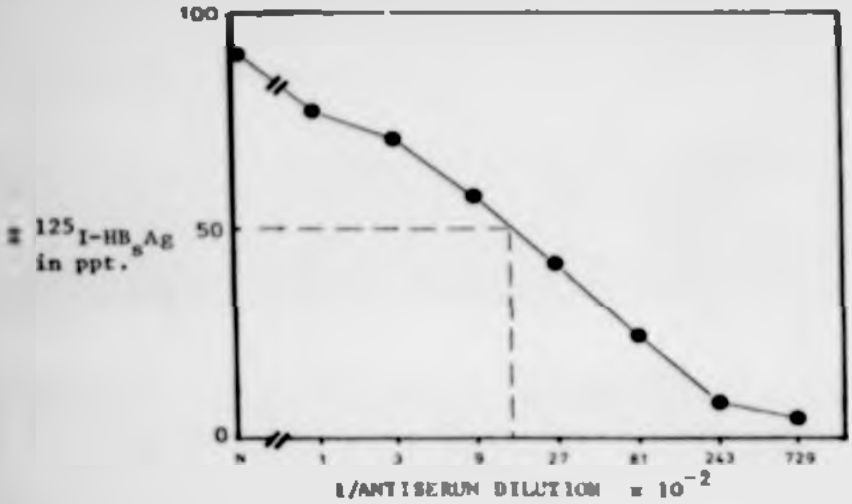


FIGURE 14

Titration of a rabbit hyperimmune serum to HB$_s$Ag. Approximately 10,000 cpm of ^{125}I-HB$_s$Ag eluted in the void volume of a Sephadex G200 column was added to an equal volume of anti-serum diluted in PBS containing 1% normal rabbit serum. After 16 hours of incubation at 4°C, 100 µl of donkey anti-rabbit serum was added and the incubation continued for 2 hours at 45°C. Immune complexes were precipitated by centrifugation at 4,200 rpm for 5 minutes and unbound ^{125}I-HB$_s$Ag assayed by gamma spectroscopy of the supernatant. Fifty percent of added radiolabel was precipitated at a serum dilution of 1:1500 as compared to a titre of 1:16 by counterimmunoelectrophoresis.

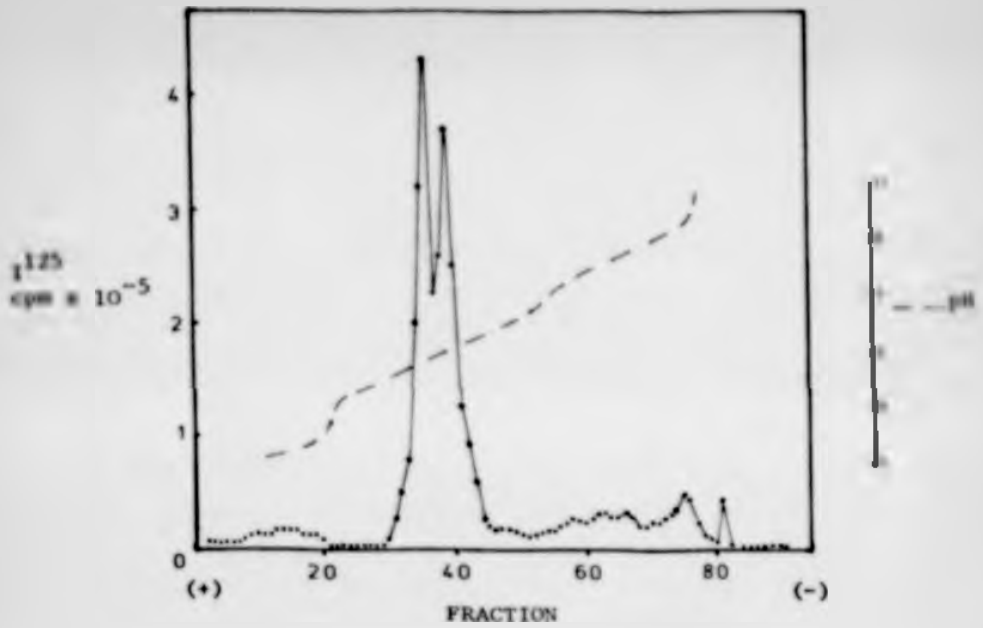


FIGURE 15

Isoelectric focusing of HB_sAg after iodination by the chloramine-T method (1). A clear heterogeneity of antigen is demonstrated at the isoelectric points of 4.7 and 4.9 in a pH 3 - 10 gradient. Focusing was for 48 hours at a maximum power output of 3 watts in a 0 - 40% (w/v) preformed sucrose gradient.

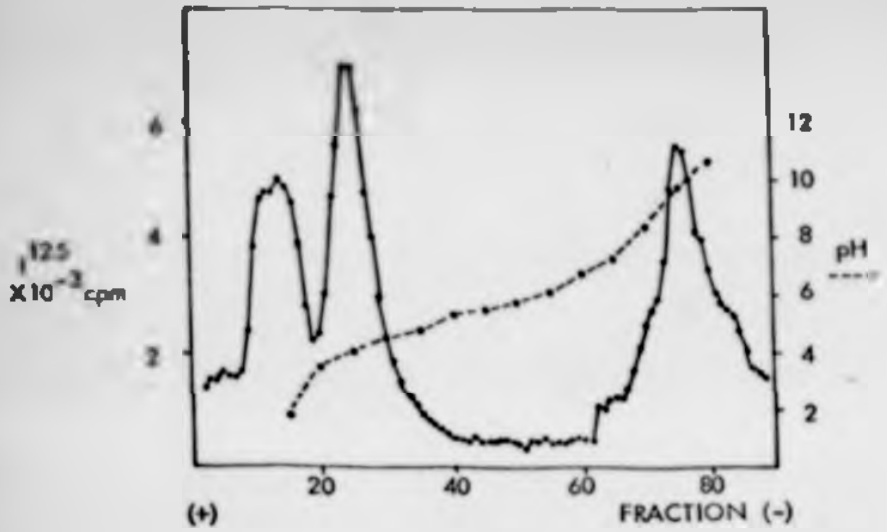


FIGURE 16

Isoelectric focusing of HB_Ag after iodination by the chloramine-T method (2). Some preparations of radiolabelled antigen were resolved into two peaks of activity, one of which was recovered from the alkaline region of the pH gradient. This material possessed an isoelectric point (9.5 - 10.0) similar to an unlabelled preparation consisting almost entirely of filaments. The first peak shown close to the anode represents free iodine.

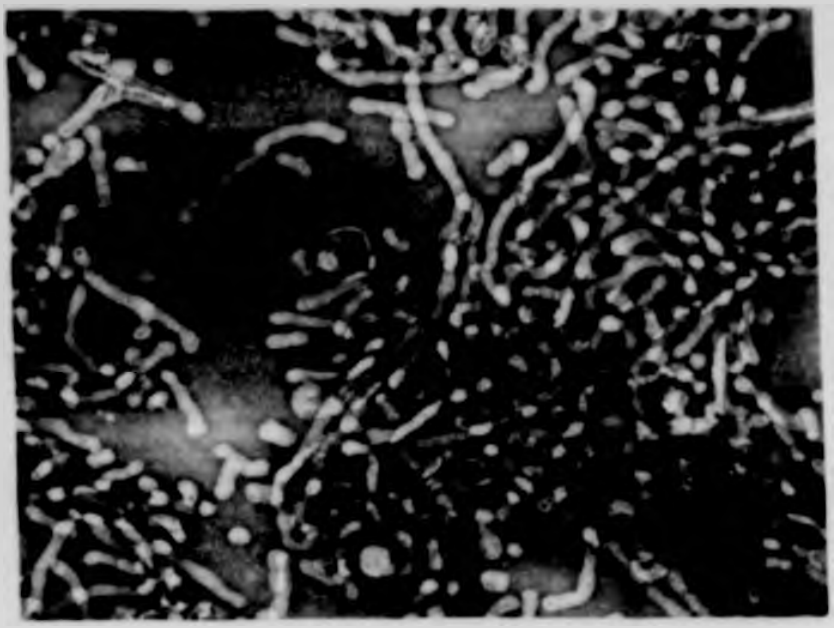


FIGURE 17

Electron microscopy of HB_sAg possessing an alkaline isoelectric point. Filamentous forms of the antigen were separated by rate zonal centrifugation and subsequently found to possess an isoelectric point of pH 9.6 - 10.

Magnification: x 126,000

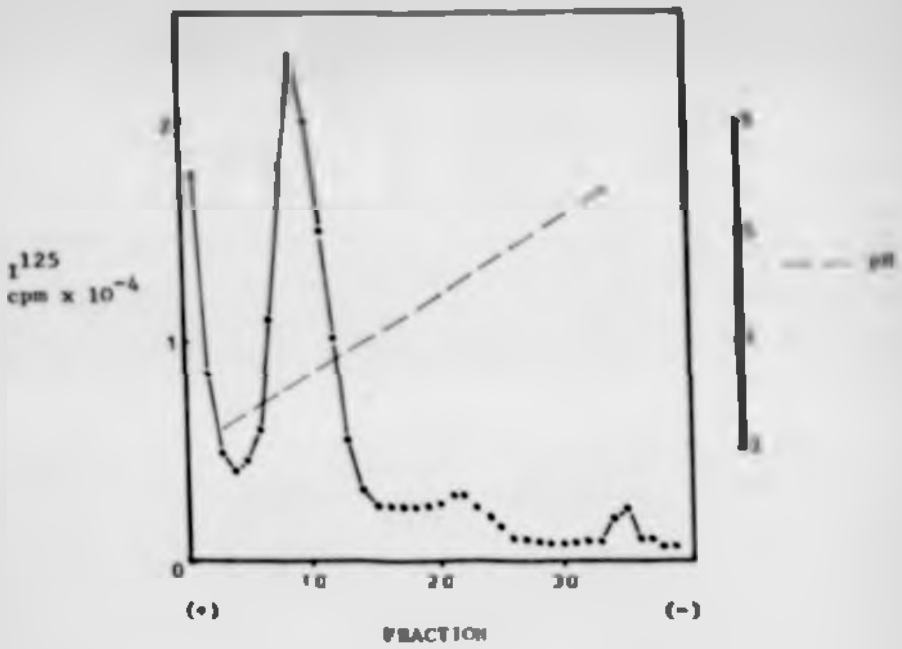


FIGURE 18

Isoelectric focusing of HB_sAg conjugated to an iodinated hydroxysuccinimide-propionic acid ester. All of the acid-precipitable material was recovered from a single symmetrical peak with an isoelectric point of 3.65.

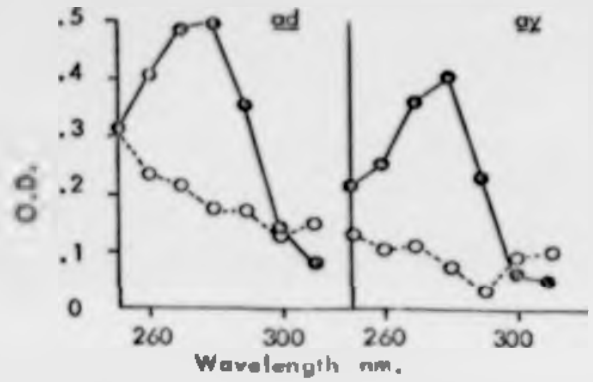


FIGURE 19

Spectrophotometric analysis of HB_sAg before and after titration with N-bromosuccinimide. The reduction in measured optical density at 280 nm permitted estimation of total tryptophan content. All measurements were made in 1 cm-path cuvettes against a similarly-treated reference solution.

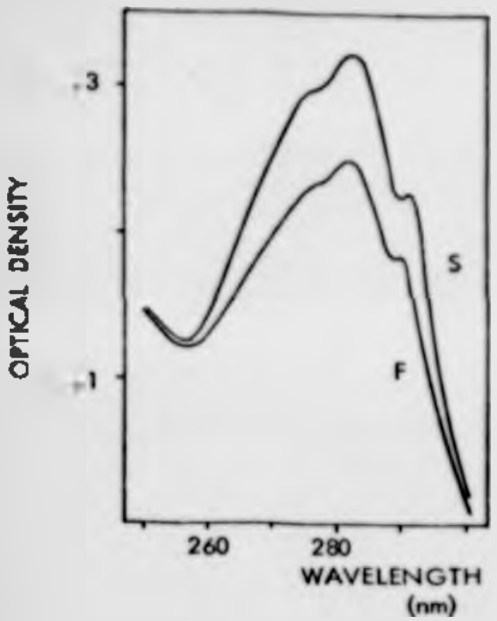


FIGURE 20

Spectrophotometric analysis of HB_sAg. Preparation containing either the small spherical (S) or filamentous (F) form of the antigen were resuspended in PBS and the optical density measured continuously from 250 nm to 300 nm in a dual-beam scanning spectrophotometer with PBS in the reference beam. Both spectra are consistent with the high content of the amino acid tryptophan (E_{max} = 290 nm) estimated by other means. The filament-rich preparation contained a higher proportion of material absorbing at 260 nm (260:280 = 0.5) when compared to the small spherical form of HB_sAg (260:280 = 0.4).

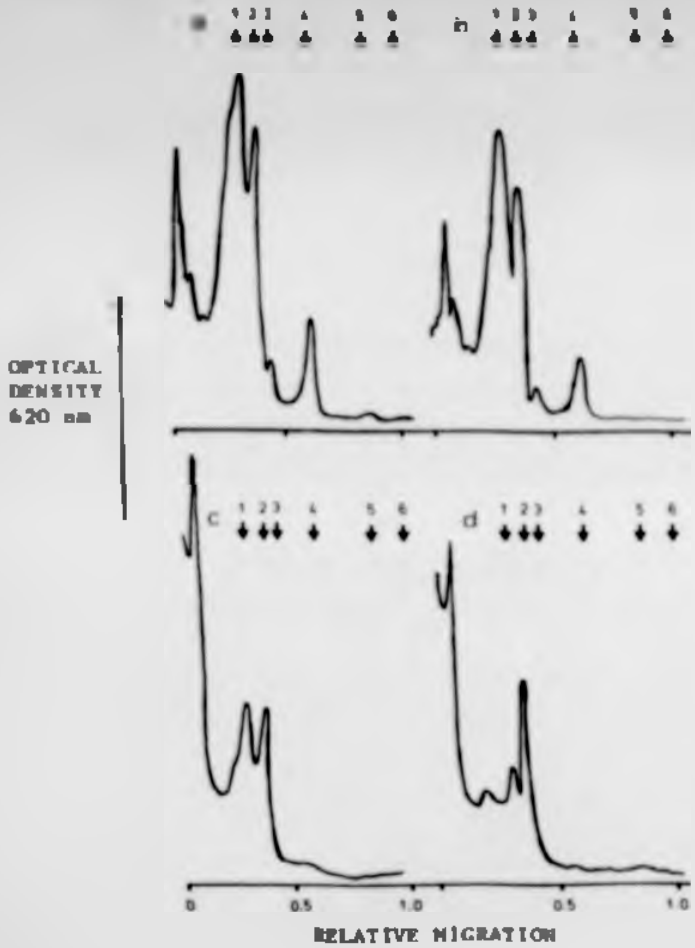


FIGURE 21

SDS-polyacrylamide gel electrophoresis of HB_sAg. Disrupted antigen was applied at the cathode in tris-phosphate pH 6.7 buffer and electrophoresed through 10% acrylamide gels containing tris-HCl pH 8.9 buffer prepared as described in Materials and Methods. Separated components were identified either by staining with Coomassie Brilliant blue (a & b) or using a Schiff-periodate procedure (c & d). Samples applied to gels a and c contained HB_sAg/ad-disrupted material, gels b and d similarly treated HB_sAg/ay material. Components were identified by their relative migrations with reference to a standard curve composed of comparative estimates for proteins of known molecular weights. The following were resolved: 1 = 90,000 m wt, 2 = 82,000 m wt, 3 = 78,000 m wt, 4 = 51,000 m wt, 5 = 30,000 m wt, 6 = phenol red. The 30,000 m wt polypeptide was resolved only with difficulty, although this component was consistently isolated from tracelabelled HB_sAg of either subtype (see figure 22).

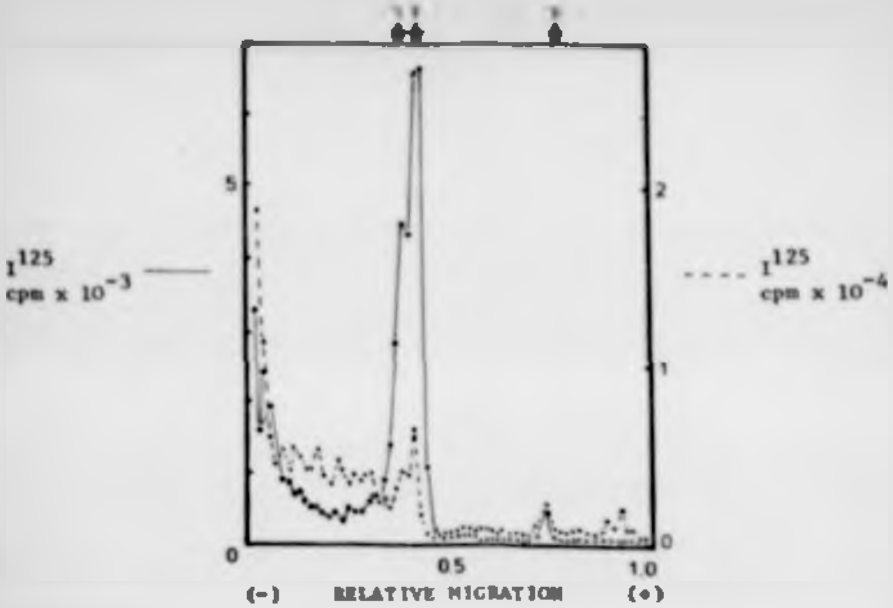


FIGURE 22

SDS-acrylamide gel electrophoresis of HB Ag iodinated by the chloramine-T (———) or the lactoperoxidase (— — —) methods. Peaks 1, 2 and 3 correspond to 90,000, 82,000 and 30,000 molecular weight components respectively.

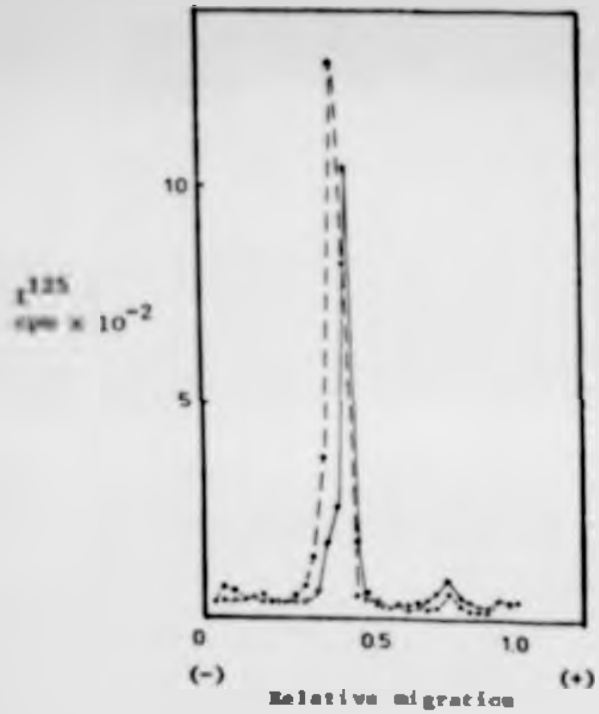


FIGURE 21

SDS-polyacrylamide gel electrophoresis of iodinated HB_sAg resolved by isoelectric focusing. Antigen of pI 4.7 contains a polypeptide of molecular weight 90,000, whilst antigen of pI 4.9 possesses the slightly smaller major polypeptide component of molecular weight 82,000. Both populations of particles contain the smaller 30,000 molecular weight component.

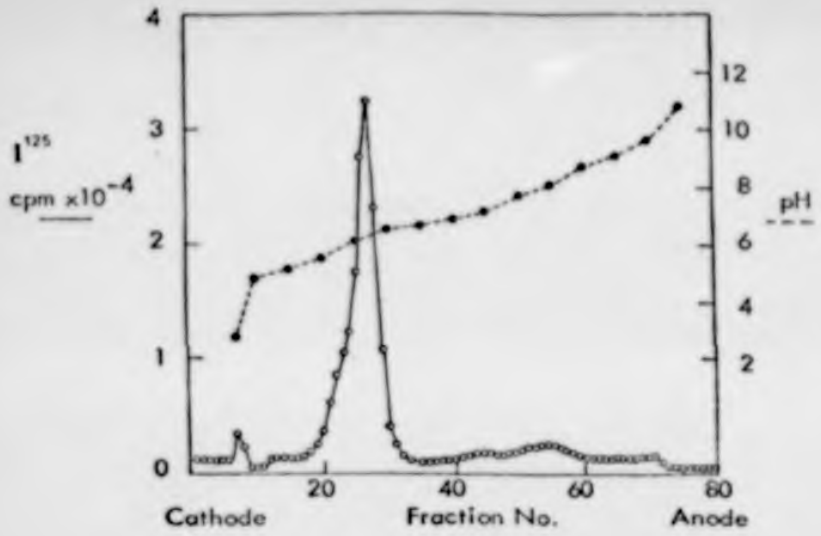


FIGURE 24a

Properties of iodinated HB_sAg after treatment with Nonidet P40, β-mercaptoethanol and urea: Isoelectric focusing. The gradient was composed of freshly-prepared 4 - 8M urea and carrier ampholutes pH 3 - 10 were added to a final concentration of 1%. The major peak of activity possessed an isoelectric point of 5.9.

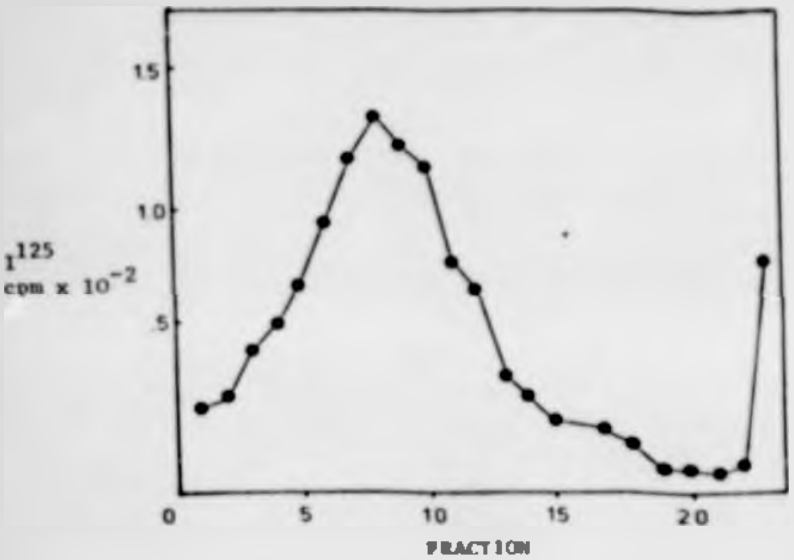


FIGURE 24b

Rate-zonal centrifugation.
Centrifugation was at 104,000 g for 5 hours at 4°C in a 5 - 35% w/v sucrose gradient. Direction of sedimentation was from left to right. The fraction containing the highest level of radio-iodine was characterized as possessing a sedimentation coefficient of 11.8S. The material at the bottom of the tube is thought to represent aggregated material.

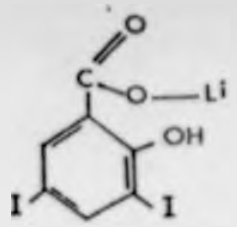


FIGURE 25

Lithium diiodosalicylate.

