HEPATITIS B ANTIGEN

AND

BLOOD TRANSFUSION

by

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Thesis presented for degree of Doctor of Philosophy of the University of Edinburgh in the Faculty of Medicine.

June, 1977.
Hepatitis B antigen in serum, showing characteristic small (22nm) spheres, rod like forms, and large spheres (42nm Dane Particles) with central cores. Magnification = 160,000
Declaration

The work reported here was designed and conducted by myself, with the following exceptions:

1. Production of spherocytes and preparation of reagents in bulk (Part Two, Chapter two and four). In this aspect I was ably assisted by Mr A.D. Watt, Chief Technician, Microbiology Division, South-East of Scotland Blood Transfusion Service.

2. Multicentre evaluation of H.A.I. for donor testing (Part Four, Chapter one). The actual testing was, of necessity, performed by the appropriate technical staff in participating centres.

Robert Hopkins
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Finally, I would express my thanks to my wife for her patience and help in many matters concerning this thesis.
PUBLICATIONS

(a) Related to thesis


(b) Other publications


5. Leslie, M., Hopkins, R. and Das, P.C. (1975). "A rapid microcapillary haemagglutination method for detection of Australia antigen (HB Ag) and its antibody". Medical Laboratory Technology, 32, 121


SUMMARY

The recognition of Hepatitis B surface antigen (HBsAg) as a marker of one of the causative agents of posttransfusion hepatitis stimulated a demand for techniques applicable to mass donor testing which would allow blood to be 'cleared' for use within hours of donation. Counter-electrophoresis was the first technique to be widely used for HBsAg testing within the blood transfusion service but, with the advance of hepatitis testing technology, was soon found to be of insufficient sensitivity. Commercial tests employing the principles of haemagglutination and radioimmunoassay became available, but at a price (particularly radioimmunoassay) which proved prohibitive to many centres. This thesis describes the development and application of a haemagglutination-inhibition (HAI) test for HBsAg designed specifically to meet the requirements of the blood transfusion service.

The optimum conditions under which fresh human erythrocytes may be tanned and coated with partially purified HBsAg are determined, and the procedure of testing established using u-bottomed microtitre plates. Sensitivity and specificity are comparable to that of commercial 'third generation' reagents.

To improve reagent economy the test has been 'miniturised' using Terasaki tissue culture trays. Reagent standardisation has improved by the introduction of glutaraldehyde-fixed spherocytes as HBsAg carriers, and 'bulk' preparation of reagents, which yielded sufficient for one million tests if conducted in Terasaki trays.

The use of antisera rendered monospecific by adsorption allowed the HAI test to be applied to antigen subtyping, thereby providing useful confirmatory evidence for the presence of HBsAg in a
positively reacting test serum. The results of a multicentre evaluation of this HAI technique demonstrated that it was an acceptable alternative to commercial third generation HB$_3$Ag tests for use in large scale blood donor testing. Thus, with the appropriate training, the technique can be introduced into most regional transfusion centres, thereby keeping reagent costs to a minimum. Re-investigation of more than one hundred donations implicated in ten cases of posttransfusion hepatitis suggests that if HAI had been used at the time of donation 40 percent of the cases may have been avoided.

Results of an 'on-going' evaluation of the efficacy of hepatitis B immunoglobulin (HB-IgG) in the prevention of hepatitis resulting from accidental exposure to HB$_3$Ag suggest that fractionation of HB$_3$Ab positive plasma provides a product which, by virtue of its high specific antibody content, provides a significant degree of protection against type B hepatitis, provided the 'challenge dose' is small.

The pursuit of a specific theme invariably stimulates new ideas aimed at further improvement. This thesis concludes with a consideration of those lines of research which are considered to be an extrapolation of the present development.
I. **Viral Hepatitis**

Viral hepatitis is the term used to describe acute inflammation of the liver caused by an infectious agent(s) presumed to be a virus. The clinical spectrum of the disease varies from asymptomatic infection through prodromal symptoms of a mild gastrointestinal nature with little or no overt hepatocellular involvement, anicteric hepatitis, hepatitis with jaundice, to acute fulminant hepatitis. Other symptoms such as urticaria, arthritis and polyarteritis nodosa may occur as a result of circulating immune complexes. Complications include chronic hepatitis which may progress to cirrhosis or hepatoma.

During and shortly after the Second World War, volunteer studies indicated two forms of viral hepatitis: infectious hepatitis (IH, virus A) and serum hepatitis (SH, virus B) (MacCallum, McFarlan, Miles, Pollock and Wilson, 1951). A number of attributes are now known to distinguish virus A infection from virus B infection and these are listed in table 1. The most outstanding difference between IH and SH comes from the observation that one does not confer immunity to the other, i.e. the causative agents will not cross-immunise one against the other (Krugman, Giles and Hammond, 1967; Krugman and Giles, 1970). The incubation period of IH is regularly 30-40 days irrespective of oral or parenteral inoculation. On the other hand, that of SH is usually longer, particularly after infection by the oral route (Giles, McCollum, Berndtson, jnr. and Krugman, 1969; Krugman and Giles, 1970). Some patients with a short incubation period are found to possess Hepatitis B Antigen (HB Ag) and it is likely that
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* In the South East Region of Scotland approximately 0.04 percent of healthy blood donors carry HBsAg for up to 3 years and probably much longer (Hopkins, Robertson, Ross, Turnbull and Das; 1975).

+ Feinstone, Zapikian and Purcell (1973) described a possible hepatitis A virus in the stool of acutely infected patients. Specific antibody was detected in the convalescent sera of these and other hepatitis A patients.