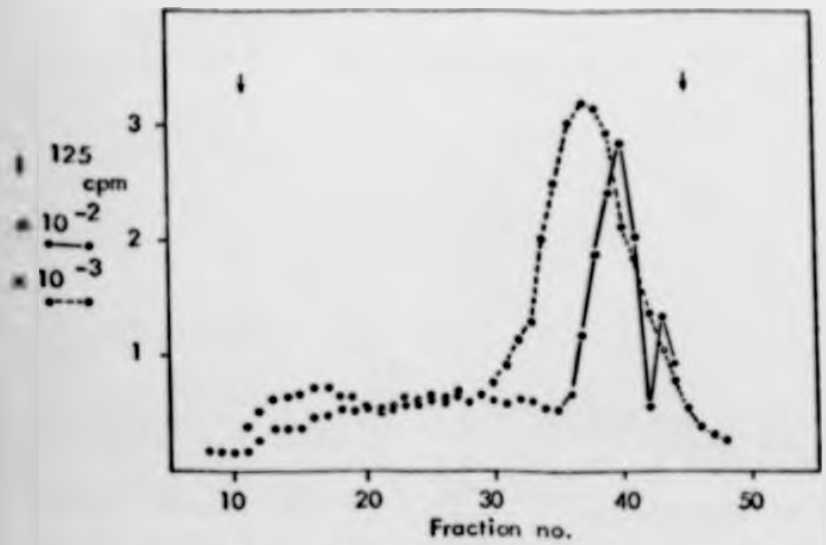


FIGURE 27

Effect of lithium diiodosalicylate on the elution of HB_sAg from Sephadex G200. Purified radioiodinated antigen was resuspended in 0.3M of the reagent for 1 hour at 37°C prior to chromatography on a 22 x 1.5 cm diameter column of Sephadex G200 equilibrated in PBS. Each fraction was assayed for total radioiodine content (open circles) and incubated on a solid surface containing immobilised guinea-pig anti-HB_s for 48 hours. After thorough rinsing, the bound radioiodine was measured (closed circles). Untreated ^{125}I-HB_sAg was eluted in fraction 11 and free, unbound iodine in fraction 44 in a separate experiment.

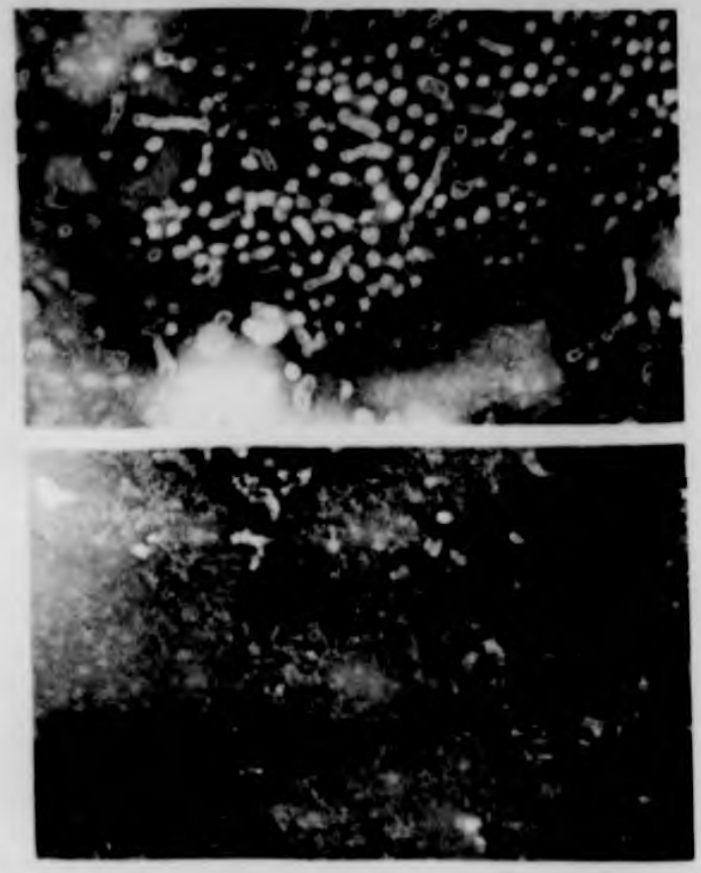


FIGURE 26

Effect of lithium diiodosalicylate on HB_sAg morphology. Purified HB_sAg was examined before (top) and after (below) treatment with 0.3M lithium diiodosalicylate for 1 hour at 37°C.

Magnification: x 126,000

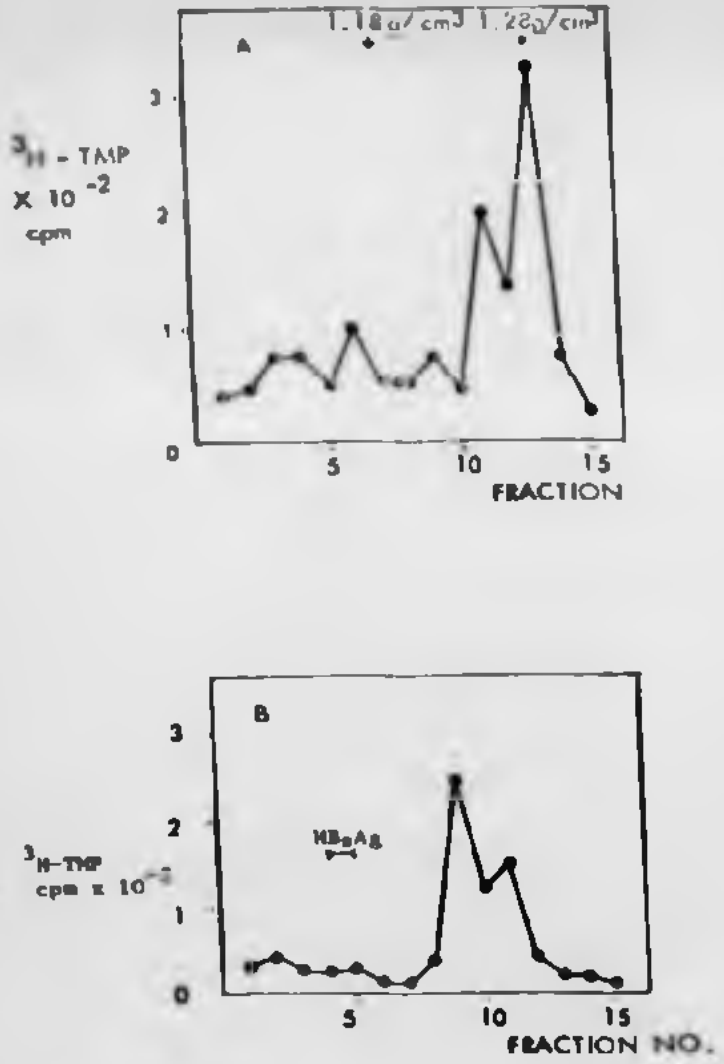


FIGURE 28

Isopycnic centrifugation of HB_cAg-containing plasma
A On a preformed sucrose gradient,
B On a composite ficoll-sucrose-deuterium oxide gradient.
In both cases, DNA polymerase activity was recovered in two incompletely resolved peaks, of average density 1.28 g cm^{-3}.

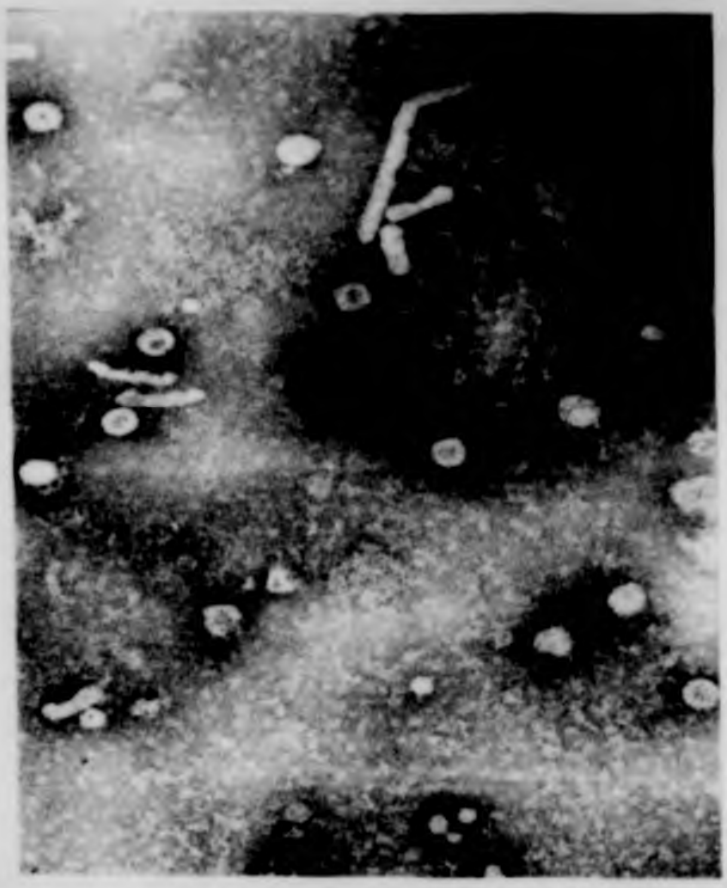


FIGURE 29

Electron microscopy of 42 nm particle-enriched HB_sAg preparation. Aliquots of plasma previously found to contain HB_cAg-associated DNA polymerase activity were concentrated by passage through 20% (w/v) sucrose solution onto a 65% (w/v) sucrose cushion at 80,000 g for 4 hours. A number of filamentous forms were also observed, together with particles 42 nm in diameter that remained unpenetrated by the negative stain.

Magnification: x 126,000

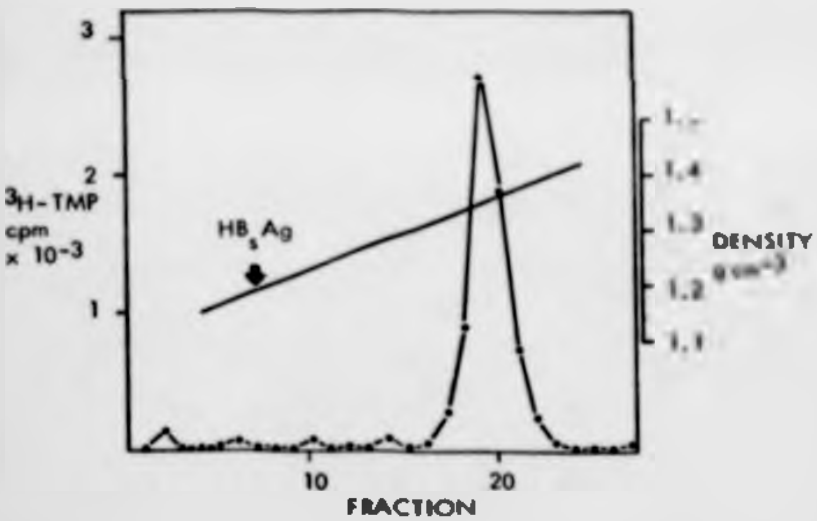


FIGURE 30

Isopycnic centrifugation of ^{3}H-HB$_c$Ag. One hundred microlitres of a 42 nm particle-enriched HB$_s$Ag preparation was incubated for 4 hours at 37°C in the presence of 1% Nonidet P40 together with the DNA polymerase reactants as outlined in Materials and Methods. The bulk of acid-precipitable material was recovered at an average density of 1.35 g cm^{-3}.

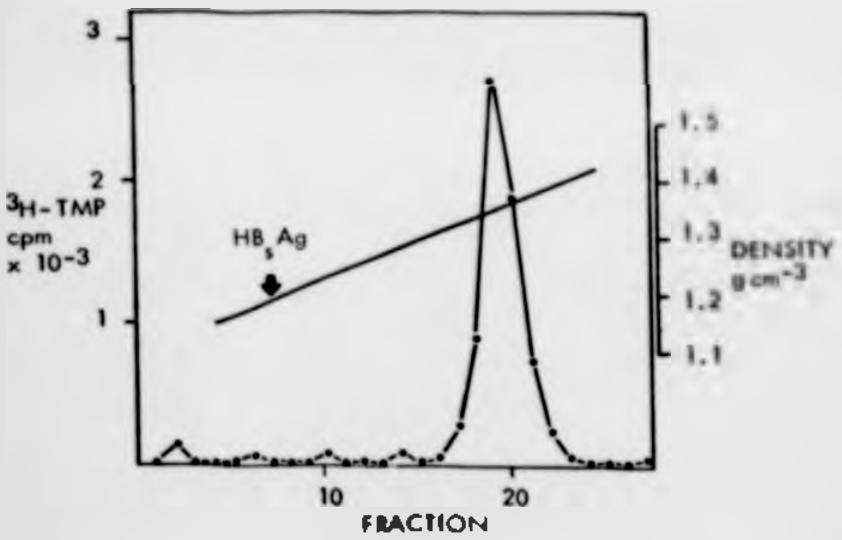


FIGURE 10

Isopycnic centrifugation of ^{3}H-HB$_s$Ag. One hundred microlitres of a 42 nm particle-enriched HB$_s$Ag preparation was incubated for 6 hours at 37°C in the presence of 3% Nonidet P40 together with the DNA polymerase reactants as outlined in Materials and Methods. The bulk of acid-precipitable material was recovered at an average density of 1.35 g cm^{-3}.

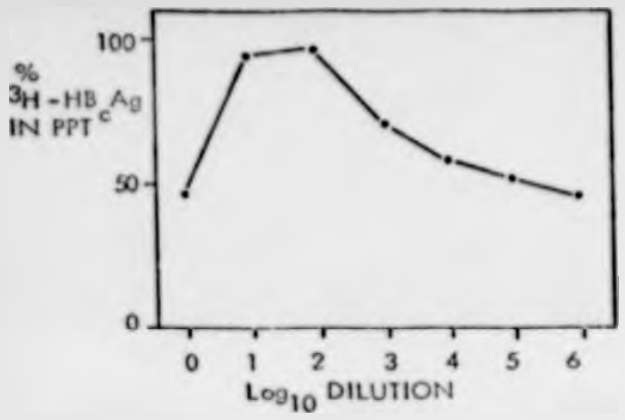


FIGURE 31

Titration of a chimpanzee hyperimmune antiserum to HB_cAg. A pool of ^3H-HB_cAg prepared by isopycnic centrifugation in CsCl (see figure 30) was used as a source of antigen. Approximately 1000 cpm of this material was mixed with an equal volume of antiserum diluted in 0.01M tris pH 7.4 containing 0.15M NaCl, 0.1% β-mercaptoethanol and 0.1% bovine serum albumin. Incubation was for 3 days at 4° prior to the addition of 10 ul of human serum and 1 mg of Staphylococcal protein A. After 2 hours of further incubation at 37°C, complexes were precipitated by centrifugation at 2,000 rpm for 15 minutes and the supernatant mixed with 0.9 ml of Nuclear Chicago Solubilizer and 10 ml of scintillation fluid. The percentage of ^3H-HB_cAg remaining in the supernatant was estimated by reference to the negative control reactions. Approximately 50% of added radiolabel was precipitated at a dilution of 1:500,000 the stock solution being a 1:5 dilution of the original serum. No significant precipitation was obtained using either a normal chimpanzee serum or a convalescent serum obtained from a chimpanzee experimentally infected with type A hepatitis virus.

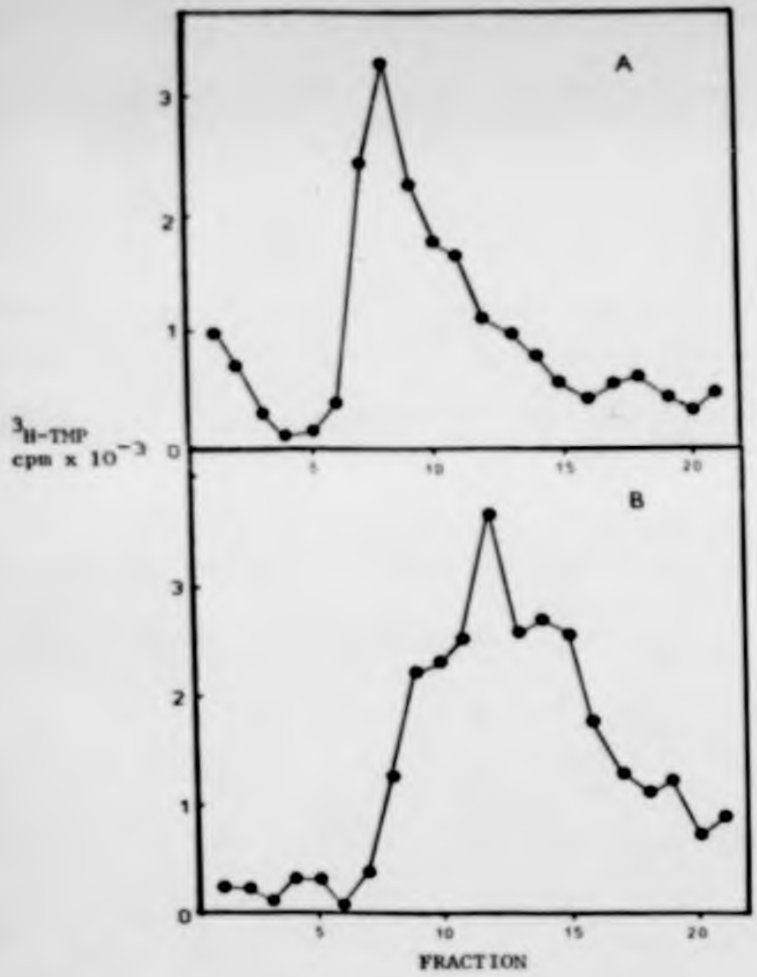


FIGURE 32

Rate-zonal centrifugation of HB_cAg in sucrose gradients.
A Recovery of ^{3}H-TMP following removal of HB_sAg and activation of the endogenous DNA polymerase activity.
B Sedimentation of HB_cAg before removal of HB_sAg.
Each fraction was assayed for the presence of HB_cAg-associated polymerase activity as an indicator of intact 42 nm double-shelled particles containing HB_cAg.

Centrifugation was from left to right in 5 to 20% w/v sucrose at 80,000 g for 60 minutes. Peak of activities correspond to sedimentation coefficients of 420S (A) and 570S (B) respectively.

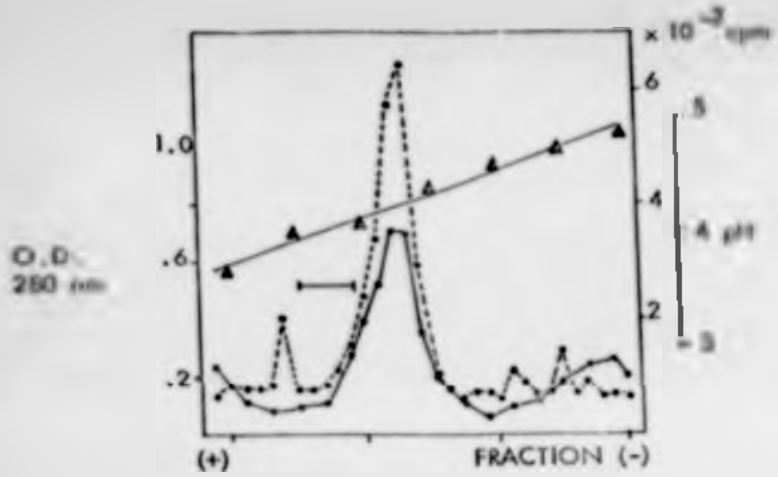


FIGURE 13

Isoelectric focusing of ^{3}H-HB$_c$Ag. Radiolabelled antigen was recovered at an isoelectric point of 4.2 - 4.3 after removal of the outer, HB$_s$Ag envelope, surface antigenicity being found at pH 3.6 - 4.0 (bar). HB$_s$Ag was not detected by radioimmunoassay in fractions containing ^{3}H-HB$_c$Ag.

Optical density: open circles, solid line.
^{3}H-HB$_c$Ag: closed circles, dashed line.

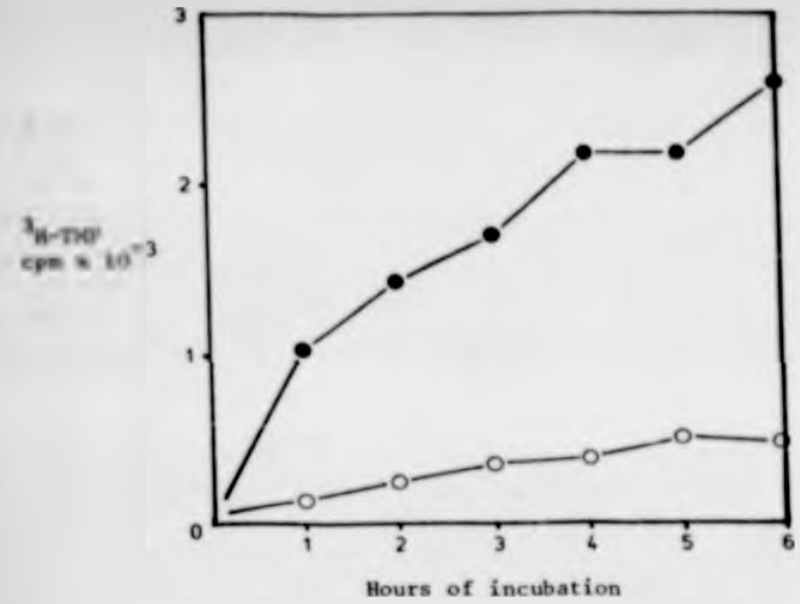


FIGURE 34

DNA polymerase activity in concentrates of HB_sAg-containing plasma. Aliquots of plasma concentrated 20-fold by centrifugation were incubated for varying lengths of time at 37°C with (closed circles) and without (open circles) the detergent Nonidet P40. Enzyme activity was measured by the extent of ^{3}H-TTP incorporation into an acid-insoluble product.

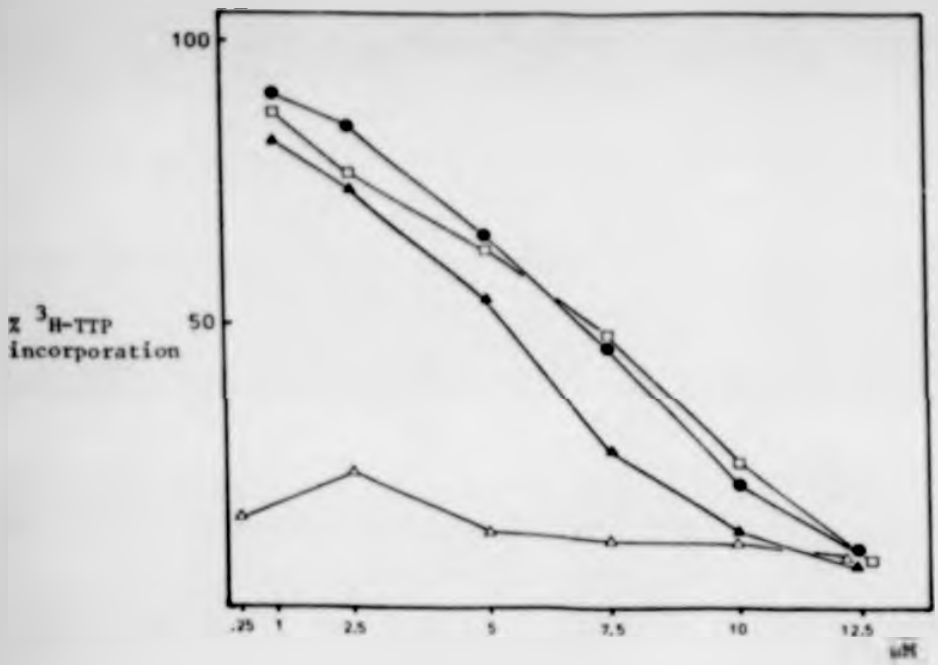


FIGURE 35

Effect of ribonucleotides on HB$_s$Ag-associated DNA polymerase activity. Complete reaction mixtures were incubated with increasing concentrations of ribonucleotides for 4 hours at 37°C. In the absence of added ribonucleotides, approximately 1600 cpm of incorporated ^{3}H-TTP was recorded.

Cytosine triphosphate		(●)
Adenosine	"	(□)
Guanosine	"	(▲)
Uridine	"	(△)

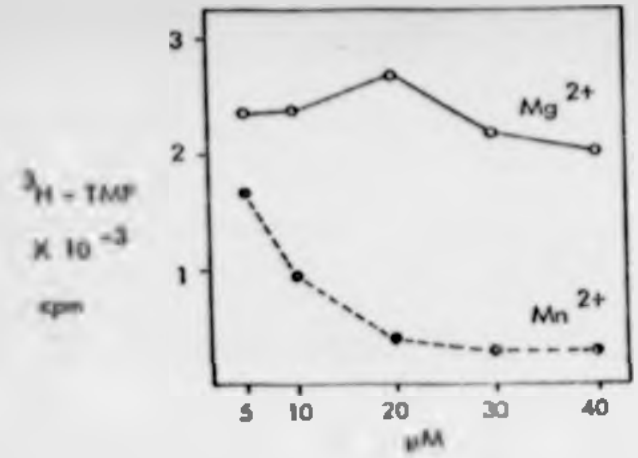


FIGURE 16

Influence of magnesium and manganese ions on DNA polymerase activity. Aliquots of resuspended hepatitis B antigens were incubated for 2 hours at 37°C in the presence of the polymerase substrates, Nonidet P40 and mercaptoethanol and increasing concentrations of magnesium or manganese chloride.

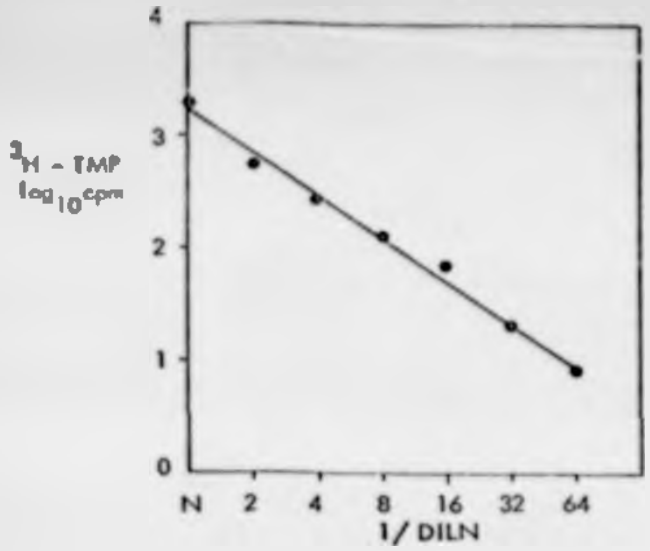


FIGURE 31

Serial dilution of HB_cAg-associated DNA polymerase activity. A concentrated preparation containing hepatitis B antigens was serially diluted in PBS and the level of HB_cAg-associated DNA polymerase activity estimated for each dilution. Each reaction mixture was incubated for 4 hours at 37°C.

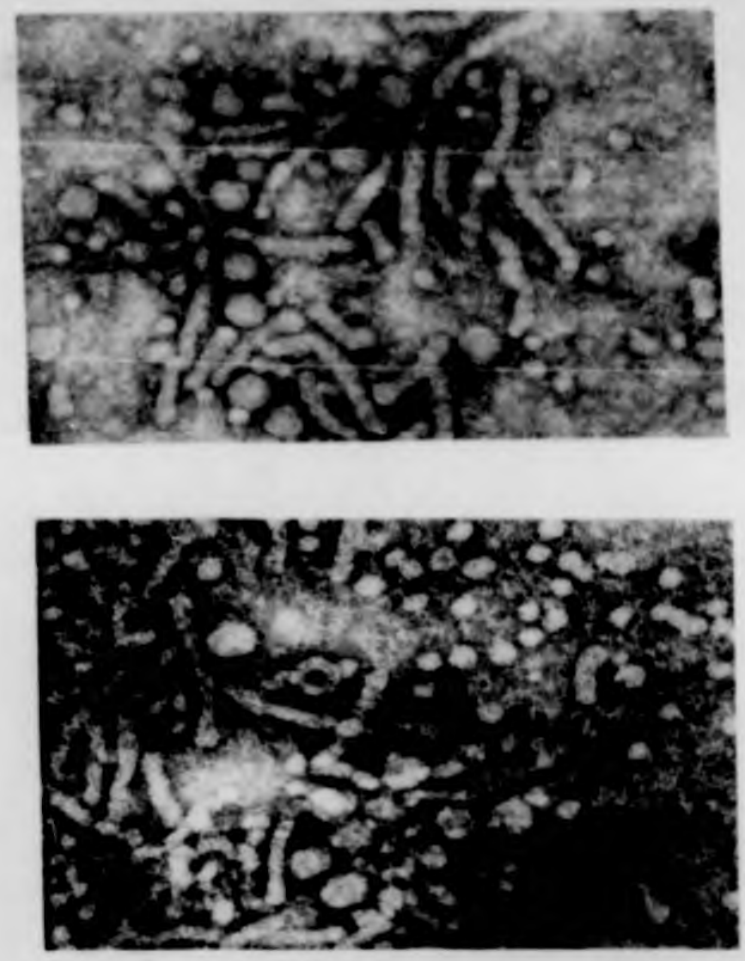


FIGURE 38

Electron microscopy of concentrated HBAg preparations. Plasma samples previously found to contain HB_cAg-associated DNA polymerase activity were concentrated 20-fold by centrifugation prior to negative staining. A high proportion of filaments was frequently found together with many 42 nm diameter particles, some being penetrated by the negative stain to reveal the inner, core component (~~top~~). Occasionally, plasma samples were found to contain a large number of particles 56 - 60 nm in diameter in which no internal structure was visible (~~bottom~~).

Magnification: x 126,000

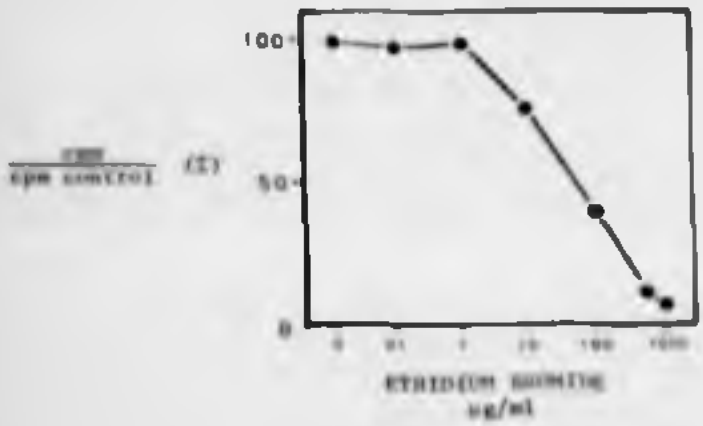


FIGURE 39

Effect of ethidium bromide on HB Ag-associated DNA polymerase activity. Reaction mixtures were incubated for 2 hours at 37°C in the presence of increasing amounts of the dye. The extent of inhibition was quantitated with respect to an untreated control reaction.

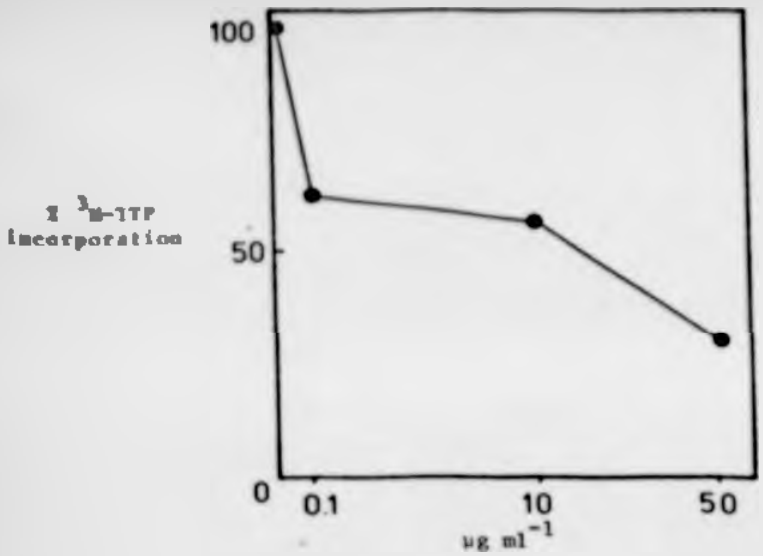


FIGURE 40

Effect of actinomycin D on HB Ag-associated DNA polymerase activity. Aliquots of concentrated HB Ag were incubated in the presence of varying concentrations of the inhibitor for 4 hours at 37°C together with Nonidet P40, β-mercaptoethanol and nucleotide precursors.

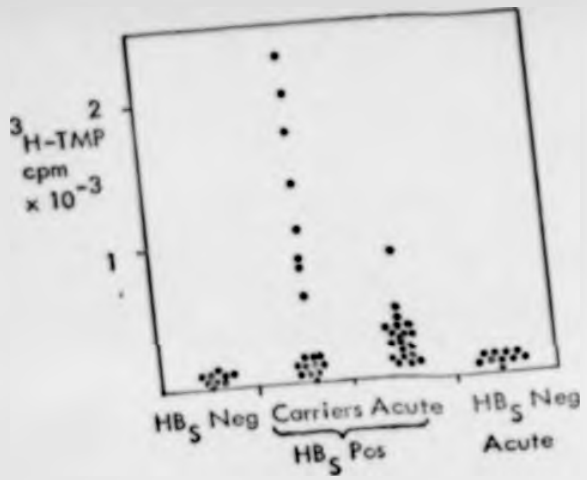
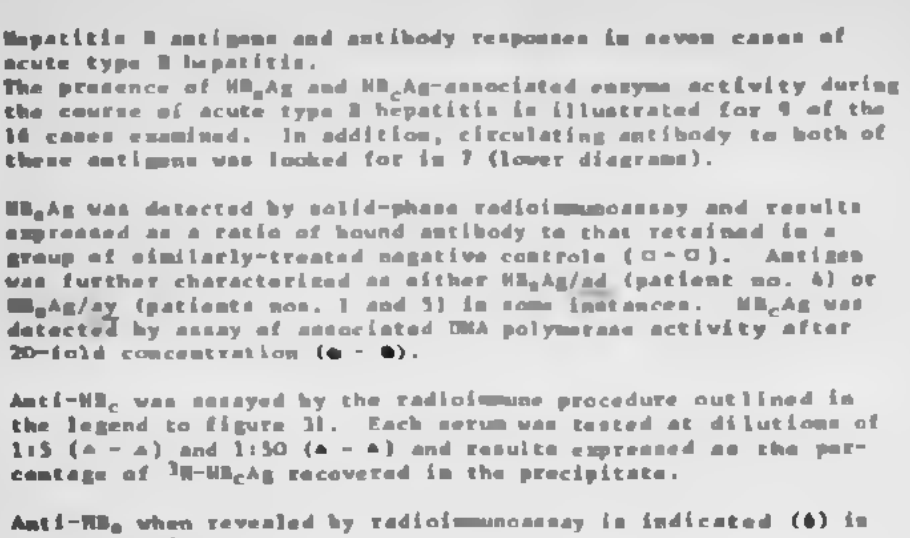


FIGURE 41

HB_cAg-associated ^{3}H-TTP incorporation in HB_SAg-positive sera and plasma. Each specimen was concentrated 20-fold by centrifugation prior to the assay of enzyme activity. HB_SAg-negative plasma selected from either a normal blood donor population or cases of HB_SAg-negative acute hepatitis consistently showed ^{3}H-TTP incorporation to be below 200 counts min^{-1} after 2 hours of incubation at 37°C.

FIGURE 42

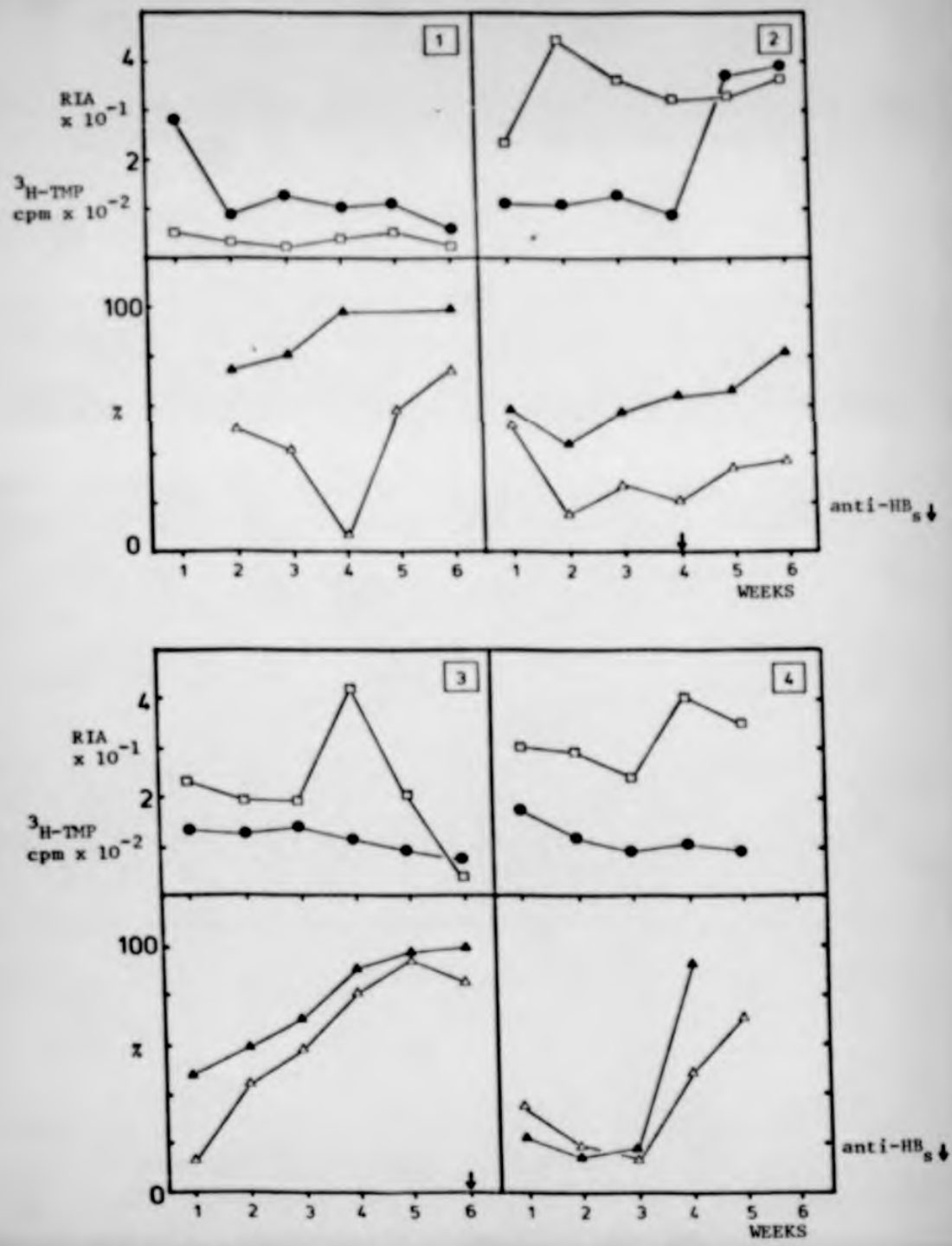


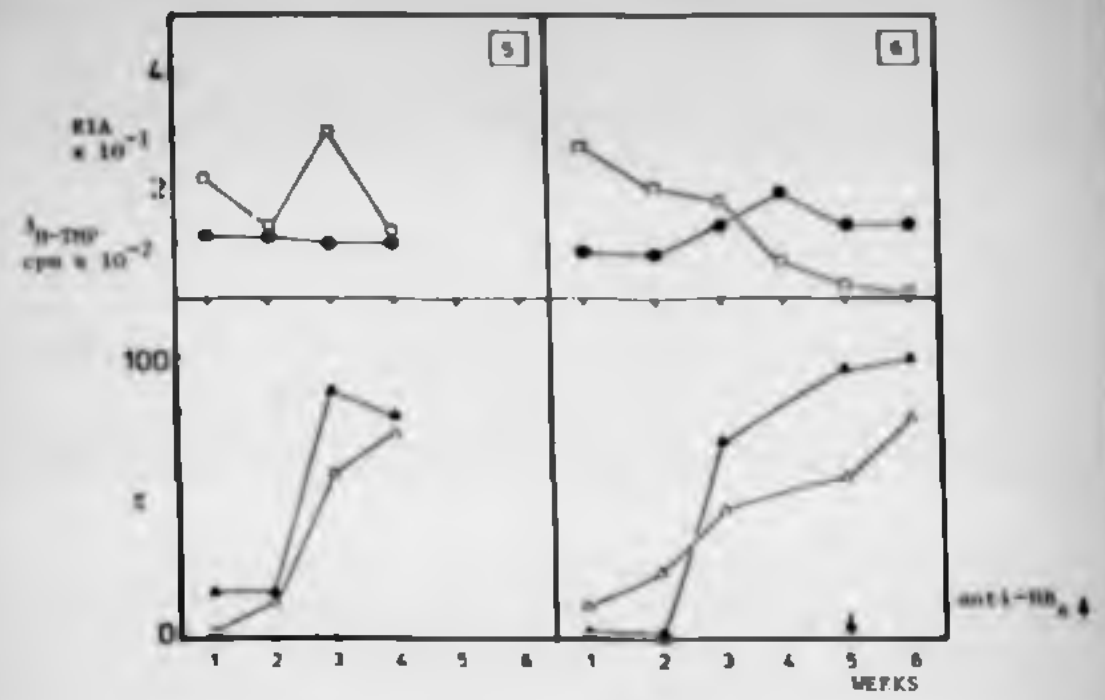
Hepatitis B antigens and antibody responses in seven cases of acute type B hepatitis.
The presence of HB_sAg and HB_cAg-associated enzyme activity during the course of acute type B hepatitis is illustrated for 9 of the 16 cases examined. In addition, circulating antibody to both of these antigens was looked for in 7 (lower diagrams).

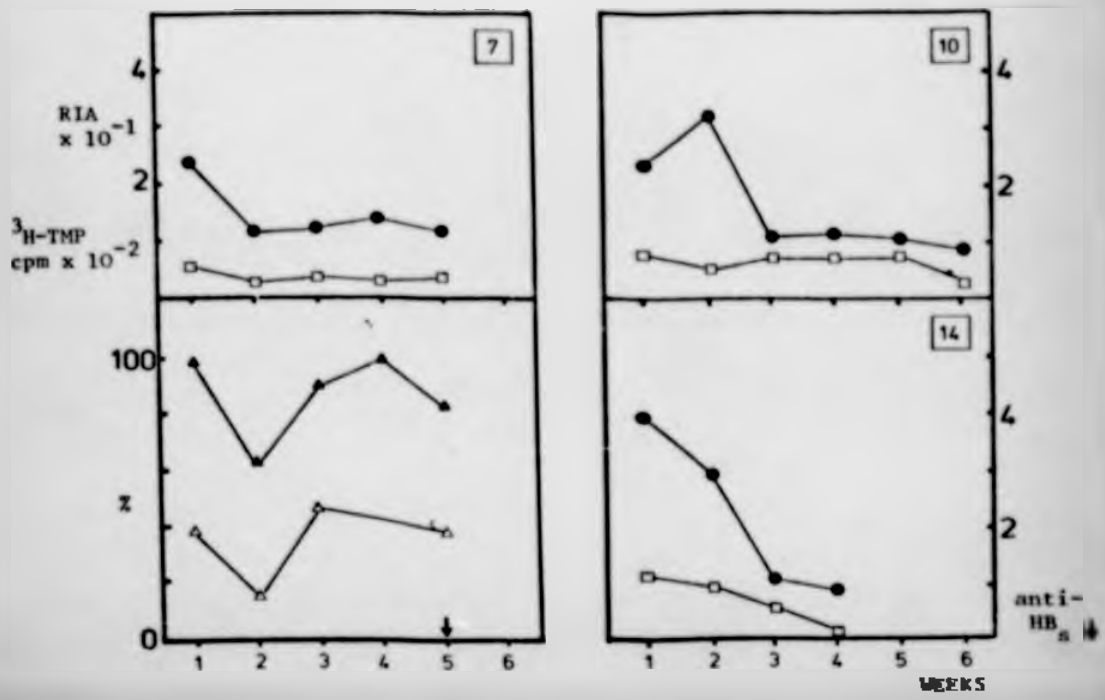
HB_sAg was detected by solid-phase radioimmunoassay and results expressed as a ratio of bound antibody to that retained in a group of similarly-treated negative controls (□ - □). Antigen was further characterized as either HB_sAg/ad (patient no. 4) or HB_sAg/ay (patients nos. 1 and 5) in some instances. HB_cAg was detected by assay of associated DNA polymerase activity after 20-fold concentration (● - ●).

Anti-HB_c was assayed by the radioimmune procedure outlined in the legend to figure 31. Each serum was tested at dilutions of 1:5 (▲ - ▲) and 1:50 (△ - △) and results expressed as the percentage of ^{125}I-HB_cAg recovered in the precipitate.

Anti-HB_s when revealed by radioimmunoassay is indicated (◊) in the lower diagrams.


1
2
3
4
RIA
x 10⁻¹
³H-TMP
cpm x 10⁻²
%
anti-HBs
WEEKS


5
6
RIA
x 10⁻¹
³H-TMP
cpm x 10⁻²
100
%
0
anti-HBs
1 2 3 4 5 6
WEEKS


7
10
14
RIA
x 10⁻¹
³H-TMP
cpm x 10⁻²
100
%
0
anti-
HBs
1 2 3 4 5 6
WEEKS

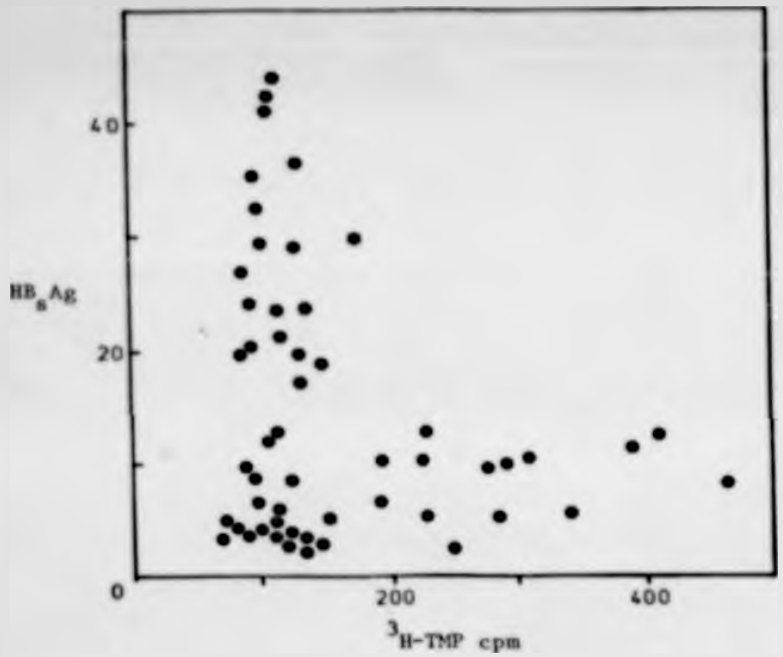


FIGURE 43

Relationship of HB_sAg titre to HB_cAg-associated DNA polymerase activity. A total of 51 sera obtained from 16 cases of type B hepatitis were examined for the presence of HB_sAg by solid-phase radioimmunoassay. The results are expressed as the ratio of bound antibody to that bound in a group of similarly-treated negative controls. The presence of HB_cAg was detected by the incorporation of ^{3}H-TTP into an acid-insoluble product after the activation of endogenous polymerase activity.