** In countries where Hepatitis B infection is common (see page 19), the highest prevalence of HBsAg is found in children 4-6 years old.

*** Depending upon HBsAb content.
The accelerated development of the illness is due to a high concentration of virus in the challenge dose (Gooner, London, Sutnick, Blumberg and Senior, 1970).

Infectious Hepatitis has been recognised for many centuries, but it has been suggested by Zuckerman (1970) that clinical SH may have a relatively short history. He makes the point that even if HB Ag has been endemic in the population for many years, outbreaks of SH only became apparent after the introduction of large scale parenteral therapy and immunisation procedures and has paralleled the increasing transfusion of blood and use of blood products. It must be borne in mind, however, that it is only within the last few years that we have been able to identify SH as a serologically distinct entity. The origin of HB Ag is uncertain. Havens jnr. (1961) suggested that it represented the emergence of a new strain derived from virus A. This seems unlikely as the causative agents show no antigenic similarities and neither confers immunity against the other (Krugman et al., 1967; Krugman and Giles, 1970). A more acceptable theory is that HB Ag has been with us for a very long time, being maintained in the population by sexual contact and ritual operations involving scarification. With the advent of parenteral therapy, it has emerged as a major complication particularly with regard to the use of blood and blood products.

II. Association of HB Ag with Posttransfusion (Serum) Hepatitis

In 1961, Allison and Blumberg reported that some patients who had received numerous blood transfusions developed precipitating antibodies against serum beta-lipoproteins. During the course of these investigations, an antigen was found in the serum of an Australian Aborigine and was designated 'Australia Antigen' (Au Ag).
It was not for some years that the association with viral hepatitis was discovered (Blumberg, Gerstley, Hungerford, London and Sutnick, 1967; Blumberg, Sutnick and London, 1968). In 1969, Giles et al. presented data to suggest that the antigen was specifically related to long incubation (serum) hepatitis. This discovery was the result of work started a few years earlier at the Willowbrook State School in New York (Krugman et al., 1967).

III. Terminology

As research efforts and publications rapidly increased, new terms were introduced. Australia antigen (Au Ag or Au(i)), serum hepatitis antigen (SH), hepatitis antigen (HA) and hepatitis-associated antigen (HAA) all referred to one and the same thing. This welter of terms and symbols has often proved confusing to clinicians, epidemiologists and laboratory workers. The priority of the term Australia Antigen is acknowledged, but if its association with hepatitis is specific, then the name could be misleading, implying an unusual association with that country. The alternative, hepatitis-associated antigen, could create confusion if other antigen-antibody systems are discovered which prove to be specific for other forms of hepatitis. The terms hepatitis A and hepatitis B were introduced as long ago as 1947 (MacCallum), and it has recently been proposed that the antigen of hepatitis B be referred to as hepatitis B antigen (HB Ag) (World Health Organisation Committee on Viral Hepatitis, 1973).

In the electron microscope, antigen positive sera are frequently characterised by the presence of three morphologic forms (see inside front cover), namely 20nm spheres, rod-like structures also 20nm in diameter, and larger 42nm spheres known as Dane particles, some of
which possess an inner electron-dense core. The discovery and subsequent confirmation of the existence of an additional antigenic specificity associated with the Dane particle core (Almeida, Rubenstein and Stott, 1971; Traavik, Kjeldsberg and Siebke, 1973; Brzosko, Madalinski, Krawczynski and Nowaslowski, 1973; Hoofnagle, Gerety and Barker, 1973* and the 'e' system (Magnius and Espmark, 1972; described in detail later) necessitated the adoption of a terminology capable of distinguishing those determinants present on the surface of the antigens' lipoprotein coat and those found in association with the Dane particle core. Hence the term hepatitis B surface antigen \( \text{HB}_s\text{Ag} \) and hepatitis B core antigen \( \text{HB}_c\text{Ag} \) are used in this thesis in reference to the surface and core antigens respectively.

As the major portion of this thesis is concerned with the serology of \( \text{HB}_s\text{Ag} \), the physical and chemical properties of the antigen will be considered in detail in the first chapter of Section One.
PART ONE

The present position.
SECTION ONE

REVIEW OF INFORMATION CURRENTLY AVAILABLE REGARDING HEPATITIS B SURFACE ANTIGEN

CHAPTER I

BIOCHEMICAL AND BIOPHYSICAL CHARACTERISTICS

1. Morphology, Stability and Chemical Composition

Bayer, Blumberg and Werner (1968) were first to observe the physical appearance of HBsAg in the electron microscope. Using gradient-purified material, they were able to identify two types of particle, 20-25nm diameter spheres and rod-like structures 20-25nm diameter, but of varying length. Both types of particle were aggregated by specific antiserum. These findings were soon confirmed by Prince (1968). Almeida, Zuckerman, Taylor and Watson (1969) observed both types of particle in the serum of a healthy carrier of HBsAg and in the acute phase sera of two patients with viral hepatitis. In a later publication, cross striations of the rod-like structures, showing a periodicity of 3nm, were noted together with an irregularity in the size of the spherical particles whose diameter varied between 16-25nm (Almeida, 1971). Dane, Cameron and Briggs (1970) reported the presence of large spherical particles, 42nm in diameter (subsequently termed 'Dane Particles'), in multiple serum specimens from three antigen positive patients. These particles were also aggregated by specific antibody and appeared to possess a complex internal structure. Gust, Gross, Kalder and Ferris (1971) and Hirschman, Vernace and Schaffner (1971) soon confirmed the occurrence of Dane particles in antigen positive sera. Almeida (1971) described Dane particles as possessing a double-shelled structure, the outer shell having a diameter of 45nm, and the inner shell, released by the action of a non-ionic detergent, having a diameter of 28nm. Similar particles were found in post-mortem liver
homogenates from serum hepatitis patients (Almeida, Waterson, Trowell and Neale, 1970), while Huang (1971) observed them in the nucleus and occasionally in the cytoplasm of liver cells from renal transplant patients who had developed antigen positive hepatitis.