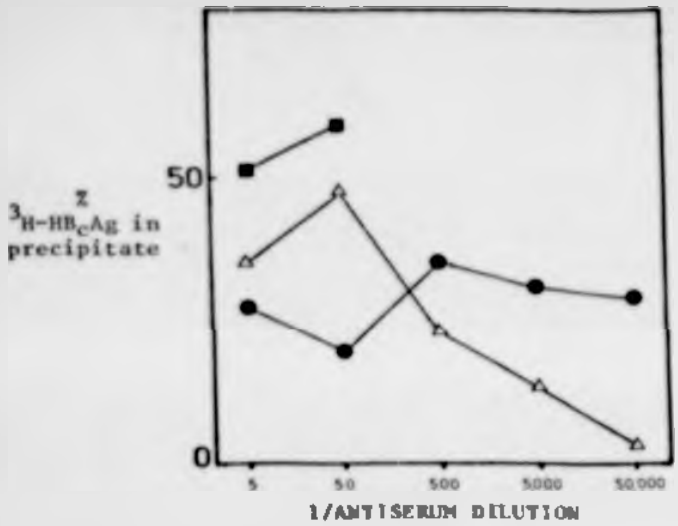


FIGURE 44

Titration of various antisera with ^{3}H-HB$_{c}$Ag.
Approximately 1000 counts min^{-1} of labelled antigen were incubated for 3 days with an equal volume of each antiserum dilution. The resulting immune complexes were separated as outlined in the legend to figure 31. All three sera were of human origin and routinely used as laboratory reagents.

Anti-HB$_{s}$ ▲—▲
Anti-HB$_{c}$ ●—●
Anti-HB$_{e}$ ●—●

TABLE 1

Physical properties of hepatitis B antigens.

A	**SMALL HB_sAg PARTICLES**		
	Diameter		
	a By electron microscopy	16 - 25 nm	Almeida et al (1969)
	b By calculation from diffusion constant	18.8 nm	See note 1
	Buoyant density		
	a In CsCl	1.20 g cm^{-3}	Gerin et al (1969)
	b In sucrose	1.16 "	Kim and Tilles (1973)
	c In potassium tartrate	1.15 "	Gerin et al (1969)
	Diffusion constant ($D^{\circ}_{20,w}$)	2.278 x 10^{-7} cm^2 sec^{-1}	Kim and Tilles (1973)
	Sedimentation coefficient	54S	Gerin et al (1971)
		34.1S	Schober et al (1971)
		30.8S	Bohne et al (1972)
		40.2S	Kim and Tilles (1973)
		40.5S	Takahashi (1975)
		33.0S (ad) 40.1S (ay)	Bourbonnais et al (1975)
	Partial specific volume		
	a From amino-acid content	0.738	See Results
	b From buoyant density measurements	0.803	Dreesman et al (1972b)
	Molecular weight		
	a By gel filtration	2.5 x 10^6	Skinhøj and Hansen (1973)
	b By centrifugation	2.4 x 10^6	Kim and Tilles (1973)
		3.6 x 10^6 (20 - 22 nm diam)	Dreesman et al (1972b)
		2.74 x 10^6	Takahashi (1975)
	Extinction coefficient ($E^{1\%}_{280\ nm}$)	37.2S (ad)	Vyas et al (1972b)
		25	Ling and Overby (1972)
		29.9	Takahashi (1975)
	Electrophoretic mobility	α_2-β globulin region	Kim and Tilles (1973)
B	**FILAMENTOUS HB_sAg PARTICLES**		
	Diameter	20 nm	Almeida et al (1969)
	Length	<60 nm to >230 nm	Bayer et al (1968)
	Buoyant density in CsCl	1.20 g cm^{-3}	Gerin (1974)
C	**HB_sAg AND HB_cAg CONTAINING DOUBLE-SHELLED PARTICLES**		
	Diameter	42 nm	Dane et al (1970)
	Buoyant density in CsCl	1.20 - 1.25 g cm^{-3}	Gerin (1974)
		1.26 - 1.27 "	Chairez et al (1974)
		1.24 "	Barinsky and Bocharov (1974)
	Sedimentation coefficient	58.5S	Barinsky and Bocharov (1974)
D	**HB_cAg CORE PARTICLES**		
	Diameter		
	a Extracellular	27 nm	Almeida et al (1971)
	b Intranuclear	27 - 28 nm	Hirschman et al (1974a)
		27 nm	Barker et al (1974)
	Buoyant density in CsCl		
	a Extracellular	1.28 - 1.30 g cm^{-3}	Lipman et al (1975)
		1.31	Gerin (1974)
		1.28 - 1.32 & 1.35 - 1.36 g cm^{-3}	Moritsugu et al (1975)
	b Intranuclear	1.30	Hirschman et al (1974a)
		1.30 - 1.33 & 1.35 - 1.36 g cm^{-3}	Barker et al (1973b)
	Sedimentation coefficient	110S	Kaplan et al (1973)
E	**e ANTIGEN**		
	Density in CsCl	1.291 g cm^{-3}	Magnius (1975)
	Sedimentation coefficient	11.6S	" "
	Isoelectric point	5.5	Ohori et al (1975)

Notes:- 1 Calculated by substitution of the experimentally determined diffusion constant into the Stokes-Einstein equation where radius of hydrated particle is: $kT/6\pi\eta_0 D^{\circ}_{20,w}$

k = Boltzmann's constant, T = absolute temperature, η_0 = solvent viscosity, $D^{\circ}_{20,w}$ = diffusion constant of small particle

TABLE 2

Polypeptides isolated from small HB_sAg particles after SDS-acrylamide gel electrophoresis.

Mol wt range	Coria et al. (1971)	Vyas et al. (1972b)	Ehrlich & Way (1972)	Druckman et al. (1972)	Coria (1973) ad	Coria (1973) ay	Chairez et al. (1973) ad	Chairez et al. (1973) ay
120,000	-	-	-	-			120,000	120,000
105,000	-	-	-	-			-	105,000
95,000	-	-	-	-	95,000	95,000	-	-
75,000	-	-	-	-	75,000	75,000	-	-
69,000	-	-	-	-	-	-	69,000	69,000
65,000	-	-	-	-	-	65,000	-	-
55,000	-	-	-	-	55,000	55,000	-	55,000
39-40,000	40,000	-	-	39,000	40,000	40,000	40,000	40,000
32-34,000	32,000	32,000	34,000	32,000	32,000	32,000	32,000*	32,000*
25-28,000	26,000	25,000	28,000	27,000	26,000	28,000	27,000*	27,000*
22-24,000	-	-	24,000	22,000	-	-	22,000*	22,000*
19,000	-	-	-	-	-	-	18,000	19,000
16,000	-	-	-	16,000	-	-	-	-
10,000	-	-	-	10,000	-	-	-	-

Only those components reported by the authors as consistently present are included. An attempt has been made to group together polypeptides of similar reported molecular weights; however, it is not necessarily implied that components included within any one grouping are identical. Major components are underlined. Chairez et al. (1973) have reported essentially similar results to those of Chairez et al. (1973). Similar analyses were later reported (Coria, 1974) with the exception that a 65,000 mol wt component was not included, although an additional 36,000 mol wt component was present. The 32-32,000 and 54,000 mol wt polypeptides were found to be glycosylated.

* indicates glycoproteins.

TABLE 3

Effect of various reagents[1] on the immunoreactivity of hepatitis B surface antigen (HB_sAg/ad) in plasma.

A	Detergents (all used at 1%)		Effect[2]
	Nonionic (Tween 80, Nonidet P40, Triton X-100, Brij 35)		0
	Anionic (sodium dodecyl sulphate)		0
	" (sodium lauryl sarconinate)		-
	Cationic (cetyltrimethylammonium bromide)		---
B	Dissociating reagents		
	Formamide	0.1M	-[3]
		1.0M	--[3]
	Urea	0.1M	0
		1.0M	0
	Guanidine-HCl	0.1M	0
		1.0M	0
C	Reducing agents		
	β-mercaptoethanol	0.1M	+
		1.0M	++
	dithiothreitol	0.1M	-
		1.0M	---

Notes:

1 One ml volumes of plasma were incubated for 1 hour at 37°C with an equal volume of the reagent dissolved in water to give the final concentrations shown. After incubation, samples were exhaustively dialysed against PBS prior to the determination of HB_sAg titre by reverse passive haemagglutination.

2 0 = titre unchanged from control; (-) = titre reduced but not by more than 50%; (--) = titre reduced by 50 - 75%; (---) = titre reduced by more than 75%; (+) = titre increased twofold; (++) = titre increased more than twofold.

3 No effect was seen on HB_sAg/ay reactivity.

TABLE 4

Precipitation of HB_sAg/ay from plasma by the addition of polyethylene glycol 6000 (Koch-Light Ltd).

Final concentration of PEG_{6000}	pH 7	pH 4
6%	20*	80
8%	33	89

* Percentage recovered from the precipitate after overnight incubation at 4°C.

TABLE 3

Purification of HB_sAg[1]

	Vol (ml)	$\frac{1}{HA\ titre}$	Amount of protein recovered (mg/ml)	% HB_sAg activity recovered	Purification factor
A BY ULTRACENTRIFUGATION					
Plasma	5.75	2048	38.5	(100)	(1)
Isopycnic centrifug. (1st)	5.00	2048	11.4	87	6
Isopycnic centrifug. (2nd)	2.10	4096	2.25	73	94
Rate-zonal Centrifugation					
i Urografin	6.3	1024	0.08[2]	55	220
ii Sucrose	6.5	128	0.1	8[3]	16
B BY AFFINITY CHROMATOGRAPHY					
i Using immobilized IgG					
Plasma	5.0	512	46.0	(100)	(1)
Eluate	14.0	32	0.04	18	72
ii Using immobilized concanavalin A					
Serum[4]	1.0	2048	46.0	(100)	(1)
Eluate	8.8	233	1.12	91	5

(1) Similar results were obtained for HB_sAg/ad and HB_sAg/ay.

(2) Estimated following precipitation from Urografin solution.

(3) Recovery was 31% in gradients containing HB_sAg-positive plasma as the sample.

(4) Recalcified with $CaCl_2$ prior to chromatography.

TABLE 6

Recovery of amino acids[1] from acid hydrolysates of HB_sAg.

		A HB_sAg purified by isopycnic centrifugation		B HB_sAg purified by isopycnic & rate-zonal centrifugation		C HB_sAg/ad purified by affinity chromatography	D Serum proteins		
		a) ad	b) ay	a) ad	b) ay		a) Albumin	b) Fibrinogen	c) Factor VIII
BASIC	Lysine	6.63	9.04	6.28	7.04	5.75	10.84	7.60	7.52
	Histidine	3.89	3.33	2.33	2.30	2.00	2.82	2.27	2.17
	Arginine	4.84	5.35	3.03	3.44	3.19	4.23	5.58	5.41
ACIDIC	Aspartic acid	9.91	9.30	9.08	9.17	10.36	9.87	12.52	11.75
	Glutamic acid	13.80	13.11	11.40	10.47	12.67	13.23	11.10	10.89
NEUTRAL	Threonine	6.30	6.22	7.45	9.17	7.20	4.67	6.62	6.07
	Serine	6.43	6.22	7.92	7.69	7.35	3.61	10.71	6.96
	Proline	4.87	5.93	6.75	7.20	2.49	6.00	3.52	4.66
	Glycine	3.64	3.64	6.52	6.22	6.40	2.12	9.73	13.23
	Alanine	4.01	8.66	6.75	7.04	8.51	9.96	5.12	4.72
	½ Cysteine[3]	2.91	3.18	ND	ND	ND	ND	ND	ND
HYDROPHOBIC	Valine	7.90	6.25	9.08	9.17	7.92	10.59	4.03	6.61
	Methionine	1.51	1.38	2.09	1.81	2.78	0.88	0.91	1.00
	Isoleucine	3.17	2.87	3.72	3.44	3.44	1.41	4.61	4.54
	Leucine	9.47	9.32	10.01	9.82	10.72	10.59	6.62	21.32
	Tyrosine	5.34	4.46	3.58	3.60	3.88	3.70	3.83	3.79
	Phenylalanine	5.38	5.90	4.19	4.42	5.29	5.47	3.24	3.23
	TOTAL CHARGED	39.07	40.13	32.12	32.42	33.97	40.99	39.07	37.54
	TOTAL HYDROPHOBIC	32.77	30.18	32.67	32.36	34.03	32.64	23.24	34.53
	TOTAL APOLAR[2]	27.43	25.72	29.09	28.66	30.15	28.94	19.41	30.74

1 Expressed as % moles recovered.

2 Total hydrophobic minus tyrosine.

3 Determined independently by performic acid oxidation.

ND = not done

T A B L E 6 (continued)

Recovery of amino-acids[1] from the acid hydrolysates of HB_sAg small spherical particles and filamentous forms separated by rate-zonal centrifugation.

	FILAMENTS	SMALL SPHERES
Lysine	2.18	1.72
Histidine	0.85	1.38
Arginine	3.03	1.84
Aspartic acid	6.31	6.89
Glutamic acid	6.06	5.62
Threonine	8.73	12.40
Serine	10.79	12.63
Proline	11.28	15.61
Glycine	8.25	9.64
Alanine	3.88	3.90
Valine	3.40	5.62
Methionine	5.22	6.89
Isoleucine	4.85	5.62
Leucine	14.92	16.30
Tyrosine	3.03	1.95
Phenylalanine	6.55	6.89
Total charged	18.43	17.45
Acidic:Basic	2.04:1	2.53:1
Total hydrophobic	37.97	43.27
Total apolar	34.94	41.32

(1) Expressed as % moles recovered.

TABLE 7

Determination of the extinction coefficient ($E_{1\%}^{280}$) of HB_sAg.

		$OD_{280\ nm}$	Protein concentration[1] ug/ml	Extinction coefficient
HB_sAg/ad	a)	1.995	563	35.44
	b)	1.225	325	37.69
	c)	0.695	208	33.41
				av. 35.51
HB_sAg/ay	a)	2.025	575	35.22
	b)	1.350	363	37.19
	c)	0.725	218	33.26
				av. 35.22

(1) Determined calorimetrically by the method of Lowry et al., (1951).

TABLE 8a

Reduction of HB_sAg activity in the presence of N-bromosuccinimide.

% concentration	% reduction in cpm[1]	
	a) with anti-ad/ay	b) with anti-d
0.1	0	9
1.0	92	83

TABLE 8b

Reduction of HB_sAg activity in the presence of cetyltrimethylammonium bromide.

% concentration	% reduction in cpm[1]	
	a) with anti-ad/ay	b) with anti-d
0.1	14	56
1.0	61	42

(1) Control assays produced results of 6588 and 855 cpm for (a) and (b) respectively.

TABLE 9

Recovery of HB_sAg activity following exposure to dissociating reagents.

Treatment*	$\frac{1}{RPHA}$ titre
1% sodium dodecyl sulphate, 1% β-mercaptoethanol, 6M urea	1
1% β-mercaptoethanol, 5M guanidine hydrochloride	16
0.5M lithium diiodosalicylate	256
Untreated control	2048

* Purified HB_sAg was treated for 1 hour at 37°C. Excess reagents were removed by dialysis prior to the assay of HB_sAg.

TABLE 10

Precipitation of HB_cAg-associated DNA polymerase with anti-$HB_{(c)}$

	^{3}H-TMP in supernatant[1] counts min^{-1}
In plasma	
75 µl + 25 µl rabbit anti-HB_c	69
75 µl + 25 µl normal rabbit serum	338
In HBAg concentrate[2]	
75 µl + 25 µl rabbit anti-HB_c	2533
75 µl + 25 µl normal rabbit serum	4588

1 After incubation for 16 hours at 4°C, 100 µl of donkey anti-rabbit serum was added and incubation continued for a further 2 hours at 45°C. Immune complexes were precipitated by centrifugation and the supernatant examined for ^{3}H-TMP activity. In later experiments, the second (precipitating) antibody was replaced with 1 mg of Staphylococcal protein A.

2 Concentrated 20-fold by centrifugation for 4 hours at 80,000 g.

TABLE II

Reaction requirements of the HB$_s$Ag-associated polymerase activity.

	^{3}H-TMP cpm	% reduction
Complete	1333	-
- Mg^{2+}	73	95
- NH_4^+	1124	16
- dATP	196	85
- dCTP	294	78
- dGTP	221	83

TABLE 12

The effect of adding purified HB_sAg to a reaction mixture containing HB_sAg-associated DNA polymerase activity.

		^3H-TMP cpm
Experiment 1	HB_sAg-containing concentrate	1096
	HB_sAg + 1:5 HB_sAg small spheres	1134
	" + 1:50 "	1324
	" + 1:5 HB_sAg filaments	1126
	" + 1:50 "	1281
	Negative control	104
Experiment 2	1:5 HB_sAg small spheres	108
	1:50 "	71
	1:5 HB_sAg filaments	69
	1:50 "	125
	Negative control	109

TABLE 13

Effect of nuclease treatment on HB_cAg-associated DNA polymerase activity.

Reaction*	^{3}H-TMP counts min^{-1}
HB_cAg + DNase	659
HB_cAg + RNase	627
HB_cAg only	661

* Incubation was for 4 hours in the presence of 100 µg of nuclease.

TABLE 1A

The nature of the HB_eAg-associated DNA polymerase activity.

	No. of counts min^{-1} precipitated by 5% trichloroacetic acid
Control	384
[a]DNase	41
RNase	305

[a] Nucleic acid was extracted from HB Ag by incubation for 2 hours at 37°C with 0.5% SDS and 0.05% pronase in 0.01M tris pH 8 containing 0.1M NaCl and 10mM EDTA. Extracted material was precipitated with ethanol. Susceptibility to nuclease treatment was carried out for 1 hour at 17°C in 0.01M $MgCl_2$ containing 250 $\mu g\ ml^{-1}$ of DNase or RNase.

PUBLISHED WORK

In 'Isoelectric Focusing' pp 281 - 287. Eds Arbuthnott, J.P. & Beeley, J.A. Butterworths, London, 1975.

21. HETEROGENEITY OF HEPATITIS B ANTIGEN

C.R. Howard and A.J. Zuckerman

The close association between hepatitis B antigen and human hepatitis B virus (serum hepatitis virus) has now been firmly established[1]. Early studies involving staining and flotation experiments showed hepatitis B antigen to be lipoprotein which was immunologically distinct from normal low density serum lipoproteins[2]. Examination of serum containing this antigen in the electron microscope by the negative staining technique revealed a remarkably heterogeneous population of virus-like particles. The principal antigenic constituent was a small pleomorphic spherical particle, measuring approx. 20 nm in diameter but with a range of between 16 and 25 nm. The presence of tubular forms, with a constant diameter of 20 nm and often a length of several hundred nanometers, was a characteristic feature[3]. The third type of particle was also spheroidal, measuring approx. 42 nm in diameter, with an inner core of 28 nm in diameter, surrounded by a 2 nm shell and an outer coat about 7 nm in thickness[4]. All three types of particles are aggregated by specific hepatitis B antibody, suggesting that there is at least one common antigenic determinant on the surface of each morphological entity. The mobility of hepatitis B antigen by immunoelectrophoresis in agar gel was found to follow closely that of α_2-globulin[5]. Kim and Tiller[6] reported that purified antigen, derived from the sera of individual patients with acute hepatitis B infection, migrated in an electrophoretic field either in the α_2-β-globulin region or in the β-globulin region with some trailing; this confirmed their earlier findings of

electrophoretic heterogeneity of serum samples obtained from such patients[7]. An antigen-positive serum from a patient suffering from post-transfusion hepatitis was examined by isoelectric focusing in large-pore polyacrylamide gel slabs[8]. The antigen in the pH range 4.8 to 5.0, was detected by subjecting slices of gel, after isoelectric focusing, to immunoelectrophoresis. By contrast, the pattern of bands found in stained gels suggested focusing of the antigen over a much wider pH range. Also, antigenic activity was not correlated with the morphology of the particles nor with a particular antigenic subtype specificity.

The close association of hepatitis B antigen with normal serum components, confirmed in our laboratory by radioimmunoassay of fractionated material, has been an acknowledged difficulty in the development of centrifugation techniques for separation of the antigen in pure form from serum. Since, unlike other separation techniques, there is almost complete recovery of total protein after separation by isoelectric focusing, we applied this technique to the purification of hepatitis B antigen. Serum containing antigen with the subdeterminants ad+y- and which was morphologically constituted almost entirely of small spherical particles, was subjected to isoelectric focusing in a sucrose density gradient in the apparatus described by Vesterberg and Svensson[9] containing carrier ampholytes (Arpholine) at a final concentration of 1% (w/v). The cathode was protected by a 2% ethanolamine solution in water and the anode by 1.4% (w/v) ortho-phosphoric acid in 60% (w/v) sucrose. Antigenic activity was found in those peaks of serum proteins which possessed isoelectric points in the pH range 4.0 to 7.0. Antigenic activity was not detected in association with separated gamma-globulins. This is in accordance with the well known epidemiological and clinical experience that the use of gamma-globulin clinically is free from the risk of transmitting hepatitis and with the failure to detect hepatitis B antigen (a marker associated with infectivity) by electron microscopy after Cohn fractionation of human plasma known to contain the antigen[10]. The low molecular weight serum proteins were removed by gel filtration on Sephadex G200 and were concentrated by ultrafiltration. Hepatitis B antigen was then separated from the remaining unwanted serum protein by isoelectric focusing in a sucrose gradient as already described. Two discrete bands of hepatitis B antigen were found[11] with

isoelectric points of 3.65 and 4.33. Normal serum proteins were not detected in the two bands by the double radial micro-Ouchterlony immunodiffusion techniques using hyperimmune horse antiserum against whole human serum. In addition, the protein in both bands was aggregated by the addition of concanavalin A after exhaustive dialysis against phosphate-buffered saline. The effect was reversed by the addition of α-methyl-D-mannoside. Each band of separated hepatitis B antigen was found by immune (using convalescent serum) electron microscopy[12] to contain the intact small spherical particles, 20 nm in diameter. This implies that there is at least one antigenic determinant common to both bands of separated antigen. It has also been noted that the small antigen particles fall into two groups[13], measuring 19 nm and 25 nm in diameter, with a corresponding difference in average molecular weights (3.56 and 4.47 x 10^6, respectively). Two isoelectric pH values were observed with iodinated, purified hepatitis B antigen; one component had a pI value of 4.0 and the other a pI value of 4.4. The relative proportions of the two particle types were dependent on the individual plasma from which the antigen was purified, certain plasma containing chiefly one or other of the particle types.

One sample of serum with antigenic subdeterminants *ayed-* was fractionated by isoelectric focusing. The sample separated into two bands of antigenic activity with pI values of 3.95 and 4.90, thus differing from those obtained previously with the *adyy-* subtype[11]. Considerable difficulty arose in a number of experiments due to isoelectric precipitation. This was overcome by employing narrow-range pH gradients and by increasing the applied voltage in small amounts at regular intervals.

Isoelectric focusing of material labelled with [^{125}I] by a modification of the chloramine T method[14], revealed trace amounts of hepatitis B antigen. Radioisotope labelling does not alter appreciably the reactivity of hepatitis B antigen and there is no significant alteration in buoyant density (Howard and Zuckerman, unpublished observations). However, the pI values of the peaks of separated antigen were raised to 4.5 and 4.8 respectively. The relatively high phospholipid content of the antigen suggested that its acidic nature, as reflected in the low pI values so far determined, might be due in part to the carboxyl group in phosphatidyl serine. Analysis of extracted lipid failed, however, to detect this

phospholipid[15,16]. Alternatively, the presence of carbohydrate may influence the surface charge of the lipoprotein moiety of the antigen, thereby imparting a hydrophilic surface when in aqueous solution[17]. It is also interesting to note that some plant viruses behave similarly when focussed in polyacrylamide gels[18].

The apoprotein constituent of hepatitis B antigen was found to be organised into a number of definable polypeptides. Aliquots from peaks I and II (Figure 11.1) of separated antigen activity were solubilised by heating for 10 min at 60°C in the presence of 1% (w/v) sodium dodecyl sulphate, 0.5 M urea and 0.1% (w/v) dithiothreitol. The resulting solution of denatured protein was then subjected to disc electrophoresis in 10% SDS polyacrylamide gels using 0.005 M Tris-glycine buffer, pH 8.0. After staining with Coomassie Brilliant Blue, eight identical polypeptides were discernable in samples of material from both peaks I and II. The range of molecular weights was approximately 2 000 to 100 000. Densitometry of the stained gels revealed that at least one polypeptide (mol. wt. 100 000) was a major component of the first peak, but it was present only as a minor component in the second peak. Conversely, the lowest molecular weight polypeptide component of peak II was not present in peak I. A similar analysis of the polypeptide components of antigen bearing the ay+d- subdeterminants has not yet been completed.