Hirschman, Schwartz, Vernace, Schaffner and Gans (1973) studied the structural polymorphism of HB Ag under different experimental conditions and concluded that the small round particles may be discs of tightly wound helical protein that can stack to form tubes of varying lengths. The interrelationship of the different morphologic forms were investigated under varying conditions of pH, ionic strength and exposure to digestive enzymes and organic denaturing agents. In water and dilute buffer, tubular structures and small round particles predominated. Large 40-60nm, multi-stranded structures with up to four lamellae-like strands appeared in 0.125M phosphate buffer and when the antigen was digested with 0.1 percent chymotrypsin at acid pH, the tubular structures disappeared, leaving small round particles. These findings indicate that the appearance of HB Ag in serum may be due to equilibria dependent on ionic strength and the concentration of certain polyvalent ions and peptides, including complement. The size distribution of HB Ag particles was found to alter in the presence of complement and specific antibody but was not affected by complement alone or by inactivated complement and antibody (Hirschman, Kochwa, Rosenfield and Schwarz, 1974).

Hollow particles measuring 15-20nm diameter have been described in one case of acute hepatitis by Ackerman, Cherche, Valet and Matte (1974), who believed them to be incomplete forms and possible precursors of normal HB Ag. Muscatello, Bianchi and Pisi (1973) have studied the effect of specific antibody on antigen morphology and
concluded that the detailed definition of the particles becomes less, or is even obscured, in the presence of surface antibody at low concentrations. High antibody concentrations result in clumping of the antigen. Such morphological changes may prove useful in determining the presence of antibody, as non-specific clumping may occur due to the negative staining procedure.

HBsAg is remarkably stable. It is unchanged morphologically and immunologically, neither does it lose infectivity, after heating at 60°C for one hour, at room temperature for six months, or at minus 20°C for twenty years (Hirschman, Shulman, Barker and Smith, 1969; Barker, Smith, Gehele and Shulman, 1969). Boiling for three minutes does not completely remove antigenicity, since one of thirteen children inoculated with this preparation produced specific antibodies (Krugman and Giles, 1970). Kim and Bissell (1971) found HBsAg to be unaltered serologically after heat treatment, freeze-thawing, putrefaction, acid-alkali digestion and treatment with most hydrolytic enzymes. They did find that subtilisin, subtilopeptidase A and sodium dodecyl sulphate treatment was followed by reduced immunoreactivity. Massar, Corbett and Blumberg (1973) found that antigenicity was destroyed by a soluble, heat labile exoenzyme produced during the growth of Pseudomonas aeruginosa and Escherichia coli. This phenomenon may have important epidemiological implications since it is conceivable that HBsAg is released into the intestine with the bile and immunologically altered by enzymes already there, such that faeces emerge carrying antigen which is no longer identifiable as HBsAg. Both P. aeruginosa* and E. coli are known bacterial inhabitants of the human gut and the enzymes they release may be equivalent to the 'faecal inhibitory factor' described by Piazza, Di Stasio and De Marco (1971) and Piazza, Di Stasio, Maio and Marzano (1973).

* P. aeruginosa is often present in soil and thus frequently infects the human gut.
Early biochemical investigations (Alter and Blumberg, 1966; Barker et al., 1969; Gerin, Purcell, Hoggan, Holland and Channock, 1969) suggested that the antigen was composed mainly of protein, but possessed a minor lipid moiety. Bouyant density was found to be 1.06-1.30 gm/cm³ in potassium bromide, 1.16 gm/cm³ in sucrose, 1.15 gm/cm³ in potassium tartrate, with a sedimentation coefficient of 110S in sucrose. Millman, Loeb, Bayer and Blumberg (1970) were unable to detect nucleic acid by spectrophotometry, chemical and radiochemical analysis. The following year, Joswiack, Koscielak, Madalinski, Brzosko and Nowaslawski (1971) reported the presence of RNA in purified, concentrated HBsAg. Hirschman et al. (1971) reported weak endogenous DNA-synthesising activity which appeared to be dependent on an RNA template, raising the possibility of an RNA-dependent-DNA-polymerase being present in concentrated HBsAg, similar to the 'reverse transcriptase' associated with some RNA oncogenic viruses (Spiegelman and Schlam, 1972; Gallo, Yang, Smith, Herrera, Ting and Fugioke, 1971). The reaction was, however, stimulated by the addition of poly (dAT) and not by poly (rA)-oligo (dT), as are the known RNA-dependent DNA polymerases (Zuckerman and Howard, 1972); furthermore, this interpretation has not been confirmed by other workers in the hepatitis field.

In 1973, Kaplan, Greenman, Gerin, Purcell and Robinson confirmed the presence of a DNA-polymerase in Dane particle-rich preparations of HBsAg. They showed that the reaction could be inhibited by actinomycin-D, suggesting that it was associated with a DNA template. Peak activity appeared to be related to Dane particle cores, no DNA-polymerase activity being found in association with the 20nm spherical particles. The following year, Robinson, Clayton and
Greenman (1974) confirmed and extended these findings by extracting radioactive DNA from labelled Dane particle cores. Electron microscopic examination revealed circular double-stranded DNA molecules approximately 0.78nm in length. Identical circular molecules were obtained when DNA was isolated by a similar procedure from particles that had not undergone a DNA polymerase reaction. The authors suggested that the circular molecules probably serve as a template for the DNA-polymerase reaction carried out by the Dane particle cores. Huang, Mao, Ling and Overby (1975) have reported the presence of free DNA in HBsAg positive plasma which showed homology with radiolabelled Dane particle DNA by molecular hybridisation. Further work is required to establish conclusively the nature of the DNA polymerase template and to identify the enzyme as viral and not host specific.

Millman, Huttenen, Marino, Beyer and Blumberg (1971) observed that purified HBsAg reacted with specific antibody but not with antibody to normal human serum components. Treatment of the purified antigen with the non-ionic detergent TWEEN 80 revealed substances which reacted with antibody to human IgG, beta 1a/1c globulin, beta lipoprotein, transferrin and albumin. In the electron microscope, the treated antigen particles appeared less dense and unfolded, yet retained dimensions similar to those before extraction. One interpretation of these findings is that the components released by detergent extraction are either intricate parts of the macromolecule, or contaminants. Sutnick, Millman, London and Blumberg (1972) showed that the extracted substances differed electrophoretically and immunologically from human serum proteins. A recent study by Burrell (1975) has shown that purified 125I-
labelled 20-25nm particles produced low affinity immunoprecipitation reactions with antisera to several normal human serum components, which are immunologically distinct from the reaction due to the classical HB Ag determinants. No evidence was found for the native material in normal serum or liver cells, to be an integral part of the structure of the 20-25nm particles.

Ultracentrifugation has indicated particles of two molecular weights, $3.6 \times 10^6$ and $4.5 \times 10^6$ (Dreesman, Hollinger, Suriano, Fujioka, Brunschwig and Melnick, 1972). Antigenic activity was detected in particles having isoelectric points at pH 4 and pH 4.4. Purified $^{125}$I-labelled antigen was found to contain six different polypeptides of molecular weights 10,000 to 39,000. The 20-25nm particles contained increased levels of cysteine amino acid residues (4.8 percent) compared with other animal viruses (0.3 percent to 2 percent), a figure which suggests that sulphhydryl groups and/or disulphide bonds may play a major role in maintaining the tertiary structure of the antigenic determinants. Sukeno, Shirachi, Yamaguchi and Ishida (1972) also emphasised the role of disulphide bonds in the structural integrity of HB Ag by showing that reduction with dithiothreitol caused irreversible loss of structure. In 1975, Dreesman, Hollinger, McCombs and Melnick showed that reduction and alkylation destroyed antigenicity, while reduction alone caused loss of morphological integrity.

Vyas, Williams, Klaus and Bond (1972) obtained an optical density of 3.726 at 280nm using an 0.1 percent solution of protein from purified 20nm particles. This protein appeared to consist of two polypeptides which contained high proportions of proline, leucine and serine, but only a small amount of tyrosine. Using
optical rotary dispersion and circular dichroism, Sukeno, Shirachi, Shiraishi and Ishida (1972) showed that HB₃Ag contained a high concentration of alpha-helices (70 to 80 percent), thus indicating a structural similarity to human lipoproteins. Burrell, Proudfoot, Keen and Marmion (1973) have obtained evidence from periodate treatment and chemical analysis of purified 20nm particles that significant amounts of carbohydrate are present and that intact carbohydrate is necessary for serological activity. This finding was supported by the work of Howard and Zuckerman (1973) who found that pure HB₃Ag, isolated by gel filtration and electrofocusing, was aggregated by concanavalin A. The major phospholipids of HB₃Ag have been characterised as phosphatidylcholine, sphingomyelin and lypophosphatydylcholine. In addition, two carbohydrate-containing lipids were observed, one of which appeared to be a non-sialic acid-containing, water soluble glycosphingolipid (Steiner, Heulner and Dreesman, 1974).