Further attempts have been made to characterise the polypeptide composition of the ady- subtype by procedures similar to those used for the isolation of adenovirus subunits[19] and the separation of the group-specific antigen of Rous sarcoma virus[20]. Hepatitis B antigen labelled with [^{125}I] was heated for 2 h at 37°C in the presence of 4 M urea, 1% (w/v) 2-mercaptoethanol and 1% (w/v) of a nonionic detergent Nonidet-P40 (B.D.H. Ltd., Poole, England). The dissociated antigen was then subjected to preparative isoelectric focusing in a urea gradient of 4 to 8 M in the absence of sucrose and containing carrier ampholytes (Ampholine) and Nonidet-P40 at a final concentration of 1% (w/v) or 0.1% (w/v) respectively. The anode was protected by 2% (w/v) ortho-phosphoric acid solution in 8 M urea and the cathode with 1.4% (w/v) ethanolamine in water. A large peak of radioactive material with a pI value of 5.9 was formed. This peak was characterised further by SDS disc gel electrophoresis as described above and the molecular weight was estimated to be 100 000 and therefore

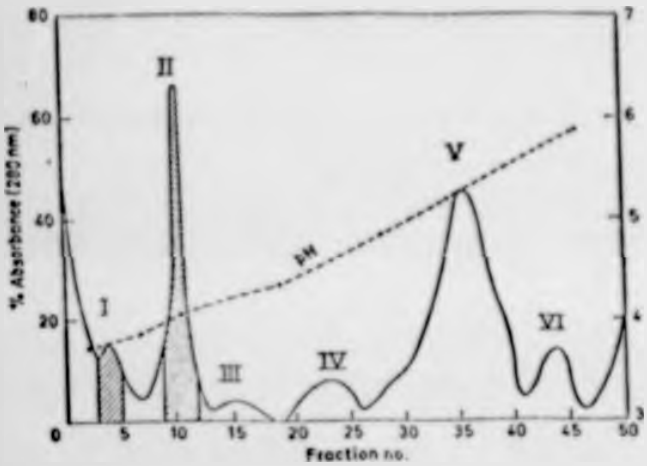


Figure 23.1 The system had a volume of 110 ml and contained 1% (w/v) Ampholine, pH range 3 to 10. The duration of the experiment was 43 h and the final potential was 600 V. pH measurements were made at 20°C. Hepatitis B antigen activity was detected by complement fixation, immunoelectrophoresis and immune electron microscopy and is indicated by the cross-hatched areas. These fractions were pooled for analysis of constituent peptides. (From Howard and Zuckerman[11])

identified as the major polypeptide component of peak I described above. This may be responsible in part for the heterogeneity of the small 20 nm antigen particles described earlier.

The heterogeneity of hepatitis B antigen has thus been confirmed by isoelectric focusing. This technique offers a convenient method for further analysis of the structure of antigens associated with viral hepatitis.

ACKNOWLEDGEMENTS

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Chapter 8

Biophysical and biochemical properties of hepatitis B surface antigen and the core

by Colin R. Howard

Early experiments by flotation centrifugation and the staining of precipitin lines revealed hepatitis B antigen to contain both lipid and protein. The location of antigenic determinants on particles of various morphological forms has facilitated the isolation of this antigen from normal serum proteins to a standard of purity necessary for its use as an immunogen in the laboratory. The degree of success achieved by isolation is dependent on knowledge of both the physical and chemical properties of the antigen. In addition, the results of biochemical studies on the isolated antigen are now making a significant contribution to our understanding of its relationship to hepatitis B virus.

Physical properties and isolation

Hepatitis B antigen is readily separated from other serum proteins by virtue of its unique buoyant density. Antigenic activity is found at a density intermediate between that of the low and high density serum lipoproteins, although the exact value may vary between sera and also according to the chemical employed in forming the density gradient. The morphology of the particles remains intact after exposure to high salt concentrations and there is no significant loss of titre after equilibrium centrifugation. Centrifugation of serum in buffered caesium chloride results in the isolation of antigen at a density of 1.20 g cm^{-3}, although the presence of immune complexes may be indicated by a second band of antigenic activity at 1.25 g cm^{-3} (Gerin et al. 1969). The use of sucrose (Kim and Tilles, 1973) and potassium tartrate (Gerin et al. 1969) gives a slightly lower value for the main band of antigen

Fig. 8.1. Serum containing hepatitis B antigen before separation by zonal centrifugation in 0·40% caesium chloride. Three distinct morphological entities are present: small pleomorphic spherical particles, tubular forms and large double-shelled spherical particles. × 252,000.

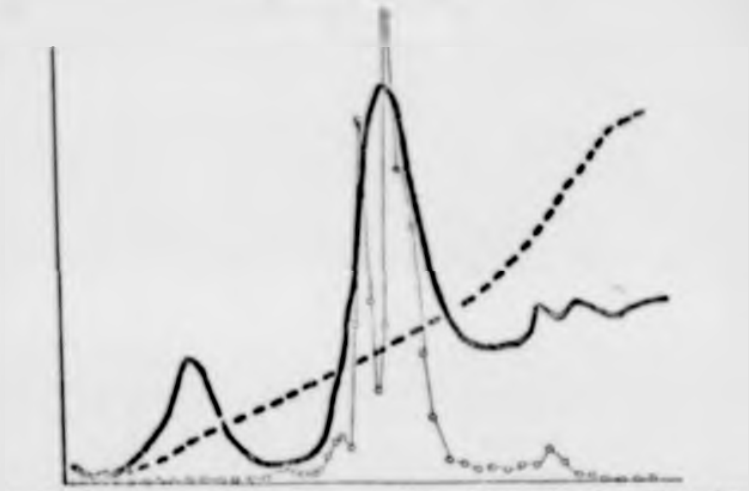

Fig. 8.2. Inverse isopycnic run of serum containing hepatitis B antigen. Heavy line represents the absorbances of the serum fractions; broken line represents the concentration of sucrose. Complement fixing activity for hepatitis B antigen is shown thus: -0-0-. (Zonal centrifugation was carried out by J. M. Leach, Esq.)

at a density of approximately 1.16 g cm^{-3}. All three morphological forms possess a similar buoyant density, but they are readily separated by rate zonal centrifugation (Figs 8.1–8.3), either in caesium chloride (Bond and Hall 1972) or in sucrose (Vyas et al. 1972). Early estimates place the sedimentation coefficient of the 20 nm particle as high as 110 S (Gerin et al. 1969), although a computer analysis of the separation obtained in a zonal rotor produced a lower estimate of 54 S (Gerin et al. 1971). More recent analyses with the model E ultracentrifuge suggest the value to be in the range of 30–40 S (Robins et al. 1972, Kim and Tillis 1973). Artifactually high values may have resulted from particle aggregation concomitant with a reduction in antigenic titre. The diffusion coefficient has been estimated by similar techniques to be in the range 2.278×10^{-7} cm^2 s^{-1} (Kim and Tillis 1973) to 4.3×10^{-7} cm^2 s^{-1}. Assuming a partial specific volume of 0.863 cm^3 g^{-1} this gives a molecular weight of approximately 2.5×10^6 for the 20 nm spherical particle. Similar estimates for homogeneous preparations of the tubular forms or the 42 nm particles are yet to be obtained.

Fig. 8.3. After separation of the serum shown in Fig. 8.1 by zonal centrifugation in 0–40% caesium chloride, the fraction showing the highest complement-fixing activity for hepatitis B antigen was found to consist of small spherical particles (20–24 nm in diameter). × 252,000.

Chemical properties

Although the lipid content of purified hepatitis B antigen may account for up to 30% of its total weight (Takahashi, T. personal communication) very few attempts have been made to determine the nature of the lipid components. Kim and Bissell (1971) found a 2:1 chloroform-methanol mixture to be an effective solvent of the lipid moiety. Some lipid is still retained by the antigen after treatment with ether (Kim and Tilles, 1973). Analysis of a chloroform-methanol extract by thin-layer chromatography in silica gel revealed a predominance of polar lipids together with cholesterol and small amounts of non-polar lipids. The major phospholipid components were phosphatidyl choline and sphingomyelin together with smaller quantities of phosphatidyl ethanolamine. Phosphatidyl serine was absent in the chromatogram suggesting that the low isoelectric point of the antigen is not the result of the carboxyl group associated with this lipid (Howard and Zuckerman 1973). Removal of the lipid leads to a decrease in immunoreactivity as assayed by immunodiffusion, although this may be indirectly the result of coagulation of protein rather than a loss of reactivity.

The use of zonal rotors in the ultracentrifuge has enabled the separation of sufficient quantities of antigen from normal serum proteins for analysis of the protein polypeptide composition of the antigen. Gerin (1972) was able to solubilize preparations of both *ad* and *ay* subtypes by treatment with sodium dodecylsulphate and mercaptoethanol. The released protein components were defined by sodium dodecylsulphate-acrylamide gel electrophoresis and the separated polypeptides were visualized by staining with Coomassie blue. Analysis of the 20 nm spherical form of antigen of subtype *ad* revealed two major polypeptides of 26,000 and 32,000 molecular weight in addition to three minor components in the molecular weight range 40,000–95,000. A similar profile was obtained with antigen subtype *ay*, although different relative densities of staining were produced. This may be due to a difference in affinities for the protein dye by the subtypes, or alternatively different ratios of certain polypeptides may indicate that not all of the polypeptides are virus specific. Several glycosylated polypeptides have been detected by Chairez et al. (1973). Periodate-Schiff staining of electrophoresed protein showed the presence of a major glycosylated polypeptide, corresponding to a molecular weight of 32,000, with two minor glycoproteins with a molecular weight of 27,000 and 22,000 respectively. The heat stability of the antigen and its resistance to proteases also suggests that the carbohydrate may be either an integral component which stabilizes

A wide range of molecular weights has been attributed to the isolated polypeptides obtained by solubilization of the antigen (Table 8.1) and this almost certainly reflects the use of differing preparative and disruptive techniques. In this context, it should be noted that some normal serum components can only be removed with difficulty, for example albumin and thyroxine (Neurath et al. 1974). Comparative electrophoresis of antigen iodinated by the lactoperoxidase and chloramine-T methods has revealed that at least 3 polypeptides may be integral components (Howard and Zuckerman 1974). Two of these polypeptide components can only be resolved with difficulty by electrophoresis but they were found to segregate, when subjected to isoelectric focusing, into two populations consisting of small 20 nm particles (Fig. 8.4). An acidic component (pI = 4.7) contained a slower-moving polypeptide with a molecular weight of 90,000, and the more basic component (pI = 4.9) contained a lower, 82,000, molecular

the antigen structure or that the carbohydrate acts as an antigenic determinant. Burrell et al. (1973) found demonstrable levels of carbohydrate by the phenol-sulphuric method in purified fractions of hepatitis B antigen. Periodate treatment subsequently reduced the antigenicity of the preparation, after 4 hours of incubation, by over 90%. Treatment of antigen-containing sera with 33% pyridine has also been found to reduce the antigen titre to a similar extent (Howard, C. R. and Zuckerman, A. J. unpublished observations), and the affinity of the antigen for concanavalin A has been utilized for its isolation (Neurath et al. 1973).

Table 8.1

Constituent polypeptides of hepatitis B antigen
Major components are in italics; those with asterisks are glycosylated.

	Mol. wt. × 10^{-3}								
Burrell et al.	120	54	*33*	*25*	20	14			
Chairez et al.	120	105	69	55	40	*32**	*27**	*22**	19
Drecsman et al.	*39*	*32*	*27*	22	16	10			
Gerlich & May	70	50	34	*28*	24				
Gerin	95	*75*	40	*32*	*26*				
Rao & Vyas	80	12	6						
Howard & Zuckerman	90	*82*	30						

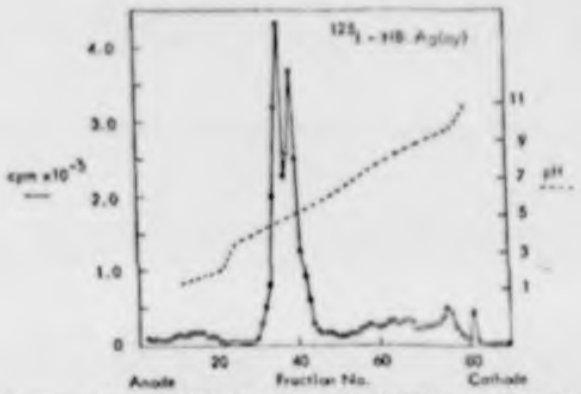

Fig. 8.4. Isoelectric focusing of HB.Ag radiolabelled with ^{125}I by the chloramine T method. Iodinated HB.Ag is recovered at pH 4.7 and pH 4.9 after focusing for 48 hours in a preformed sucrose gradient containing ampholytes in the pH 3–10 range.
(Reproduced by kind permission of the Editor of Intervirology)

weight polypeptide. Dreesman et al. (1972) reported a similar heterogeneity, but considered that there was an additional segregation yielding spherical particles with a diameter of 19 ± 2 nm and 25 ± 2 nm respectively. Recent studies from the same laboratory (Chairez et al. 1974) have shown a polypeptide heterogeneity among these diverse morphological forms.

Rao and Vyas (1973) recovered an antigenically active peptide with a molecular weight of 6,000 after sonication of hepatitis B antigen in the presence of 2-mercaptoethanol and urea. Amino acid analysis of this peptide after fractionation by high voltage electrophoresis revealed a high content of tryptophan. The presence of this amino acid, normally destroyed by acid hydrolysis, was previously indicated by the high extinction coefficient of purified antigen ($E_{1cm}^{1\%}$: 37.26) and the appearance of a shoulder on its absorbance spectrum at 290 nm (Vyas et al. 1972) (Fig. 8.5). This was further confirmed by spectrophotometric titration with the reagent N-bromosuccinimide. The absence of cysteic acid was somewhat surprising since analyses of intact particles showed high levels of cysteine (Dreesman et al. 1972, Vyas et al. 1972). However, the presence of disulphide linkages may be important for demonstrating at least one specificity. Suleno et al. (1972) found that hepatitis B antigen lost completely its serological activity after reduction and alkylation, although it still retained a capacity to induce

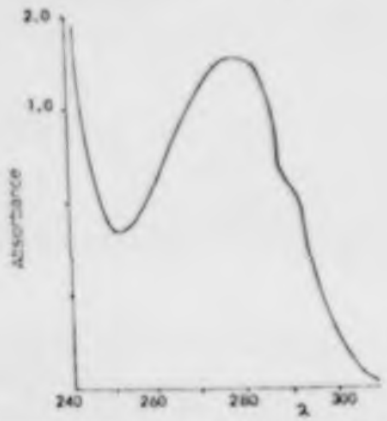

Fig. 8.5. Spectrophotometric analysis of purified HB$_s$Ag. The absorbance spectrum closely resembles that of protein with a maximum peak of absorbance at 280 nm. The ratio of absorbance at 260 nm :280 nm is indicative of the absence of nucleic acid. An additional shoulder of absorbance at 290 nm is suggestive of a high concentration of the amino acid tryptophan.

a cell-mediated response in guinea pigs (Vyas et al. 1972). It has been suggested that only the *a* determinant is sensitive to the reducing agent dithiothreitol, whereas the *d*, *y*, *w* and *r* subdeterminants are relatively resistant to such treatment (Imai et al. 1974). Yet, both the group-specific determinant *a* and the subdeterminants appear to be present on the same antigen particle.

It is clear that considerable effort is still required to correlate the presence of specific polypeptides with each antigenic specificity. Very little is known about the biological function of the components of the antigen. The amino-acid composition and acidic nature of hepatitis B antigen have a certain similarity to the non-basic regulatory proteins of the mammalian nucleus. Interestingly enough, a close involvement of the nuclei of cultured human embryo liver cells inoculated with sera containing hepatitis B antigen has been observed by immunofluorescence (Brighton et al. 1971). A further indication of the biological role of hepatitis B antigen may be inferred from preliminary experiments indicating that a kinase, capable of phosphorylating basic proteins in the presence of ATP, may be closely associated with the

antigen particles (Howard, C. R. and Zuckerman, A. J. unpublished observations).

Nucleic acid and the core

The examination of purified 20 nm spherical particles and the tubular forms of the antigen by ultraviolet absorption spectroscopy produces an absorption spectrum typical of protein, the ratio of absorption at 260 and 280 nm being approximately 0.7. It is unlikely, therefore, that any nucleic acid is present in these preparations in amounts greater than a few percent. As yet, a spectrum has not been reported for a preparation homogeneous for the 42 nm double-shelled Dane particles. Jozwiak and Koscielak (1972) reported the finding of up to 5% of RNA in antigen purified by affinity chromatography. This finding, however, still remains to be confirmed.

More progress has been achieved in the search for a possible virion-associated nucleic acid polymerase. Hirschman et al. (1971) first demonstrated a polymerase activity to be closely associated with hepatitis B antigen. Crude pellets of antigen obtained by high-speed centrifugation of sera from a few patients with clinical and histological evidence of hepatitis were found to stimulate the incorporation of d[^{3}H]TTP into an acid-insoluble product in the presence of all four deoxynucleoside triphosphates, although the level of incorporation was very low. This endogenous activity was abolished by pretreatment of the samples with RNAase and it was concluded that the template or primer was RNA. However, the reaction was stimulated by the addition of poly(dA-dT) and not by poly-(ra)-oligo dT, as are the known RNA-dependent DNA polymerases. The preparations were found to consist largely of the small 20 nm spherical particles, although tubular forms and the larger 42 nm spherical particles were present. Gerin (1972) examined similar preparations in an assay system which had previously detected a similar activity in a wide range of viruses and concluded that there was neither RNA- nor DNA-dependent DNA polymerase associated with the small 20 nm antigen particles. However, Gerin pointed out that the preparation procedure of Hirschman et al. would tend to concentrate the filamentous forms together with the larger 42 nm spherical particles.

Kaplan et al. (1973) demonstrated a DNA polymerase activity to be associated with the core component of the 42 nm Dane particle after removal of the outer or surface antigen coat by treatment with the non-ionic detergent Nonidet-P40 (Fig. 8.6). The enzyme appeared to function in the absence of any exogenous template, suggesting that there is an

endogenous template within the core. The incorporation of tritiated dTTP was dependent on the presence of all four deoxynucleoside triphosphates and magnesium ions. The reaction was inhibited in the presence of the intercalating agents actinomycin D and daunomycin but not by rifampicin. The enzyme product was found to be associated with material possessing a sedimentation coefficient of 110 S. This preparation was subsequently reduced to 15 S after incubation with sodium dodecylsulphate. The nature of the tritiated enzyme product was investigated after digestion with pronase. Approximately 20% of the acid-precipitable label was found in the aqueous phase after extraction of the digest with phenol, and found to possess a buoyant density typical of DNA, banding at 1.71 g/cm^3. Rate-zonal centrifugation confirmed the close association of the polymerase with a proportion of the 42 nm particles which migrated slightly ahead of the peak of the cores. Kaplan and his associates concluded that if the DNA polymerase is a virion enzyme and if the reaction is DNA dependent, then the enzyme would be unique because similar virion enzymes have not been described so far.

A major advance with this work has been the observation by electron microscopy of circular DNA molecules (Fig. 8.7) by shadow casting

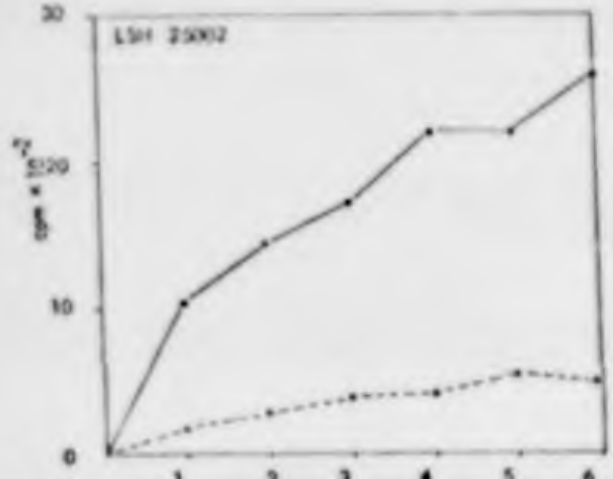
30
LSH 25302
20
10
0
1
2
3
4
5
6

Fig. 4.7. Electron micrograph of circular double-stranded DNA molecules separated by the aqueous technique. (Electron micrograph kindly provided by Dr W. S. Robinson, Stanford University School of Medicine and reproduced by permission of the Editor of *Journal of Virology*.)

(Robinson et al. 1974). The 42 nm hepatitis B antigen particles were concentrated by centrifugation followed by purification by repeated equilibrium centrifugation in sucrose density gradients. The preparations were exposed to DNAase in order to eliminate free DNA. After treatment with the detergent Nonidet-P40, the core pellets were disrupted with sodium dodecylsulphate to release any nucleic acid. Incubation with DNAase I before electron microscopy removed all of the circular molecules, whereas RNAase had little effect. The smooth, open configuration of the molecules suggested that they are double-stranded and additional molecules of single-stranded nucleic acid were not found after spreading in formamide. The circular structures were not supercoiled, possibly as a result of a nick in one of the two strands. Size-distribution analysis gave a mean length of the circular molecule of 0.78 μm, corresponding to an estimated molecular weight of the DNA of about 1.6×10^6. This is smaller than double-stranded DNA found in any known 'complete' virus and it is similar in molecular weight to adeno-associated virus. The thermal denaturation kinetics of the isolated DNA confirmed its double-stranded nature and gave a result consistent

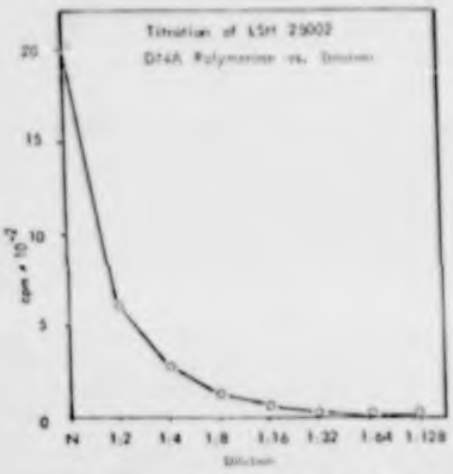

Fig. 8.8. Incorporation of [^{3}H]thymidine triphosphate after 4 hours of incubation in the presence of varying concentrations of pelleted HBAg. Two-fold dilutions of the pellet were made in phosphate-buffered saline and the presence of HB$_s$Ag monitored by a red cell agglutination test (end-point 1:512).

with a G:C content of 48–49%. This is a somewhat unexpected finding in view of the similarity to the base composition of mammalian DNA, and if this is representative of part of the whole of hepatitis B virus genome it is more consistent with those viruses possessing the ability to integrate their own genome into that of their host. It is also difficult to envisage that this molecule is capable of coding for more than 5 or 6 proteins and it would therefore almost certainly not contain sufficient information to satisfy the requirements for active virus replication and the apparent complexity of the surface determinants of hepatitis B antigen particles. It is also difficult to imagine the active association of the enzyme and template within the confines of the core of the 42 nm particle (reviewed by Zuckerman and Howard 1974). Titration of the polymerase activity reveals a rapid non-linear decline with dilution, which may indicate the segregation of template and enzyme (Fig. 8.8). There is the possibility of their segregation as components of different morphological forms, in a manner perhaps similar to the Fraenkel-Conrat covirus model for some plant viruses which require

[illegible] particles. This concept, which was extrapolated by Zuckerman (1970) to human hepatitis type B, cannot yet be ruled out. There is also the possibility that the circular DNA is derived from a defective virus, and infectious particles with larger DNA [illegible] which may not yet have been detected, might be present. Alternatively, a helper virus might be required for replication (Kennea, Finch, and, although a second or helper virus has not yet been identified, such models for hepatitis have been proposed (Zuckerman et al 1967).