II. Antigenic Heterogeneity

Levene and Blumberg (1969) were among the first to discover the existence of antigenic heterogeneity in HB₃Ag. They immunised a rabbit with HB₃Ag-positive serum from a leukaemic patient, whose serum had given an unusual and inconsistent reaction with specific antisera. The resultant rabbit antiserum, after absorption, had at least one specificity in common with other HB₃Ab-containing sera (anti-Au(1)) and one specificity not shared with these. This was demonstrated by the production of a spur in Ouchterlony double diffusion. The rabbit antiserum was designated anti-Au(2). Since anti-Au(1) possessed at least two specificities which they called 'a' and 'c', those of anti-Au(2) they called 'a' and 'b'. In 1970,
Raunic, London, Sutnick, Millman and Blumberg reported the findings of a study in which HBsAg had been collected from different parts of the world and compared in respect to specificity against a number of antisera. All the antigens appeared to have one specificity in common, but by selection of appropriate reagents, differences could be demonstrated. Kim and Tilles (1971) observed three sub-specificities in combinations 'a', 'ab' and 'abc'. The latter showed electrophoretic mobility equivalent to that of beta-globulin, while 'a' and 'ab' were intermediate between alpha- and beta-2 globulin. The 'b' and 'c' of Kim and Tilles (1971) were not the same as the 'b' and 'c' of Levene and Blumberg (1969). Analysis of the surface antigens of HBsAg by Le Bouvier (1971) revealed one common specificity 'a' and three additional determinants 'd', 'x' and 'y', which were not shared by all positive sera. It was possible to divide sera into three groups depending upon the possession of 'd' and 'y', i.e., group A (=a+dy-); group D (=ad+y-); group Y (=ay+d-). It is of interest to note that 'x' and 'd' are equivalent to 'b' and 'c' of Kim and Tilles (1971), but are distinct from the 'b' and 'c' of Levene and Blumberg (1969). It has been suggested (Le Bouvier, 1972; Le Bouvier, McCollum, Hierholzer, Irwin, Krugman and Giles, 1972) that 'a', 'd' and 'y' reflect the genotype of the antigen, and that 'x' may be a component of the host which has become integrated into the antigen or firmly bound to its surface. Occasionally, the 'd' and 'y' specificities have been found on the same particle (Nordenfelt and Le Bouvier, 1973/74), the most likely explanation for this being that a dual infection has occurred, resulting in the combination of viral genomes with the formation of a new genome representing both specificities. Recent studies by
Soulier and Courouce-Pauty (1973) suggest a subdivision of group Y into three categories \((a^1y, a^2y, a^3y)\) and of group D into two categories \((a^4d, a^5d)\) with a further group containing a genuine association of D and Y (ady).

In 1972, Bancroft, Mundon and Russell described three immunodiffusion patterns of antigenic reactivity. One showed identity with Y and one with D, while one showed partial identity with both. Absorption with heterologous material showed that the third type of reaction pattern was distinguishing two additional antigenic determinants subsequently designated 'w' and 'r'. This study also suggested that 'w' was more common in the U.S.A., while 'r' predominated in sera from Thailand. Bar-Shany, Edwards, Moseley and Bancroft (1973) confirmed and extended these findings to show that 'w' occurred almost exclusively in the U.S.A., Europe, North Africa and the Middle East. An investigation of antigen subtypes among asymptomatic carriers in Canada (Fienman, Berris, Sinclair, Krobel, Alter and Holland, 1973) showed that D and Y appeared to be related to country of origin, implying infection at an early age. These findings were confirmed by Mazzur and Blumberg (1973), who studied the determinants 'd', 'y' and 'w' in 34 chronic carriers and concluded that they represented good long term epidemiological markers.

A global pattern of subtype distribution appears to be emerging with zones where there is an excess of one subtype and regions where a mixture of subtypes is common. Mazzur, Burgert and Blumberg (1974) have suggested that a partial explanation of this distribution may be that HBsAg-positive mothers infect their children who then become carriers of the same subtype. Daughters transmit the antigen to their children and so on through many generations. This mechanism could maintain the subtype brought into a new environment by an
immigrant population. The introduced subtype would not necessarily predominate since there are other mechanisms which produce carriers.

As previously described, treatment of the Dane particles with Tween 80 released an inner 27nm diameter core (Almeida et al., 1971). Specific antibody to this core (HB\textsubscript{o} Ab) may be found in patients convalescing from antigen positive viral hepatitis, some possessing HB\textsubscript{o} Ab in the absence of HB\textsubscript{Ag}. Such findings, which have been confirmed by a number of other workers (Traavik et al., 1971; Brzosko et al., 1973; Hoofnagle et al., 1973), imply the presence of a further antigenic specificity normally hidden by the stable outer protein coat. Hoofnagle et al. (1973) found HB\textsubscript{o} Ab in all chronic HB\textsubscript{Ag} carriers studied and suggested that it may be a sensitive marker of viral replication even when HB\textsubscript{Ag} is not detectable.

Magnius and Espmark (1972) reported the existence of a specificity 'e', distinct from 'a', 'd', 'y', 'w', 'r' and 'x', and possibly related to contagiousness. This antigen was one of a group of precipitating antigens found in HB\textsubscript{Ag}-positive sera. The 'e' antigen was frequently present in sera from patients on haemodialysis, and anti-'e' was found in serum specimens from healthy carriers. The presence of 'e' has been confirmed by Nielsen, Dietrichson and Juhl (1974), who suggest that it may be a marker of continued viral replication and abnormal liver function, and therefore of prognostic value.

Neurath, Prince, Lippin and Chen (1973) used immunoabsorbants coated with antibody to Apolipoprotein C to show that HB\textsubscript{Ag} particles possess this protein moiety on their surface, and are therefore immunologically related to LP-X, an abnormal plasma lipoprotein detected in the serum of patients with obstructive jaundice. In a
letter to the Editor of Lancet, Goudeau, Houwen and Dankert (1974) have suggested that these results may be due to non-specific adsorption of HB$_s$Ag onto the antibody-coated gel.

There is increasing evidence (Holland, Purcell, Smith and Alter, 1972; Iwarson, Magnus, Lindholm and Lundin, 1973), that in the U.S.A. and Europe group Y is more likely to be associated with clinical hepatitis, while group D is more frequently found in chronic asymptomatic carriers of HB$_s$Ag. In a study of 63 asymptomatic carriers of antigen in Toronto, Fienman et al. (1973) found elevated serum glutamic pyruvic transaminase (SGPT) equally common in 'adr', 'adw' and 'ayw' specificities. Gold, Alter, Holland, Gerin and Purcell (1974) used a modified haemagglutination test to subtype HB$_s$Ab in terms of anti-d and anti-y activity. Blood donors from the U.S.A. and Egypt showed frequencies of antibody that closely parallel the frequency of antigen groups in each population. An interesting result of this study was that antibodies against both 'd' and 'y' were not detected together in any patient, including those who had been repeatedly transfused.