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HEPATITIS-B ANTIGEN AND TOBACCO PLANTS

SIR,—Considerable interest has been generated by the reported finding of Banatvala and Payne [1] that certain sera containing hepatitis-B antigen (HBAg) may induce necrosis in the leaves of tobacco plants (*Nicotiana sylvestris*). In particular, the effect was readily seen using sera from 13 of 14 patients in the acute phase of hepatitis-B infection, although the rate of success was somewhat less when sera from apparently healthy carriers of HBAg were used. Beasley and Su [2] tried without success to repeat these results, but suggested their failure may be related to the HBAg subtype, since the overwhelming majority of HBAg carriers in Taiwan are of the *ad* subtype, although this was confirmed in only 1 of the 29 HBAg-positive sera used. We now report a lack of success in inducing either necrosis or stunting in tobacco plants in our laboratories, and we suggest this to be unrelated to the subtype of HBAg employed as the inoculum. The effect of purified HBAg was tested as well as sera obtained from patients with acute hepatitis.

Seeds of tobacco plants (*Nicotiana tabacum* L. cv. Xanthi, *N. tabacum* L. cv. White Burley, and *N. sylvestris*) were germinated and grown in a sterilised soil substitute ("Vermipeat") under glasshouse conditions. Briefly, there were an ambient temperature of 25–30°C, a relative humidity of 50–60%, and supplementary lighting (Philips MBTL fluorescent lamps) to provide a 15-hour-light, 9-hour-dark cycle. Plants grown in this manner have a characteristic "soft" appearance and are highly susceptible to infection by plant viruses. When the largest leaf was between 1·7 and 3·0 cm. long (approximately 5–7 weeks after sowing), the inocula were carefully spread over the two largest leaves by means of a glass rod whilst supporting the underside of the leaf with a petri-dish or microscope slide; this technique is well established as a reproducible means of obtaining infection with plant viruses. Plants grown and inoculated in this way were found to be readily infected with a local lesion-forming strain of tobacco (aucuba) mosaic virus. After inoculation plants were kept either under glasshouse conditions or on a laboratory bench for at least 2 weeks. Each serum was confirmed as positive for HBAg by at least two serological tests, usually reverse passive haemagglutination and sandwich-type radioimmunoassay, and inoculated into from two to ten plants. The antigenic subtype of each was identified by radioimmunoassay using monospecific radiolabelled guinea-pig antibody to *ad* and *ay* subtypes respectively. Inocula containing purified HBAg in phosphate-buffered saline supplemented with 0·5% bovine serum albumin were prepared by polyethylene glycol precipitation and followed by two cycles of equilibrium centrifugation. Likely additives to sera were assessed for their potential effect on tobacco plants. Our results are as follows:

Inoculum	*No. tested*	*Subtype*	*Effect on N. sylvestris*
Sera from patients with acute hepatitis	5	*ad*	none
	2	*ay*	none
Sera from apparently healthy carriers	10	*ad*	none
	14	*ay*	none
	3	N.T.	none
Purified HBAg (1 mg./ml.)	—	—	none *
Tobacco (aucuba) mosaic virus	—	—	Localised necrotic lesions
Sodium azide (1 mg./ml.)	—	—	none
Heparin (0·2 mg./ml.)	—	—	none
Sodium citrate (3 mg./ml.)	—	—	Slight bronzing of inoculated leaf

* Tested on *N. tabacum* vars. White Burley and Xanthi.
N.T.—not tested.

We have no explanation of our failure to produce any effect on inoculation with any of the above sera. In our work it soon became clear that young plants inoculated mechanically can be readily damaged in an irreversible manner resulting in large areas of collapse which may be mistaken for necrosis, and could further lead to a retardation of growth. The failure to produce any effect with purified HBAg at high concentration makes it unlikely that any toxic effect is a direct result of introducing HBAg into plant tissues.

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TRANSMISSION OF HEPATITIS B TO THE RHESUS MONKEY

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ABSTRACT

The rhesus monkey does provide a suitable laboratory animal model for studying hepatitis B virus and for investigating various aspects of interaction between this infectious agent and the host. This species of non-human primate, however, offers a less sensitive animal model for hepatitis B infection than the chimpanzee.

There have been many attempts to transmit hepatitis B virus to non-human primates and these studies have yielded, until recently, equivocal or negative results. The finding of hepatitis B surface antigen and antibody in the serum of a small proportion of chimpanzees, orangutans and gibbons renewed interest in the possibility that these primates might serve as a suitable experimental model for hepatitis B.

Hepatitis B surface antigen and surface antibody have been detected in 6-12% of captive chimpanzees when tested by relatively insensitive techniques. Most of the animals appear to be healthy carriers of the antigen. Hepatitis B surface antibody was found in a significant number of captive non-human primates when sensitive techniques such as passive hemagglutination and radioimmunoassay had been used. Antibody was detected in the chimpanzee, orangutan, gibbon, baboon, Celebes ape, patas monkey, vervet, several species of macaque, mangabey and langur and in a number of species of New World monkey. Antibody was found in about 90% of chimpanzees examined but in less than 10% of most Old World and New World monkeys (5, 7). It appears that much of the difficulty which had been experienced in the past was due to the unknown susceptibility of these animals before experimentation and the relatively mild nature of the infection.

Recent studies have shown that although hepatitis B in chimpanzees is consistently mild, as shown by modest serum transaminase enzyme elevations without jaundice or overt signs of illness, the serological responses are identical to those seen in man and further that susceptible chimpanzees are almost as

sensitive to infection with hepatitis B virus as man (1). However, the practical obstacles to the use of chimpanzees for studying infection with hepatitis B virus include the strictly limited availability and expense of these non-human primates.

Transmission of hepatitis B to the rhesus monkey

London *et al.* (1972) reported the successful serial transmission in rhesus monkeys (*Macaca mulata*) of an infectious agent, which stimulated an antibody response to hepatitis B surface antigen. The infection was inapparent and it was not associated with biochemical evidence of liver damage nor with histological changes in the liver. Hepatitis B surface antigen was demonstrated in low titre and transiently in some of the sera by solid-phase radioimmunoassay. The antibody responses measured by radioimmunoprecipitation and passive hemagglutination were similar in pattern to those observed in man following either natural or artificial infection with hepatitis B virus. Successful transmission was achieved through five serial passages in rhesus monkeys. Although the rhesus monkey is less susceptible to human hepatitis B virus, it does provide a more accessible animal species for the experimental study of this infection.

Zuckerman (1972) postulated that the high prevalence of hepatitis B antigen in many tropical countries may be related to changes in the immunological response associated with a background of repeated parasitic infection and in particular to the immunosuppressive effect of malaria. An extension of this hypothesis was the induction of chronic infection with *Plasmodium inuei* in the rhesus monkey in an attempt to enhance the susceptibility of this species of non-human primate to hepatitis B virus.

Malaria and hepatitis B infection in the rhesus monkey

Sera from young rhesus monkeys were screened for hepatitis B surface antigen and hepatitis B surface antibody by solid-phase radioimmunoassay before they were admitted to the study. The animals were divided into small groups consisting of uninoculated controls, those infected chronically with *Plasmodium inuei*, a group inoculated intravenously with 0.25 ml of a well-characterized serum known to have induced clinical post-transfusion hepatitis in man and containing hepatitis B surface antigen subtype *adw*, and a small number of animals inoculated with the same hepatitis B infective serum after chronic infection with malaria was fully established.

Serum samples were tested for hepatitis B surface antigen and antibody by solid-phase radioimmunoassay. Results for antigen were considered positive only after neutralization with specific human antibody. Liver biopsies were examined histologically and sections were also stained for cytoplasmic hepatitis B surface antigen after Shikata *et al.* (1974). Finally, cellular hypersensitivity was examined by the mixed leucocyte-macrophage migration test (3) whole blood lymphocyte transformation and by intradermal tests and skin biopsy. The results of the studies of cell-mediated immunity will be the subject of another report.

Hepatitis B surface antigen was detected in the serum of four out of six rhesus monkeys previously infected with malaria. The antigen was demonstrated in one animal on the day of inoculation, presumably due to the antigen present

Fig. 1. HB$_s$Ag and anti-HB$_s$ response in rhesus monkeys infected with malaria and hepatitis B.

Hepatitis B surface antigen was not found by radioimmunoassay in any of four animals inoculated with hepatitis B only. An antibody response was elicited in only one of these rhesus monkeys, 54 days after infection, and the antibody has persisted for some months.

Serum containing hepatitis B antigen was collected from two of the successfully infected rhesus monkeys and 1ml was administered intravenously to three animals previously infected chronically with *Plasmodium inui*. Hepatitis B surface antigen was detected in the serum of one of these animals 49 days after inoculation, but an antibody response has not been observed. Further passages are in progress.

Infection with hepatitis B in these rhesus monkeys was subclinical, specific histological changes in the liver were not seen and the surface antigen was not detected in the liver by staining with orcein or with aldehyde fuchsin.

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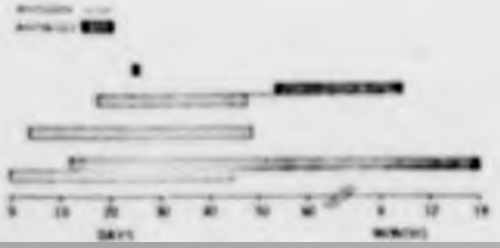
in the original inoculum, and the antigen was subsequently found repeatedly in the serum of this subject for 45 days. Surface antibody was first detected 12 days after inoculation and it has persisted in the serum so far for 18 months. The presence of circulating hepatitis B antigen-antibody complexes was confirmed by electron microscopy. In another monkey the antigen was first detected 18 days after inoculation and it persisted for 30 days. An antibody response followed 54 days after inoculation. In the third animal the antigen was first found 4 days after inoculation and it remained in the circulation for 45 days without a detectable antibody response. Antigen was detected for one day only 25 days after inoculation in another animal, again without an antibody response (Figure 1). There was no detectable antigen or antibody in the remaining two monkeys in this group.



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THE NATURE OF HEPATITIS-B VIRUS

SIR,—We have read with great interest Professor Hirschman's hypothesis (Sept. 6, p. 436) on the possible mode of replication of hepatitis-B virus (H.B.V.). However, a few further points should be considered. Firstly, several workers have measured the circular D.N.A. molecule in the electron microscope after its extraction from the circulating Dane particle.[1-3] The finding of a considerable variation in observed length is similar to the variable genome size previously reported for the SV40 virus serially passaged at high multiplicity of infection, suggesting that H.B.V. is defective in its mode of replication. Secondly, it is unlikely that the low molecular weight of the extracted D.N.A. would contain sufficient information to code for a specific D.N.A.-polymerase activity in addition to the antigenically complex HB surface antigen. This raises the interesting possibility of the HBcAg-associated enzyme being acquired from the infected host cell. As was pointed out by Professor Hirschman, this may be added to the maturing HBcAg particle in the host-cell cytoplasm: a host enzyme possessing a D.N.A.-polymerase activity in addition to a requirement for a circular D.N.A. molecule is present within the mitochondria. Both the mitochondrial enzyme and that associated with HBcAg are stimulated in the presence of salt, are functional in the presence of sulphydryl reagents, and are thought to have an endonuclease activity. A common sensitivity to the mutagenic dye ethidium bromide seems to distinguish both of these activities from the larger cytoplasmic D.N.A. polymerases. This hypothesis may be taken further and the *e* antigen postulated as representing this host enzyme. Dilution experiments of polymerase-containing HBAg preparations in our laboratory have shown a multicomponent effect to be operating, which may be explained by the presence of a bound polymerase within the circulating D.N.A. particle in close association with its template, and a similar activity free in the serum that has access to the same template. The *e* antigen has been suggested to exist both free[3] in the serum and bound to the Dane particle.[4] The proposed size of the *e* antigen is also consistent with his hypothesis. The appearance of antibodies to this antigen may then be explained as an autoimmune response to an antigen normally sequestered within the liver-cell mitochondria but released in large quantities in H.B.V. infection as a result of extensive mitochondrial degradation. The apparent lack of antibodies to the cellular organelle in HBsAg-positive cases of acute[5] and chronic[6] hepatitis is consistent with this view. Some experimental data demonstrate that naturally occurring and carbon-tetrachloride-induced heat-labile and heat-stable autoantibodies are directed towards unique autoantigenic determinants on rat-liver inner and outer mitochondrial antibodies.[7] Studies with viruses are yet to be carried out.

Finally, it should be emphasised that there is as yet no convincing evidence that the Dane particle is indeed the infectious virion. It must therefore remain a "candidate H.B.V." until more conclusive experimental data become available.

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Electrofocusing of Hepatitis B Antigen

(*Accepted* 3 *May* 1973)

SUMMARY

Hepatitis B (Australia) antigen was isolated from normal serum proteins by gel filtration and electrofocusing. The latter technique revealed a heterogeneity of the small spherical particles associated with hepatitis B antigen activity, there being a variance in determined isoelectric points according to the serological subtype studied. Hepatitis B antigen isolated in this manner was aggregated by concanavalin A.

Early studies by staining and flotation experiments disclosed the lipoprotein nature of the hepatitis B (Australia) antigen which is closely associated with the human hepatitis type B virus (serum hepatitis virus). Several techniques for the isolation of hepatitis B antigen from normal serum proteins by equilibrium and rate zonal sedimentation have been described (Gerin *et al.* 1969; Vyas *et al.* 1972), but these methods are time-consuming and there is evidence to suggest the isolated hepatitis B antigen is still closely associated with normal human serum proteins (Millman *et al.* 1970). A procedure is described which offers the possibility of isolating hepatitis B antigen by a simple two-step procedure which negates the need for equilibrium and rate-zonal sedimentation and reveals a heterogeneity of the predominant morphological form as viewed in the electron microscope.

Isoelectric focusing may be used for analytical or preparative separation from heterogeneous mixtures of individual ampholytes, particularly proteins, and the technique has recently been extended to the study of the protein components of viruses (Shortridge & Biddle, 1970; Hung, Robinson & Robinson, 1971). The technique offers almost complete recovery of total protein after separation. Prior concentration of the sample is rarely necessary.

The plasma samples used for electrofocusing were obtained from a number of apparently healthy carriers of hepatitis B antigen, one of whom had recently been implicated in two deaths from post-transfusion hepatitis in transfused recipients. The identity of the antigen in each sample was confirmed by several tests including immunodiffusion, complement fixation and radioimmunoassay. The predominant morphological type was the small spherical particle 20 to 25 nm diam., as revealed by immune electron microscopy (Memorandum, 1970). Plasma was clarified by preliminary centrifuging at 15000*g* for 30 min at 4 °C and the supernatant fluid was subjected to gel filtration in order to remove the major portion of the normal plasma proteins. Samples of 7·5 ml were applied to a 100 × 5 cm diam. Sephadex G 200 column and the hepatitis B antigen eluted in the void vol. with 0·05 M-tris-hydrochloric acid buffer at pH 7·4. Total protein content of the void vol. was found to be 5% of that in the applied sample as estimated by the method of Lowry *et al.* (1951). There was little or no loss of complement fixation titre using the above conditions.

Samples of the partially purified hepatitis B antigen containing not more than 10 mg of protein were used in the formation of a 0 to 40% (w/v) sucrose gradient. A 40% (w/v) solution of a mixture of carrier ampholytes (Ampholine) was added to the gradient at a final concentration of 1% (w/v). The anode was protected with a 1·4% solution of orthophosphoric acid in 60% (w/v) sucrose and the cathode with a 2% solution of ethanolamine in water. In some experiments, the final concentration of carrier ampholytes was increased

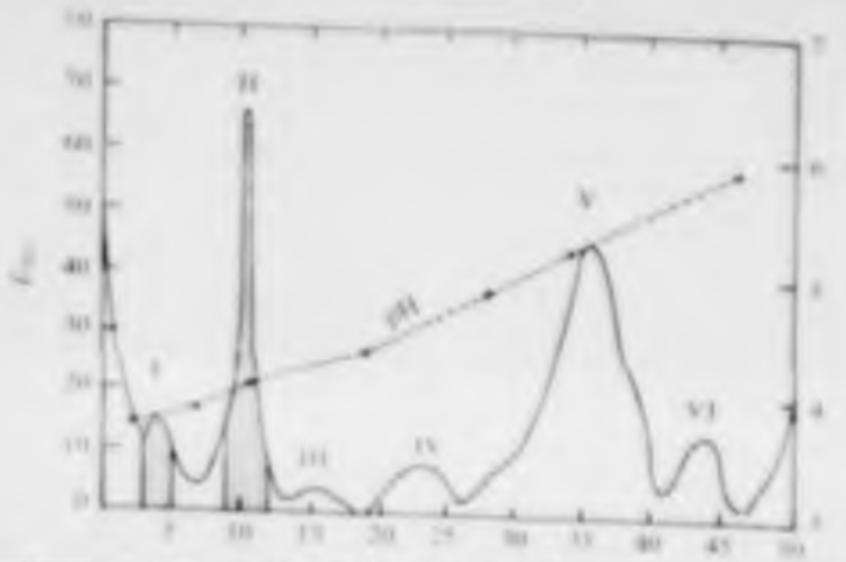
II
III
IV
V
VI

Fig. 1. Isoelectrofocusing of hepatitis B antigen. Fractionation profile obtained 48 h after applying partially purified hepatitis B antigen to a preformed pH 3–10 (w/v) sucrose gradient containing carrier ampholytes. The shaded areas under peaks I and II were found to contain antigen.

to 2% (w/v) in order to enhance the solubility of fixused proteins, or the nonionic detergent Brij 35 added to a final concentration of 0·1%. The pH gradient was established in an electric field maintained at an output of 3 W during the initial 24 h and the sample was fractionated in the gradient after a further period of 10 h.

The elution profile of fixused protein from a typical experiment is shown in Fig. 1. The areas under peaks I and II were found to be positive for hepatitis B antigen by immunodiffusion, counter-immunoelectrophoresis (Memorandum, 1970), latex particle agglutination and solid-phase radioimmunoassay. In addition, the hepatitis B antigen was detected by complement fixation after exhaustive dialysis of separated proteins against phosphate-buffered saline, pH 7·2, and when examined by immune electron microscopy. Normal serum proteins were not detected in either peak of hepatitis B antigen by immunodiffusion against horse whole human protein antiserum. The protein in both peaks was aggregated upon addition of concanavalin A (330 μg/mg separated protein) at room temperature in phosphate-buffered saline. The effect was reversed upon addition of α-methyl-D-mannoside (0·1 mol).

The appearance of the separated hepatitis B antigen particles by immune electron microscopy is shown in the plate. There is thus at least one antigenic determinant in common with both peaks of antigenic activity. There is no significant difference in diam., although the particles isolated in peak I (Fig. 2b) appear to be not as clearly defined when compared to peak II (Fig. 2a). There has recently been some suggestion that the small spherical particles may be either 20 nm or 25 nm in diam. with a corresponding difference in determined mol. wt. (Dreesman *et al.* 1972). The isoelectric points of isolated hepatitis B antigen were determined as 3·65 and 4·33 for peaks I and II, respectively, for the material initially characterized as being of the "ad" subtype. One sample of the "ay" subtype was available for study, the values being determined as 3·95 and 4·90. This difference almost certainly reflects the antigenic composition of the particle surface. The low values of determined isoelectric points could be due also to the presence of phosphatidyl serine in the lipid component of the antigen, but lipid analyses of hepatitis B antigen purified by sedimentation precludes this possibility (Kim & Bissell, 1971; T. Takahashi, personal communication). Interestingly

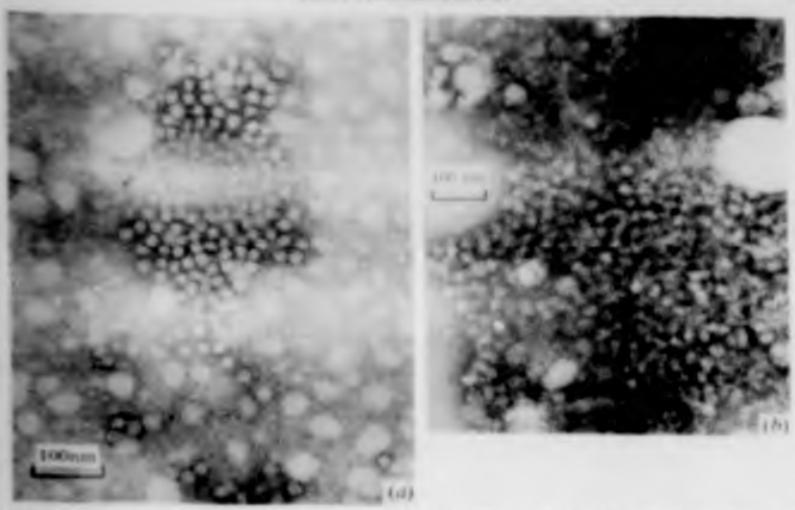

Fig. 2. Electron microscopic appearance of hepatitis B antigen isolated in (*a*) peak II and (*b*) peak I.

enough, similar values of determined isoelectric points have recently been obtained for Qβ phage and several plant viruses (Rice & Horst, 1972) as well as for Rous sarcoma virus (Hung *et al.* 1971).

The extinction in peak I increased at the expense of peak II after prolonged storage of partially purified material. This may be due to an ageing effect on the apoprotein. Also of interest is the observation that purified hepatitis B antigen in both peaks is aggregated by concanavalin A. Carbohydrate has been reported to be present (Bond, 1972) and this may contribute to the antigenic composition of the particle surface (Burrell *et al.* 1973), as well as to the overall ionic properties of the antigen.

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Characterization of Hepatitis B Antigen Polypeptides

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Key Words. Hepatitis B antigen · Polypeptides, hepatitis B · Antigenic variation, hepatitis B

Summary. The close association of hepatitis B antigen with serum proteins was demonstrated. The small morphological form of the antigen, measuring approximately 20 nm in diameter, was separated by isoelectric focusing and the heterogeneity of the antigen was confirmed by the finding of unique polypeptides in each preparation. Comparative trace-labeling techniques showed the integral nature of the constituent polypeptides, two of which were found to be mutually exclusive in the separated particles. Release of these polypeptides was achieved by treatment with Nonidet P40 and 2-mercaptoethanol. The significance of these findings in relation to hepatitis B virus is discussed together with the importance of surface antigenic variation as a function of protein heterogeneity of this virus.

The close association between hepatitis B antigen and human hepatitis B virus has now been firmly established [1]. Early studies employing staining and flotation experiments revealed hepatitis B antigen to be a lipoprotein of density 1.21 g/cm³ (CsCl), which was immunologically distinct from normal low density serum lipoproteins [2]. The lipid component plays no apparent role in its antigenicity [3]. Examination of serum containing this antigen in the electron microscope by negative staining revealed remarkable morphological heterogeneity of virus-like particles. The principal antigenic constituent is a small pleomorphic spherical particle, measuring approximately 20 nm. The presence of tubular forms, with a constant diameter of 20 nm and often several hundred nanometers in length, is a characteristic feature [4]. The third type of particle is also spheroidal, measuring about 42 nm in diameter, with an

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inner core of 27 nm in diameter, a 2-nm shell and an outer coat about 7 nm in thickness [5]. The antigenic reactivities of the core have not yet been studied in detail, but it has been shown that antibody to the core has an entirely different specificity from antibody to the outer (hepatitis B antigen) coat [6–8]. There is some evidence now which is consistent with the view that the 42-nm particle is the human hepatitis B virus, the core being the virion nucleocapsid and hepatitis B antigen the outer protein coat [9].

Various workers have analyzed the protein moiety of hepatitis B antigen, but there has been no close agreement as to the number or size of the component polypeptides isolated [10–13]. The existence of at least two high molecular weight components is now reported. These two polypeptides appear to be responsible for a surface heterogeneity of the 20-nm hepatitis B antigen particle. The significance of the isolated subunits is discussed.

Materials and Methods

Hepatitis B antigen. Serum was obtained from the routine screening of blood donors for hepatitis B antigen by discontinuous counter-immunoelectrophoresis and radioimmunoassay. The principal antigen subspecificities, **d** and **y**, were determined (phenotypes **ad** + **y** − and **ad** − **y** +) by solid-phase radioimmunoassay using monospecific guinea pig antisera. Specific hepatitis B antibody was not found by either of these methods in any of the samples. All the sera examined contained hepatitis B antigen consisting almost entirely of the small 20- to 22-nm spherical form. Sera were stored at − 20° in 100-ml volumes until required.

Serological methods. Four techniques, counter-immunoelectrophoresis, latex particle agglutination, radioimmunoassay, and reverse passive hemagglutination [14], were employed for the detection of hepatitis B antigen in the original samples and in the various fractions. Counter-immunoelectrophoresis is a widely used, relatively simple and specific method. A discontinuous buffer system increases the sensitivity and ease of reading of the precipitin lines. Latex particles coated with hepatitis B antibody prepared in guinea pigs provided a rapid and simple screening procedure for each fraction. This technique is slightly more sensitive than complement fixation. The solid-phase radioimmunoassay system employed ^{125}I-labelled guinea pig hepatitis B antibody, and results were confirmed as positive only if neutralization tests with human hepatitis B antibody showed specific blocking. Reverse passive hemagglutination, using erythrocytes from various species coated with the 7S fraction of human or animal hepatitis B antibody, proved to be a sensitive method for the quantitative estimation of the antigen. Tests on comparative sensitivity indicated a much greater sensitivity than complement fixation and passive hemagglutination inhibition.

Separation of hepatitis B antigen from serum proteins. Hepatitis B antigen was initially precipitated by the addition of a 30% w/v polyethylene glycol 6000 (Koch-Light Ltd., Colnbrook, England) stock solution in distilled water to serum at room temperature to give a final concentration of 5%. After gentle mixing for 15 min, the precipitate was collected by centrifugation at 500 *g* for 10 min at 4°. The precipitate was redissolved in 0.05 M Tris-HCl buffer (pH 7.2) to one fifth of the original volume. This step precipitated over 94% of

the antigen present in the original serum, as quantitatively assessed by discontinuous counter-immunoelectrophoresis and reverse passive hemagglutination.

Aliquots of the redissolved precipitate were then layered on 20-ml volumes of cesium chloride at an initial density of 1.20 g/cm^3 and buffered to pH 7.5 with 0.05 M Tris-HCl. The antigen was separated from polyethylene glycol by centrifuging at 100,000 *g* for 18 h at 4° in an SW30 MSE swing-out rotor. The gradients were collected from the top in 1-ml volumes and the position of hepatitis B antigen detected by at least two serological tests. Fractions containing antigenic activity were pooled, concentrated in a Minicon B15 ultrafiltration unit (Amicon Ltd.) and rebanded in cesium chloride for at least a further 18 h. Fractions containing the antigen were again pooled, concentrated, and exhaustively dialyzed against 0.05 M phosphate buffer, pH 7.5 (for iodination), or 0.01 M phosphate-buffered saline, pH 7.2, before storage.

Trace-labelling techniques. (a) Iodination by the chloramine-T method was accomplished by a modification of the method of HUNTER and GREENWOOD [15]. The purified hepatitis B antigen was quantitated by optical density measurement, assuming an $E^{1\%}_{280\,nm}$ of 37.5 [16]. The concentration was adjusted to 1 mg/ml by dilution with 0.05 M phosphate buffer. The reaction mixture containing 1 mg of protein consisted of 1 mCi of ^{125}I-Na (spec. act. 100 mCi per ml; Radiochemical Centre, Amersham) in 10 µl and 100 µg of chloramine-T in 100 µl of phosphate buffer, added dropwise through a No. 25 gauge syringe needle. After 90 sec at room temperature, the reaction was terminated by the addition of 100 µg of sodium metabisulfite. Iodinated protein was separated from unbound iodine by immediate gel filtration through a 25 × 0.9 cm column of Sephadex G-25 equilibrated with 0.1 M phosphate-buffered saline (PBS). The iodinated antigen was eluted with PBS in the void volume. A repeat of this step resulted in a final preparation of which at least 97% of ^{125}I was precipitable by trichloroacetic acid. The specific activity of the final preparation was never less than 15 µCi/mg. Iodinated antigen was stored after the addition of solid bovine serum albumin at a final concentration of 0.5% and passage through a 0.25-µm (Millipore) filter.

(b) Iodination of surface protein was carried out using the method of STANLEY and HASLAM [17]. The antigen preparation was standardized as in (a) above, and the reaction mixture consisted of 100 µg of lactoperoxidase (Sigma Chemical Co.) in 100 µl, 1 mCi of ^{125}I-Na in 10 µl, and 100 µl of hydrogen peroxide previously diluted 1 : 3 in 0.05 M phosphate buffer. The reaction was allowed to continue for 15 min at room temperature before terminating the reaction by the addition of 160 µg of cysteine-hydrochloride. Free iodine was removed as before. The specific activity of preparations iodinated by this method was about 30% less than iodination by the chloramine-T method.

(c) Fixation of protein with ^{14}C-formaldehyde: Radioactive labelling of proteins was achieved by the attachment of ^{14}C-methyl radicals to amino groups by the reductive alkylation technique of RICE and MEANS [18]. Briefly, 100 µl of PBS (pH 9.0), containing 0.1 mg of purified antigen, was mixed rapidly at room temperature with 10 µl of 0.04 M ^{14}C-formaldehyde (spec. act. 10 mCi/mmole; Radiochemical Centre, Amersham). After 30 sec and after 1 min, 10 µl of 0.132 M sodium borohydride was added to ensure complete reduction of the formaldehyde. Low molecular weight components of the reaction mixture were then removed by chromatography as for the iodination procedures outlined above.

Gel electrophoresis. Constituent polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were prepared to the required final concentration by dilution of a 28% w/v acrylamide solution containing 1.4% methylene bisacrylamide as cross-linking reagent. The gels were buffered with 0.375 M Tris-HCl buffer

(pH 8.9) and contained 0.5 M urea and 0.1% SDS. Polymerization was brought about in 5-mm diameter tubes by the addition of N,N,N,N¹-tetramethylethylenediamine and ammonium persulfate at final concentrations of 0.03 and 0.075%, respectively. The gels were allowed to polymerize for at least 3 h before polymerizing ions were removed by a pre-run with 0.375 M Tris-HCl buffer containing urea and SDS at the same concentrations [19]. The gels were removed from the tubes and soaked overnight in the same solution containing in addition 0.1% dithiothreitol. This step was found to be essential for optimum resolution. Immediately before electrophoresis, the gels were returned to fresh running tubes and trimmed to the required length.

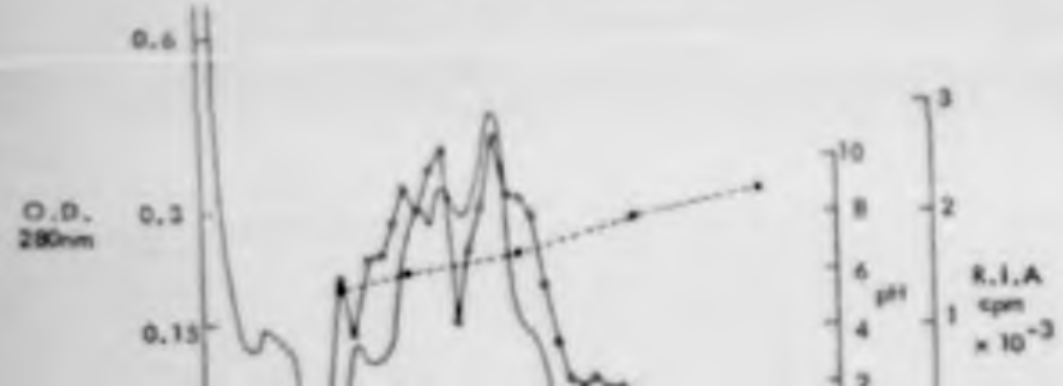
Samples for analysis were disrupted in 1% SDS, 0.5 M urea and 0.1% dithiothreitol at 80° for 15 min. A one-fifth volume of 0.47 M Tris-phosphate buffer (pH 6.7) was added and the density increased by adding a two-fifths volume of 80% w/v sucrose. Samples containing approximately 250 μg of protein were electrophoresed at 2.5 mA/gel in 0.005 M Tris–0.038 M glycine, pH 8.6, containing 0.1% SDS, 0.05 M urea and 0.02% dithiothreitol. Phenol red was added to the samples as a tracking dye.

After electrophoresis, gels were extruded into 5% trichloroacetic acid, and fixation was allowed overnight. Bands were detected either by staining with 0.5% Coomassie Brilliant Blue or by cutting the gels into 1-mm slices for trace-label detection. Iodinated polypeptides were detected by placing slices directly into a Gamma-Guard well-type scintillation counter. Slices containing ^{14}C-polypeptides were dried at 50° overnight in scintillation vials before the addition of 0.1 ml hyamine hydroxide in methanol and 15 ml of PPO/POPOP scintillant (5 and 0.5 g/liter, respectively). Vials were heated for 10 min in a 50° water-bath before counting at ambient temperature in a Corumatic 200 scintillation counter. Stained gels were scanned at 620 nm in a Joyce-Loebl Chromoscan 200 densitometer.

Isoelectric focusing. Separation of protein in sucrose gradients was carried out in a column designed by VESTERBERG and SVENSSON [20] using the method of HOWARD and ZUCKERMAN [21], the gradients being collected by downward displacement with water. The final concentration of carrier ampholytes was 1%. Fractions containing radioiodine were counted directly in a well-type scintillation counter. Disruptive isoelectric focusing was carried out by replacing the sucrose gradient with a linear gradient of urea containing 0.1% mercaptoethanol and 0.1% Nonidet P40 [22]. The concentration of carrier ampholytes remained at 1%. Samples subjected to isoelectric focusing were previously disrupted by heating for 2 h at 37° in 1% Nonidet P40, 1% mercaptoethanol and 4 M urea.

Results

Isoelectric focusing of serum containing hepatitis B antigen. The close association of hepatitis B antigen with serum proteins is demonstrated by isoelectric focusing of 0.1 ml of serum in a preformed sucrose gradient (fig. 1). The presence of the antigen was detected after fractionation by solid-phase radioimmunoassay. An approximate linear relationship has been reported to exist over the range of results obtained [23]. Antigenic activity was found to correspond closely to the isolation of the major serum protein components


0.6
O.D.
280nm
0.2
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10
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6
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1
R.I.A
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x 10⁻³

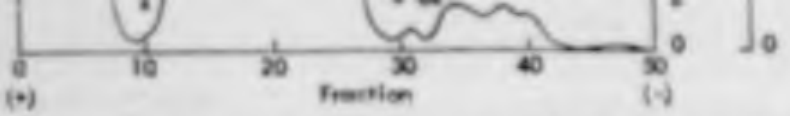

Fig. 1. Isoelectric focusing of serum containing hepatitis B antigen in a 0–60% w/v sucrose gradient containing carrier ampholytes (Ampholine) to establish a pH 3–10 gradient at a final concentration of 1%. After 72 h, fractionation of the separated proteins was followed by radioimmunoassay of each fraction. Only results positive for the antigen are shown. pH readings (dashed line with points); radioimmunoassay readings, cpm × 10^{-3} (solid line with points); optical density (solid line without points).

over the pH range 4.0–7.0. Antigenic activity was not detected in those fractions containing separated γ-globulins.

Polypeptide composition of separated hepatitis B antigen. Hepatitis B antigen iodinated by both chloramine-T and lactoperoxidase methods was analyzed for trace-labelled polypeptides by electrophoresis in parallel in 10% SDS-acrylamide gels. The profiles obtained are illustrated in figure 2, and bear a close resemblance to gels stained for protein with Coomassie Brilliant Blue (fig. 3). As a control for the iodination procedure affecting the overall polypeptide composition, a further series of gels contained samples labelled with ^{14}C (fig. 4). The average molecular weight of the isolated polypeptides was estimated from a series of experiments according to the method of SUMMERS and MAIZEL [24], and these results are summarized in figure 5. Comparison of the electrophoretic profiles reveals at least two polypeptides, of molecular weights 82,000 and 90,000, to be major internal components of the small 20-nm particles.

Isoelectric focusing of isolated hepatitis B antigen. Partially purified hepatitis B antigen, separated from serum proteins by gel filtration, may be resolved into at least two populations of intact morphological forms, according to surface charge [21].

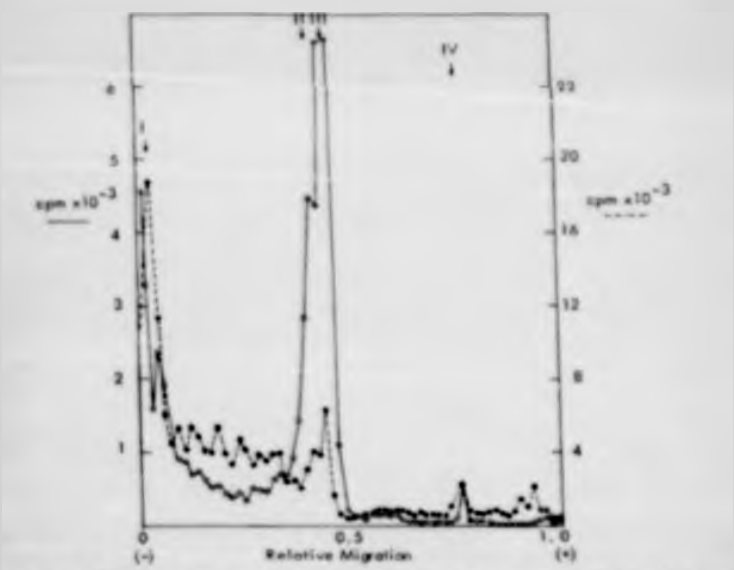

Fig 2. SDS-acrylamide gel electrophoresis of purified hepatitis B antigen (subtype a+d−y+) iodinated by the chloramine-T (——) or the lactoperoxidase (– – –) methods. Peaks II, III and IV are believed to be integral protein components of the small 20-nm diameter particles

Hepatitis B antigen separated as described above and trace-labelled by the chloramine-T method resolves the two peaks over a higher pH range (fig 6). SDS-acrylamide analysis of these two peaks (fig 7) illustrates that the two major polypeptides are segregated into each population as demonstrated by isoelectric focusing. The more acidic component (pI = 4.7) having the bulk of radioiodine in a slower moving 90,000-molecular-weight polypeptide and the more basic (pI = 4.9) containing the lower 82,000-molecular-weight component. Both contain a 30,000-molecular-weight component, as indicated by iodination.

Partial disruption of hepatitis B antigen. Separation of the protein moiety of the antigen was attempted by treatment with the nonionic detergent

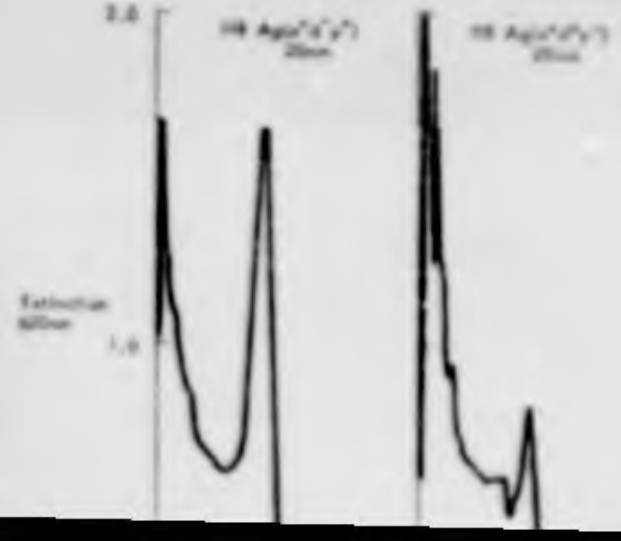
2.0
1.0

Fig. 3. SDS-acrylamide gel electrophoresis of purified hepatitis B antigen (a) subtype a + d − y + and (b) subtype a + d + y −. Both preparations consisted almost entirely of the 20-nm diameter particle. Separated polypeptides were detected by staining in Coomassie Brilliant Blue and destained in 7% acetic acid.

Nonidet P40 and 2-mercaptoethanol followed by isoelectric focusing in a urea gradient. The radiolabel was found to focus in a band with a shoulder towards the anode (fig. 8). The increase in isoelectric point may reflect the release of non-protein components responsible for the rather low value obtained with intact antigen, e.g., carbohydrate.

Discussion

The mobility of hepatitis B antigen by immunoelectrophoresis in agar gel was found to follow closely that of α_2-globulin [25]. Kim and Tillis [26] reported that purified antigen obtained from the serum of individual patients with acute hepatitis B infection migrated in an electrophoretic field either in the α_2-globulin region or in the β-globulin region with some trailing, confirming

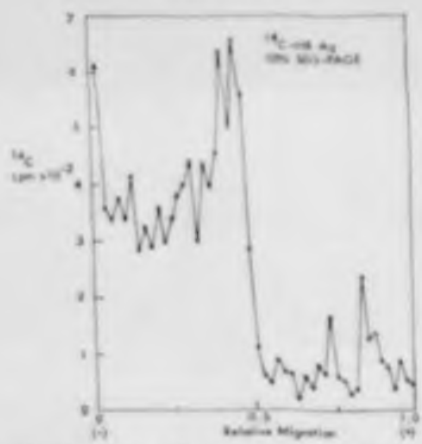

Fig. 4. SDS-acrylamide gel electrophoresis of purified hepatitis B antigen as obtained after trace-labelling with ^{14}C-formaldehyde. Both major polypeptide peaks are seen to be preserved after an independent method of trace-labelling.

their earlier findings of electrophoretic heterogeneity of serum samples obtained from such patients. JEANNEL *et al.* [27] examined an antigen-positive serum from a patient suffering from post-transfusion hepatitis by isoelectric focusing in large pore polyacrylamide gel slabs. The antigen was detected by immunoelectrophoresis in slices of gel in the pH range 4.0–5.0, although the staining of separated proteins suggested focusing of the antigen over a broader pH range. Antigenic activity was not correlated with the morphology of the particles, with a particular antigenic subtype specificity, or with chemical heterogeneity.

The close association of hepatitis B antigen with normal serum components, confirmed in our laboratory by radioimmunoassay of fractionated material, has been an acknowledged difficulty in developing purification techniques for separation of the antigen from serum prior to biochemical and serological characterization. The association with other proteins in unfractionated serum was confirmed by isoelectric focusing in a sucrose gradient. The antigen carrying the subdeterminants ad - y and morphologically consisting almost entirely of the small 20- to 22-nm particles was found to be

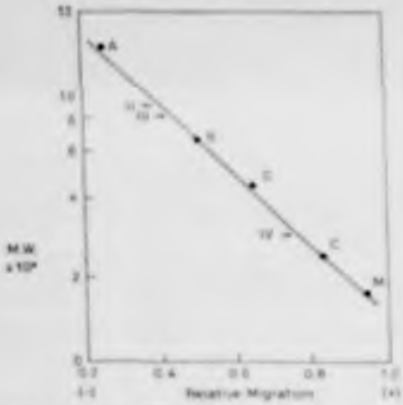

Fig. 5. The molecular weights ($\times 10^5$) were obtained for polypeptides II, III, and IV by comparison in a series of experiments with the relative migration of the following: B, bovine serum albumin; O, ovalbumin; C, chymotrypsinogen A; and M, myoglobin. Polypeptides were estimated as being 90,000 (II), 82,000 (III) and 30,000 (IV) in molecular weight.

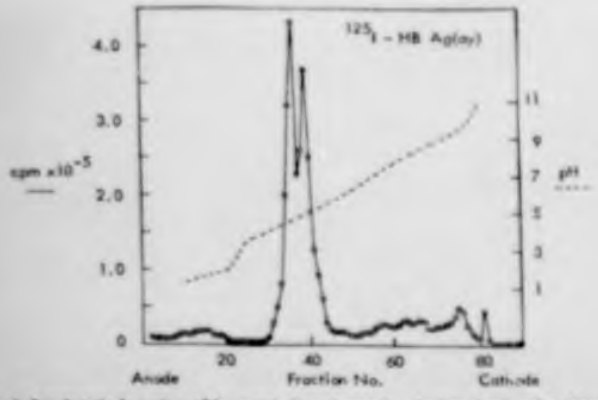

Fig. 6. Isoelectric focusing of hepatitis B antigen after iodination by the chloramine-T method. A clear heterogeneity of antigen is demonstrated at the isoelectric points of 4.7 and 4.9 in a pH 3–10 gradient.

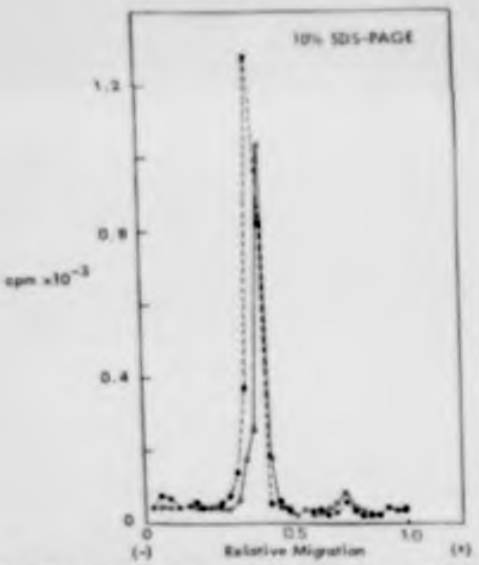

Fig. 7. SDS-acrylamide gel electrophoresis of separated bands of iodinated hepatitis B antigen. Antigen of pI 4.7 (——) contains a polypeptide of molecular weight 90,000, while antigen of pI 4.9 (——) possesses the slightly smaller major polypeptide component of molecular weight 82,000. Both populations of particles contain the smaller 30,000-molecular-weight component, as revealed by iodination.

associated with serum proteins over a pH range of 4.0–7.0 (fig. 1). Of interest is the observation that antigenic activity was not detected in association with separated γ-globulins, which is in accord with the long epidemiological and clinical experience that γ-globulin is free of the risk of transmitting hepatitis and with the failure to detect hepatitis B antigen, a marker associated with infectivity, by electron microscopy after Cohn fractionation of human plasma known to contain the antigen [28]. We have previously shown that if the major portion of serum proteins was removed by gel filtration, the technique of electrofocusing separates the antigen from the remaining unwanted serum proteins and reveals a heterogeneity in the surface properties of the small antigen particles [21]. The isoelectric points of the two isolated

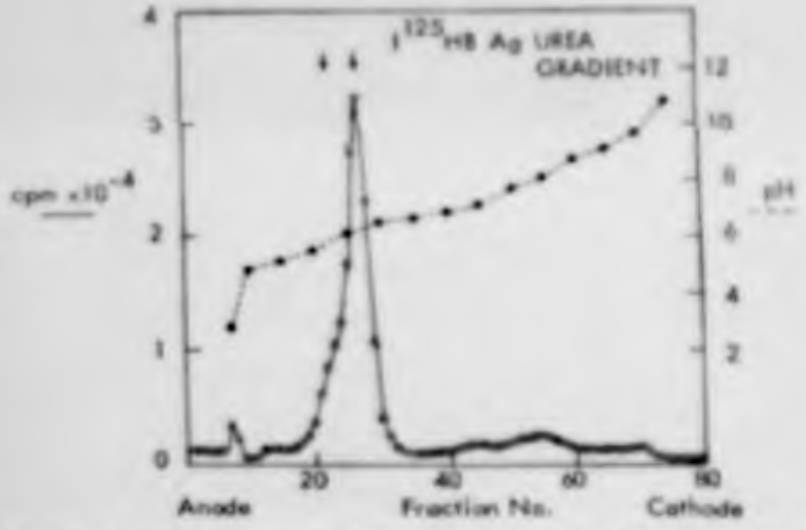

Fig. 8. Isoelectric focusing of iodinated hepatitis B antigen after treatment with Nonidet P40 and 2-mercaptoethanol. The gradient was composed of freshly prepared 4–8 M urea, and carrier ampholytes pH 3–10 were added to a final concentration of 1%. The shoulder on the main peak of activity was separable with the use of a carrier ampholyte over a narrower pH range.

bands were found to differ according to the nature of the sub-determinants and to share at least one common antigenic determinant as demonstrated by immune electron microscopy [22]. In a further series of experiments, purified hepatitis B antigen was iodinated with the radioisotope ^{125}I after separation from serum proteins by polyethylene glycol precipitation and equilibrium centrifugation in cæsium chloride. Figure 6 illustrates iodinated antigen to focus at slightly higher isoelectric points. The two heterogeneous bands were analyzed for their constituent polypeptides by PAGE (fig. 7). Each band was found to contain one of the major iodinated polypeptides, together with the smaller, 30,000-molecular-weight polypeptide. Both of these major peaks of activity are integral components of the structure as revealed by comparison with similarly prepared antigen iodinated by the lactoperoxidase method (fig. 2). With a molecular weight of 87,000, lactoperoxidase specifically labels those components whose tyrosyl residues lie on or close below the surface of the antigen particle [17]. Both of the major iodinated polypeptides were isolated independently by the introduction of ^{14}C into the antigen in a parallel experiment. We also found that antigen trace-labelled with ^{14}C behaved in isoelectric focusing experiments in a similar way to iodinated hepatitis B antigen. Removal of the lipid component by prior incubation of the sample

Table I. [illegible]

[illegible]	[illegible]								
[illegible] et al. [10]	100	54	*33*	*25*	20	14			
[illegible] et al. [11]	120	105	*69*	55	40	*32*[b]	*27*[b]	*22*[b]	[illegible]
[illegible] et al. [11]	*39*	*32*	*27*	*22*	16	10			
[illegible] and [illegible] [12]	70	50	34	*28*	24				
[illegible] [10]	95	75	40	*32*	*26*				
[illegible] and [illegible] [13]	80	12	6						
This study	90	*82*	30						

[illegible]
[b] [illegible]

in the nonionic detergent Nonidet P40 and mercaptoethanol before isoelectric focusing in a urea gradient released at least two components from the antigen. Each component possesses a higher unique isoelectric point, compared to the intact original particles (fig. 8). The increase in isoelectric point was paralleled by a significant increase in the sedimentation coefficient of the radiolabel, presumably as a result of a loss of the lipid moiety and the alteration of the secondary structure of the separated protein.