**III. Nature of HB Ag**

There seems little doubt that HB$_s$Ag is a viral product, while the 'full' Dane particle represents the infectious viroin. In the electron microscope HB$_s$Ag exhibits a morphology which is so characteristic as to be of diagnostic value, and possesses the size, pleomorphic symmetry and negative staining characteristics found among other viruses. Furthermore, its close association with type B hepatitis endows it with the epidemiological peculiarities of an infectious agent. The work of Kaplan et al. (1973) and Robinson et al. (1974) provides strong evidence for the association of a DNA-genome with HB$_c$Ag. Early attempts to 'grow' the virus in tissue and organ
culture proved singularly unsuccessful, possibly due to the use of the surface antigen as marker. Subsequent studies suggest that it may be possible to infect cultures with Dane particle-rich material and to show an increase in the amount of core antigen (Fauouze-Perrin, Rachman, Courouce-Pauty and Dupuy, 1973). Despite the mounting evidence in favour of the viral nature of the antigen, two features still distinguish it from the majority of other viruses.

1. The amount of DNA associated with HBcAg (\(1.6 \times 10^6\) daltons) is unlikely to be sufficient to code for the DNA genome; the core and e antigens; in addition to the complex lipoprotein coat that surrounds and presumably protects the core proteins and associated enzymes.

2. The high alpha-helical content of HBsAg suggests analogy to that of human tissues.
I. Blood

The incidence of HB Ag in the blood of donors from several countries is listed in Table 2.1. Of those studied, the highest incidence is found in countries near the equator, with the exception of Greenland. The common feature of both polar and tropical populations studied is a poor socio-economic environment with associated overcrowding and poor hygiene, suggesting that this factor is of primary importance concerning the spread of HB Ag.

In 1969, Lewkonia and Finn reported an increased susceptibility to serum hepatitis among group A blood donors, although subsequent reports have shown there to be no significant relationship between the antigen carrier state and the A, B, O or Rh blood groups (Hersch, Goyal, Grubb, Warch and Melnick, 1971; Sammessa, Prince and Cherubin, 1971; Nadzynamis, Pappasvengiou and Vissalis, 1973; Vale, Thomas, Haskes and Kelly, 1974). However, it is now apparent that certain groups of blood donors are associated with a higher risk of HB Ag transmission than others. These groups include commercial blood donors (Kunin, 1959; Grady and Chalmers, 1964; Cohen and Dougherty, 1968; Walsh, Purcell, Morrow, Chanock and Schmidt, 1970; Cherubin and Prince, 1971), institutionalised blood donors such as prisoners or military personnel (Kliman, Reid, Lilly and Morrison, 1971; Nelson and Cooke, 1971) and blood donors from lower socio-economic communities where overcrowding, poor hygiene, and occasionally drug addiction may be found. Homosexuals
<table>
<thead>
<tr>
<th>Country/population</th>
<th>Donor source</th>
<th>No. tested</th>
<th>HBs Ag %</th>
<th>Author(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweden</td>
<td>Paid</td>
<td>11,000</td>
<td>0.18</td>
<td>Iwarson et al., 1972</td>
</tr>
<tr>
<td>Norway</td>
<td>Volunteer</td>
<td>3,200</td>
<td>0.16</td>
<td>Solas, 1970</td>
</tr>
<tr>
<td>Denmark</td>
<td>Volunteer</td>
<td>10,600</td>
<td>0.18</td>
<td>Banke et al., 1971</td>
</tr>
<tr>
<td>Germany</td>
<td>Volunteer</td>
<td>22,800</td>
<td>0.32</td>
<td>Fiedler, 1971</td>
</tr>
<tr>
<td>Holland</td>
<td>Volunteer</td>
<td>10,900</td>
<td>0.09</td>
<td>Brummelhuis, 1970</td>
</tr>
<tr>
<td>Scotland</td>
<td>Volunteer</td>
<td>73,800</td>
<td>0.12</td>
<td>Wallace et al., 1972</td>
</tr>
<tr>
<td>Switzerland</td>
<td>Volunteer</td>
<td>7,000</td>
<td>0.36</td>
<td>Frey-Nettstein, 1972</td>
</tr>
<tr>
<td>Austria</td>
<td>Volunteer</td>
<td>39,700</td>
<td>0.46</td>
<td>Newalka et al., 1970</td>
</tr>
<tr>
<td>Spain</td>
<td>Paid</td>
<td>800</td>
<td>1.0</td>
<td>Guardia et al., 1970</td>
</tr>
<tr>
<td>Italy</td>
<td>?</td>
<td>1,800</td>
<td>1.5</td>
<td>Constantino et al., 1970</td>
</tr>
<tr>
<td>Yugoslavia</td>
<td>Volunteer</td>
<td>14,600</td>
<td>0.6</td>
<td>Dejanov et al., 1971</td>
</tr>
<tr>
<td>Greece</td>
<td>Mixed</td>
<td>5,100</td>
<td>3.3</td>
<td>Hadziyannis et al., 1972</td>
</tr>
<tr>
<td>Turkey</td>
<td>?</td>
<td>1,600</td>
<td>3.0</td>
<td>Ertugrul and Say, 1971</td>
</tr>
<tr>
<td>Israel</td>
<td>Mixed</td>
<td>10,000</td>
<td>0.9</td>
<td>Bar-Shany et al., 1972</td>
</tr>
<tr>
<td>Canada</td>
<td>Volunteer</td>
<td>411,000</td>
<td>0.22</td>
<td>Moore and Path, 1972</td>
</tr>
<tr>
<td>U.S.A.</td>
<td>Volunteer</td>
<td>2,596,000</td>
<td>0.10</td>
<td>Dodd et al., 1973</td>
</tr>
<tr>
<td>Africa</td>
<td>?</td>
<td>8,400</td>
<td>6.6-8.7</td>
<td>Saimot et al., 1972</td>
</tr>
<tr>
<td>(Nigeria, Kenya, Malian, Senegal, Ivory Coast)</td>
<td></td>
<td></td>
<td></td>
<td>Francis and Smith, 1971</td>
</tr>
<tr>
<td>Australia</td>
<td>Volunteer</td>
<td>34,600</td>
<td>0.11</td>
<td>Bagahave et al., 1971</td>
</tr>
<tr>
<td>Japan</td>
<td>Paid</td>
<td>5,200</td>
<td>1.0</td>
<td>Nelson and Cooke, 1971</td>
</tr>
<tr>
<td>Singapore</td>
<td>?</td>
<td>2,600</td>
<td>0.1</td>
<td>Okochi and Murakami, 1968</td>
</tr>
<tr>
<td>Papua New Guinea</td>
<td>?</td>
<td>3,500</td>
<td>7.5</td>
<td>Simons et al., 1972</td>
</tr>
<tr>
<td>Greenland</td>
<td>Not donors</td>
<td>2,900</td>
<td>7.1</td>
<td>Woodfield et al., 1972</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>Volunteer</td>
<td>375,323</td>
<td>0.06</td>
<td>Skinhøj et al., 1974</td>
</tr>
</tbody>
</table>

(pooled data from five centres, 1971, Appendix F)
may also constitute a high risk group (Jeffries, James, Jeffress, MacCleod and Wilcox, 1973). Young adult males show the highest incidence of antigen carriage (Blumberg, Sutnick, London and Melartin, 1972; Sutnick, London, Gerstley, Cronlund and Blumberg, 1968; London, Sutnick and Blumberg, 1969; Mason, Shaw, Harding and Witney, 1972). It could be argued that their greater social mobility makes this group more likely to become infected and to infect others, although it is difficult to see how this principle could operate in all populations studied.

Most of the early data relating to the incidence of HB$_s$Ag were obtained using the relatively insensitive techniques of counter-electrophoresis (C.I.E.O.P.) and immunodiffusion (I.D.). It has been estimated (Chalmers and Alter, 1971; Kliman et al., 1971) that these methods will detect only 20-40 percent of HB$_s$Ag carriers, and evidence is beginning to accumulate which suggests that haemagglutination and radioimmunoassay are capable of detecting circulating antigen at a concentration below the sensitivity of the above techniques (Levy and Hawrisiak, 1972; Geogini Jr., Hollinger, Leduc, Issarescu, George, Blackman, Thayer Jr., 1972; Shaffer, Vyas, Shahed, Chen and Perkins, 1972; Ling, Irace, Decker, and Overby, 1973).

Carrara, Bryant and Leonard (1973) have reported HB$_s$Ab and HB$_s$Ag simultaneously in 26 of 10,470 blood donations, suggesting that the presence of antibody may also identify potential antigen carriers. Milman, London, Sutnick and Blumberg (1970) showed that when some HB$_s$Ab positive sera were pelleted, HB$_s$Ag could be detected in the pellet.

The incidence of HB$_s$Ab in most volunteer blood donor populations studied by I.D. or C.I.E.O.P. is similar to that for
HB$_s$Ag; e.g., 0.28 percent in Copenhagen (Banke, Dybkjaer, Nordenfelt and Reinicke, 1971), 0.06 percent in the West of Scotland (Wallace, Milne and Barr, 1972), the principal exception being an incidence of 6.7 percent among volunteer blood donors in New York (Szmuness, Prince, Etling and Pick, 1973). The use of haemagglutination and/or radioimmunoassay techniques lead to an increase in detectable antibody to between 1 percent and 20 percent (Ni, Shoff and Lama, 1972; Alter, Lander and Purcell, 1971). No direct evidence has been presented to implicate HB$_s$Ab-positive blood with HB$_s$Ag-positive jaundice (Gocke and Fanick, 1972; Reinicke, Poulson, Banke and Bybkjaer, 1973; Aach, Alter, Hollinger, Holland, Lander, Melnick and Weiler, 1974). However, in a recent letter to Lancet, Renton and Wadsworth (1975) have pointed out that blood containing HB$_s$Ab appears to be four times as infectious as blood negative for antigen and antibody, although this interpretation may not reflect the true situation since their data were derived from an invalid reconstruction of population statistics.

II. Faeces

In 1970, Ferris, Kaldor, Gust and Cross reported a particulate antigen similar in appearance to HB$_s$Ag yet serologically distinct from it, in faecal extracts from cases of HB$_s$Ag-negative hepatitis. These findings were confirmed by Cross, Waugh, Ferris, Gust and Kaldor (1971), who went on to show that the larger particles (40-45 nm) of faecal antigen appeared to possess an antigenic component in common with the Dane particles observed in serum. It was suggested that the faecal antigen represented serum antigen degraded as a result of passage through the intestinal tract. Grob and Jemelka (1971) reported HB$_s$Ag in faeces from eleven cases of sporadic acute hepatitis, whose serum were all HB$_s$Ag positive. Using two-dimensional I.D., Grob and Jemelka (1972) were able to show
that \( \text{HB}_\text{s} \text{Ag} \) in faeces was immunologically and electrophoretically similar to that in serum. Despite these findings, many workers have been unable to identify \( \text{HB}_\text{s} \text{Ag} \) in faeces of serum-antigen-positive patients. Moodie, Stannard and Kipps (1974) suggested that this was due to the action of hydrolytic enzymes found in the human gut, capable of degrading the surface lipoprotein of \( \text{HB}_\text{s} \text{Ag} \) and exposing the inner core of Dane particles which Almeida et al. (1971) have shown to possess their own peculiar antigenicity.