There is at present some disagreement on the estimated number and concomitant molecular weights of isolated polypeptides reported by various workers (table I). The conditions of electrophoresis gave reproducible results only when the separating gels were exhaustively soaked in dithiothreitol. Coupled with prior separation by the isoelectric focusing, the procedure outlined allows fine resolution of constituent polypeptides. Several estimates contain at least one polypeptide in the molecular weight range of 80,000 to 100,000. A slower migrating component may be resolved into at least two polypeptides after trace-labelling, each being partly responsible for an apparent surface heterogeneity of the small 20-nm particles. Iodination of the antigen in our experiments followed closely the analysis obtained by staining separated polypeptides. The results obtained with iodinated antigen are applicable to the treatment of unlabelled material used for preparative work.

Although we consider that the proteins constituting a large part of the outer coat antigen are virus-coded, it is likely that some host lipoproteins, including various pre-existing structures of the liver cells, are incorporated into the protein coat of hepatitis B virus. Alternatively, surface antigenic variation may be a function of protein heterogeneity of the virus. Subdetermi-

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3 Kim, C.Y. and Bissell, D.M.: Stability of the lipid and protein of hepatitis-associated (Australia) antigen. J. infect. Dis. *123:* 470–476 (1971).

4 Zuckerman, A.J.: Viral hepatitis and the Australia-SH antigen. Nature, Lond. *223:* 569–572 (1969).

5 Dane, D.S.; Cameron, C.H., and Briggs, M.: Virus-like particles in serum of patients with Australia antigen-associated hepatitis. Lancet *i:* 695–698 (1970).

6 Almeida, J.D.; Rubenstein, D., and Stott, E.J.: New antigen-antibody system in Australia-antigen positive hepatitis. Lancet *ii:* 1225–1227 (1971).

7 Barker, L.F.; Chisari, F.V.; McGrath, P.P.; Dalgard, D.W.; Kirschstein, R.L.; Almeida, J.D.; Edgington, T.S.; Sharp, D.G., and Peterson, M.R.: Transmission of type B viral hepatitis to chimpanzees. J. infect. Dis. *127:* 648–662 (1973).

8 Hoofnagle, J.H.; Gerety, R.J., and Barker, L.F.: Antibody to hepatitis B virus core in man. Lancet *ii:* 869–873 (1973).

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nants **d** and **y** of the antigen are specified by the viral genotype and not by the host, and it is likely that subdeterminants **w** and **r** are similarly specified. These two pairs of subdeterminants, **d**:**y** and **w**:**r**, are mutually exclusive. Recently, however, a new variant of hepatitis B antigen carrying both the **d** and **y** subdeterminants was found [29]. The emergence of distinct new genotypes of hepatitis B virus would be reflected by the production of different structural antigenic sites, which would also account for the heterogeneity of the protein components of the antigen complex. Such events could be of great epidemiological and practical importance.

Acknowledgements

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mate d and y of the antigen are specified by the viral genotype and not by the host, and it is likely that subdeterminants a and y are similarly specified. These two pairs of subdeterminants, d, y and a, y, are mutually exclusive. Recently, however, a new variant of hepatitis B antigen carrying both the d and y subdeterminants was found [39]. The emergence of distinct new genotypes of hepatitis B virus would be reflected by the production of different structural antigenic sites, which would also account for the heterogeneity of the primary specificity of the antigen complex. Such events could be of great epidemiological and practical importance.

Acknowledgements

This investigation was supported by grants from the Medical Research Council, the World Health Organization and the Wellcome Trust. We are also indebted to Mrs. M. Barnes for technical assistance.

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3 Kim, C.Y. and Bissell, D.M.: Stability of the lipid and protein of hepatitis-associated (Australia) antigen. J. infect. Dis. *123:* 470–476 (1971).
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TOWARD HEPATITIS B VACCINES

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TOWARD HEPATITIS B VACCINES*

A. J. Zuckerman, M.D., D.Sc., and C. R. Howard, M.Sc.

A [illegible] hepatitis B [illegible] by [illegible] Tabor, and Hollinger[1] and Krugman and Ward,[2] using as the immunogen a known infective human serum (MS-1 serum) that contains hepatitis B virus and was diluted 1 in 10 and heated at 60° C for 10 minutes. The serum treated in this manner was non-infective and successfully protected or modified hepatitis B illness in [illegible] of 19 children who were challenged after four to eight months with the untreated ineffective MS-1 serum. The illness in the remaining nine children was generally mild; jaundice occurred in only a volunteer. Abnormal levels of serum glutamic oxaloacetic transaminase were noted in 12 [illegible] of 19 children. Hepatitis B antigen was detected in the serum of 11 children and the antigen persisted in three of these children, giving a chronic carrier rate of about 16%, whereas in a control group of 24 nonimmunized children a chronic carrier rate of [illegible]% was observed after exposure to MS-1 serum. Hepatitis B antibody, assayed by sensitive techniques, was induced in [illegible] of 19 children but it was transient in six of the children and, further, one of the children with [illegible]-induced antibody became a persistent carrier of the antigen after challenge. The results with the heat inactivated serum were essentially the same after one, two, or three inoculations.

Diluted and heat-treated [illegible] serum may be regarded as an inactivated vaccine, but it is a crude way of inducing immunity and it is improbable that such material will be licensed for general use.

The repeated failure to passage hepatitis B virus serially in tissue or organ cultures[3] has hampered progress toward the development of a safe

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and effective vaccine. Attention has therefore been directed more recently toward the use of other preparations for active immunization against hepatitis B.

Hepatitis B Virus

The close association between hepatitis B antigen and human hepatitis B virus is now firmly established.[5] The description by Dane, Cameron, and Briggs[6] of distinct virus-like double-shelled 42 nm. spheroidal particles in the serum of some patients with acute illness associated with hepatitis B antigen was followed by the finding by immune electron microscopy of a second antigen-antibody system in this infection.[7] After detergent treatment of pellets of antigen obtained by ultracentrifugation of whole serum, the 42 nm. Dane particles separated into an outer coat which possessed hepatitis B antigen activity and an inner component or core, 27 nm. in diameter, resembling morphologically the enteroviruses. Antibody in convalescent hepatitis B serum reacted with the core to yield immune aggregates resembling those previously seen in homogenates of the liver obtained at autopsy from patients with hepatitis B. The core antibody was found to have an entirely different specificity from antibody to the outer (hepatitis B surface antigen) coat. Hoofnagle, Gerety, and Barker[8] demonstrated that all patients who had infections associated with hepatitis B surface-antigen developed complement-fixing antibody to the core. The titre of the core antibody fell to low levels after recovery from natural hepatitis B infection, but in chronic carriers titres of core antibody remained high. These and other observations suggested that core antibodies are produced in response to replication of the virus. Kaplan and his co-workers[9] demonstrated DNA-dependent DNA polymerase activity in association with the Dane particles and evidence was provided suggesting that the polymerase activity was associated with the cores, released spontaneously or by detergent treatment. The data are consistent, therefore, with the view that the Dane particle is the human hepatitis B virus, the core being the virion nucleocapsid and hepatitis B antigen the outer protein coat.

Krugman and his co-workers[10] recently examined serial serum samples for hepatitis B-specific DNA polymerase and for core antibody. These sera were obtained from volunteers exposed to the MS-2 strain of hepatitis B virus and to heated-inactivated MS-2 serum. Hepatitis B surface antigen appeared first in the serum of infected volunteers, fol-

lowed by DNA polymerase activity then core antibody. In rare or at the time of elevation of serum transaminases DNA polymerase activity persisted for days or weeks in acute cases and for months or years in chronic carriers, while core antibody persisted in all cases. Hepatitis B surface antigen, DNA polymerase, and core antibody were not found in persons inoculated with heat inactivated MS-2 serum. It was concluded that DNA polymerase activity identified the period of peak replication of hepatitis B virus and that the core antibody reflects recent or continuing replication of the virus.

Hoofnagle[7] reported that titres of core antibody in three patients were not raised by re-exposure to hepatitis B antigen-positive serum. These and other findings suggest that core antibodies are produced in response to replication of the virus in the liver, these antibodies were not raised by re-exposure to serum containing hepatitis B antigen and, unlike antibody to the surface antigen core, antibodies did not correlate with resistance to reinfection nor did they signal recovery from infection.

Subunit Hepatitis B Vaccine

Isolated viral coat protein challenges the body's immune mechanism in the same way as the whole infectious agent and the possibility of using purified hepatitis B surface antigen particles, which are free of nucleic acid and therefore of infectivity, appears attractive. However, such an approach may be precluded by the amounts of host protein that may form complexes with the viral protein in quantities which appear to be far in excess of the protein coat of most recognized viruses. These host proteins may include various pre-existing structures of the liver cell and may thus induce undesirable immunological reactions.[3] Subunits of the antigen, in the form of small polypeptides, on the other hand, offer a much greater promise as possible immunogens.

The close association of hepatitis B surface antigen with normal serum components, confirmed in our laboratories by radioimmunoassay of fractionated material, has been an acknowledged difficulty in the development of purification techniques for separation of the antigen from serum prior to biochemical and serological characterization. This association with other proteins in unfractionated serum was confirmed by nucleotrix focusing in a sucrose gradient. The antigen carrying the subdeterminants ad / ay and morphologically consisting almost entirely of

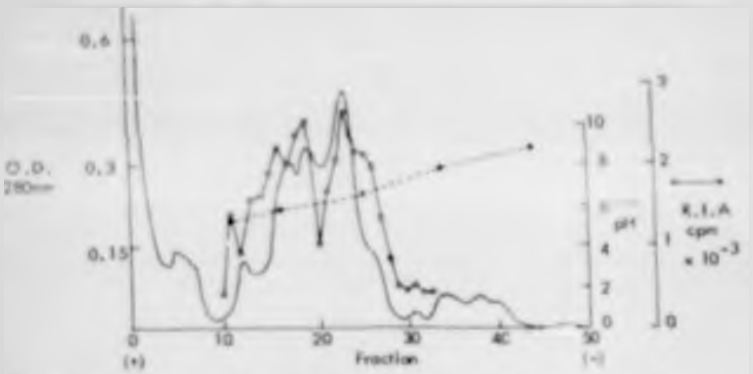


Fig. 1. Isoelectric focusing of serum containing hepatitis B antigen in a 0.66% sucrose gradient containing carrier ampholytes (Ampholine) to establish a pH 3-10 gradient at a final concentration of 1%. After 72 hours, fractionation of the separated proteins was followed by radioimmunoassay of each fraction. Only positive results for the hepatitis B surface antigen are shown.

the small 20 nm particles was found to be associated with serum proteins over a pH range of 4.0 to [illegible] (Figure 1). Howard and Zuckerman[7] have previously shown that if the major portion of serum proteins was removed by gel filtration, the technique of electrofocusing separates the antigen from the remaining unwanted serum protein and reveals a heterogeneity in the surface properties of the small antigen particles. The isoelectric points of the two isolated bands were found to differ according to the nature of the subdeterminants and to share at least one common antigenic determinant as demonstrated by immune electron microscopy.[8]

In a further series of experiments, purified hepatitis B antigen was iodinated with the radioisotope iodine-125 after separation from serum proteins by polyethylene glycol precipitation and equilibrium centrifugation in caesium chloride. The iodinated antigen was found to focus at slightly higher isoelectric points. The two heterogeneous bands were analysed for their constituent polypeptides by polyacrylamide gel electrophoresis (Figure 2). The peptides obtained were found to bear a close resemblance to gels stained for protein with Coomassie Brilliant

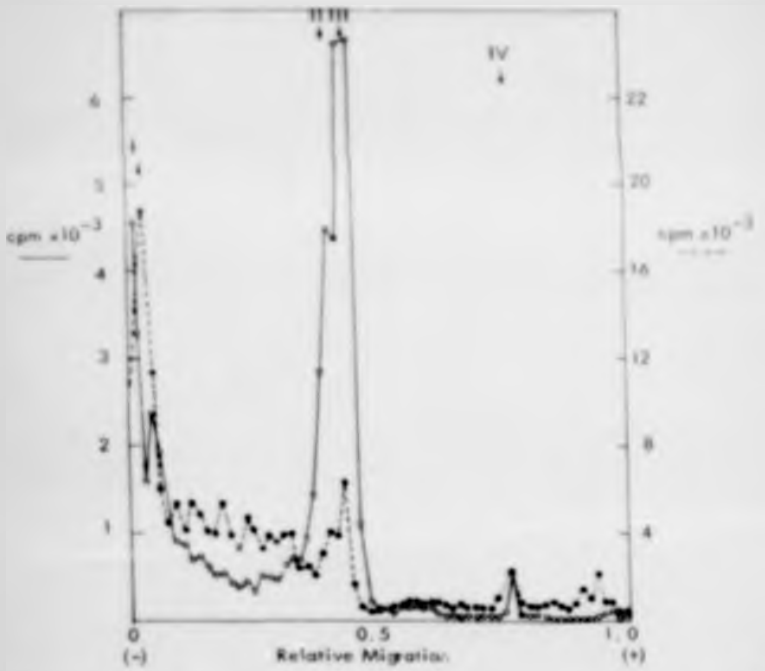


Fig. 2. SDS-acrylamide gel electrophoresis of separated bands of solubilized hepatitis B surface antigen. Antigen of pI 4.7 contains a polypeptide of molecular weight 99,000, whilst antigen of pI 4.9 possesses the slightly smaller major polypeptide component of molecular weight 82,000. Both populations of particles contain the smaller 20,000 molecular weight component, as shown by subunits.

Blue. The average molecular weight of the isolated polypeptides was estimated from a series of experiments according to the method of Summers and Maizel[11] and shown in Figure 3. Each band was found to contain one of the major solubilized polypeptides, together with a smaller polypeptide which had a molecular weight of 20,000. Both of these major peaks of activity are integral components of the antigen as shown by comparison with standard purified antigen sedimented by the Tacon peroxidase method (Figure 4). With a molecular weight of 82,000,

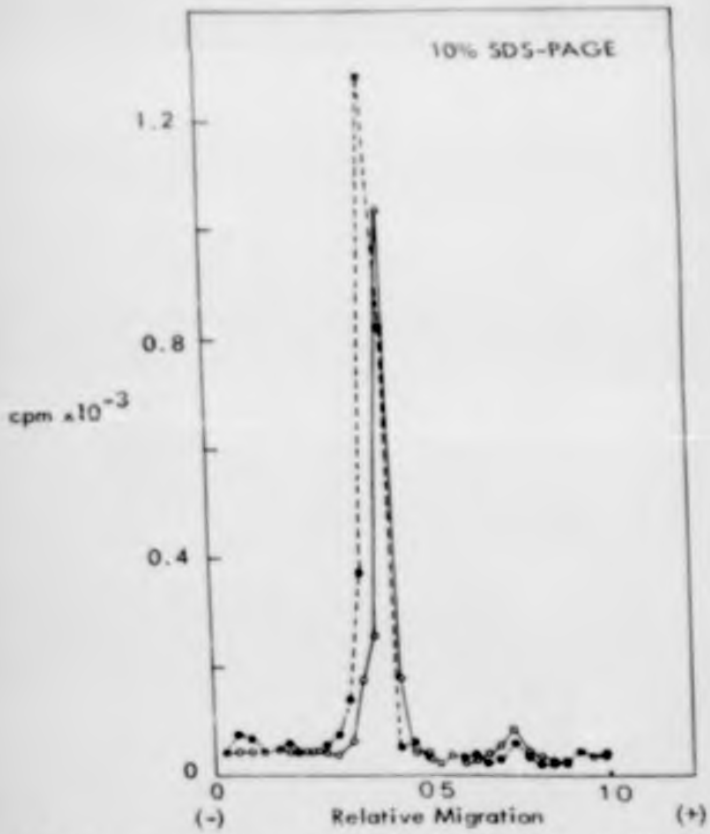


Fig. 3. The molecular weights (x10³) were obtained for polypeptides II, III, and IV by comparison in a series of experiments with the relative migration of the following: B, bovine serum albumin; O, ovalbumin; C, chymotrypsinogen A; and M, myoglobin. Polypeptides were estimated as being 80,000 (II), 85,000 (III), and 50,000 (IV) in molecular weight.

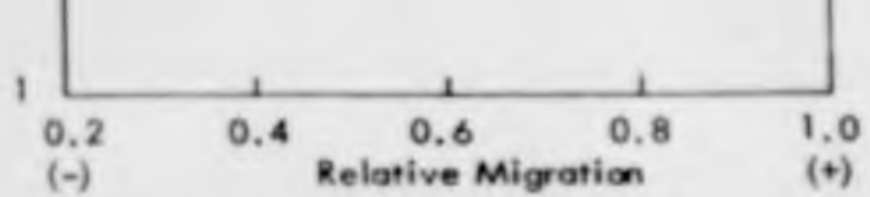
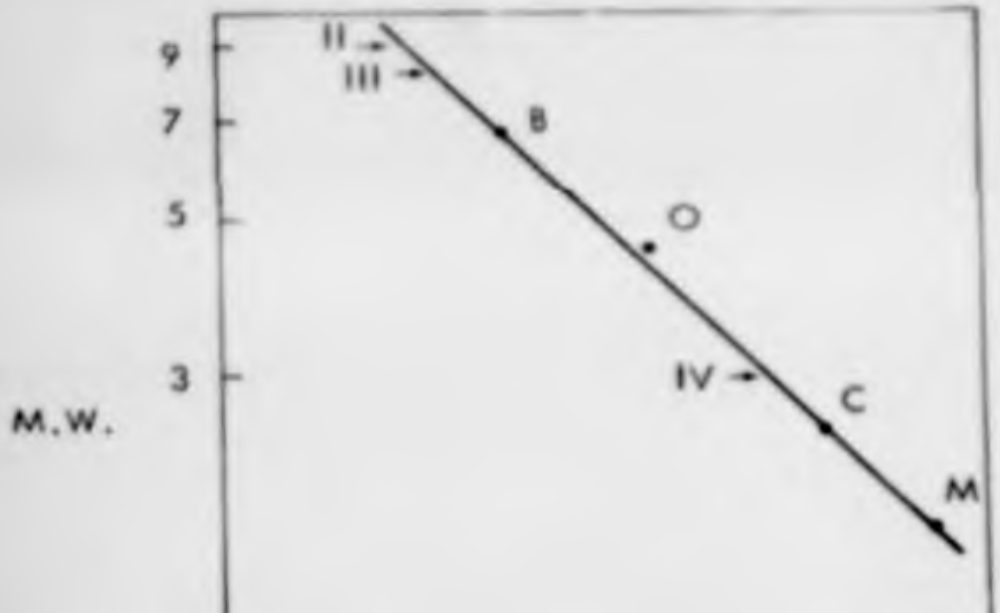


Fig. 4. SDS-acrylamide gel electrophoresis of purified hepatitis B surface antigen (Subtype ay) iodinated by the chloramine T (solid line) or the lactoperoxidase (dashed line) methods. Peaks II, III, and IV are believed to be integral protein components of the small 20 nm. diameter particles.

lactoperoxidase specifically labels those components whose tyrosyl residues lie on or close below the surface of the antigen particle.[15] Both of the major iodinated polypeptides were isolated independently by the introduction of ^{14}C into the antigen in a parallel experiment. It was found that antigen trace-labeled with ^{14}C behaved in isoelectric-focusing experiments in a similar way to iodinated hepatitis B antigen. Removal of the lipid component by prior incubation of the sample in the nonionic detergent Nonidet P_{40} and mercaptoethanol before isoelectric focusing in a urea gradient released the two major components from the antigen. Each component possesses a higher unique isoelectric point, compared to the intact original particles. The increase in isoelectric point was paralleled by a significant increase in the sedimentation coefficient of the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radiolabel, presumably as a result of loss of the lipid moiety and the alteration of the secondary structure of the separated polypeptides.

There is disagreement on the estimated number and concentration molecular weights of isolated polypeptides reported by various workers.[8-11] Prior separation by isoelectric focusing and the procedures for electrophoresis used in our laboratory permitted the resolution of the constituent polypeptides of hepatitis B antigen. Several estimates contain at least one polypeptide in the molecular weight range of between 30 and 40. A slower migrating component may be resolved into at least two polypeptides after trace labeling, each being partly responsible for an apparent surface heterogeneity of the small to mid particles of the antigen. Inhibition of the antigen in our experiments followed closely the analysis obtained by staining separated polypeptides. The results obtained with the radiolabeled antigen can be applied to the treatment of antibodied material used for preparative work.

Such polypeptide preparations should be investigated as potential vaccines for hepatitis B by defining the immunogenic moiety by studies in appropriately selected nonhuman primates such as chimpanzees.

Synthetic Hepatitis B Vaccine

An immunochemical study of purified hepatitis B antigen is essential for the understanding of this unique antigen. Analogous to the FMDVP decapeptide, the primary sequence of the hepatitis peptide of hepatitis B antigen may provide another approach for a development of a synthetic peptide, which, when coupled to a macromolecular carrier, could serve as a suitable immunogen. Once detailed data are available on the protein, peptide, and amino acid composition of this antigen, it should be possible to define by careful minimization the moiety responsible for the antigenic activity.

There are reports in the literature to support the feasibility of such an approach. For example, in 1966 Stewart[7] defined the antigenic moiety of FMV protein decapeptide. Arnon[7] showed that it was possible to use a synthetic macromolecule for eliciting antibodies reacting exclusively with a specific region of a native egg white lysozyme. This was achieved by synthesizing a particular segment of the enzyme from its amino-acid components, attaching the peptide to a synthetic polypeptide carrier, and using the conjugate for immunization. The resulting antibodies reacted with native lysozyme in a unique, conformation-

dependent antigenic determinant. Of course, in the case of lysozyme both the amino-acid sequence and the three-dimensional structure are known, and the synthesized peptide was designed on the basis of previous information concerning its contribution to the antigenic specificity of the molecule.

A similar approach might be attempted for type B hepatitis depending largely on the success in elucidating the structure of hepatitis B antigen, since there is little doubt that polypeptides and other moieties such as specific lipoproteins can be attached to a macromolecular carrier[22] for subsequent immunization. The prospects of active immunization against hepatitis B appear brighter.

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