### III. Urine

Blainey, Earle, Flewett and Williams (1971) were first to report the presence of \( \text{HB}_\text{s} \text{Ag} \) in urine. Addition of specific antiserum resulted in the formation of characteristic immune complexes when viewed by electron microscopy (E.M.). These findings were confirmed by Apostolov, Bauer, Selway, Fox, Dudley and Sherlock (1971) and Trapitsis and Horst (1971), who demonstrated immunological identity between \( \text{HB}_\text{s} \text{Ag} \) in urine and in serum. Heathcote, Talanides and Sherlock (1973) detected a substance in the urine of some antigen positive patients, which reacted with \( \text{HB}_\text{s} \text{Ab} \) by Complement Fixation (C.F.). However, morphologically characteristic \( \text{HB}_\text{s} \text{Ag} \) could not be seen in the urine by electron microscopy.

### IV. Saliva and Other Body Fluids

Ward, Borchert, Wright and Kline (1972) detected \( \text{HB}_\text{s} \text{Ag} \) in saliva in 50 percent of mentally handicapped, institutionalised patients. Antigen positive saliva has been reported by Tanno, Fay and Roncoroni (1972) in cases of acute and chronic hepatitis and postnecrotic cirrhosis, while Kistler, Sonnabend and Krech (1973) were able to detect \( \text{HB}_\text{s} \text{Ag} \) by I.D. and E.M. in mixed saliva concentrates.
from a similar group of patients which included two HBs Ag positive kidney transplant recipients. Antigen has been observed in the saliva and semen of carriers with high serum antigen titres (Heathcote, Cameron and Dane, 1974). Ogra (1973) employed C.I.E.O.P. and immunofluorescence (I.F.) to study patients with icteric and asymptomatic infections. She found antigen in the naso-pharangeal washings of some subjects over a short period. Other workers have reported HBs Ag in synovial fluid (McKenna, O'Brien, Scheinman, Delaney, Pellechioa and Lepore, 1971), amniotic fluid (Matsuda, Tada, Shiraohi and Ishida, 1972), gall bladder bile (Serpeau, Mannoni, Dhumlack and Berthelot, 1971; Bose, Bahu, Hammond and Lubbat, 1971), and sweat (Telatar, Kayhan, Kes and Karacadaq, 1974).

It should be remembered, however, that any biological fluid contaminated with infected blood will appear antigen positive if the test is sufficiently sensitive.

V. Plasma Fractions

The cold alcohol procedure of Cohn, Strong, Hughes, jnr., Mulford, Ashworth, Melin and Taylor (1946) has been most commonly used for industrial scale fractionation of plasma. Several investigators have fractionated HBs Ag-rich plasma and looked for antigen in the resulting fractions (Andrassey, Ritz and Sanwald, 1970; Schroeder and Mozen, 1970; Zuckerman, Taylor, Bird and Russell, 1971). HBs Ag has been detected in all fractions except immune serum globulin, a finding in accordance with the well-recognised non-infectivity of this fraction. Such findings may reflect removal of the antigen by fractionation or more likely, antibody present in the fraction may have masked HBs Ag by complexing with it. In a recent report, Lewis, Maxwell and Brandon (1974)
found concentrates of factor IX to be associated with serum hepatitis in haemophiliacs three times more frequently than concentrates of factor VIII.
CHAPTER 3

HB\_s Ag LIVER DISEASE

I. Asymptomatic Carriers: Clinical Hepatitis

As early as 1971, Singleton, Merrill, Fitch, Kohler and Rettberg suggested that the majority of HB\_s Ag positive blood donors might be suffering from some form of underlying liver disease. However, Reinecke, Dybkjaer, Poulsen, Banke, Lylof and Nordenfelt (1972) reported that none of 24 antigen positive Danish blood donors showed histological evidence of hepatitis. Subsequent studies have shown that some HB\_s Ag positive donors exhibit histological and/or biochemical evidence of liver dysfunction (Casal and Robinet-Levy, 1972; Woolfe, Boyes, Dymock, Renton and Stratton, 1972; Papadopoulos, Traianos, Thomakos and Tiniakos, 1973; Anderson, Sun, Berg, and Chang, 1974), and it has been suggested that the best approach to apparently healthy HB\_s Ag carriers is to observe clinically, with regular biochemical tests of hepatic function as long as no evidence of chronic liver disease occurs, reserving biopsy for those patients with persistent hepatic dysfunction (Griffin, 1973). An increased incidence of Dane particles in the serum of patients with abnormal liver function tests has been reported (Neilsen, Neilsen and Elling, 1973; Moulias, Couceru and Goust, 1973).

The specificity of HB\_s Ag for serum hepatitis has been confirmed by the Willowbrook experiments of Giles et al. (1969). Initial reports indicate a frequency of HB\_s Ag in acute hepatitis of 12 percent in Australia (Gust and Lucas, 1971; Ferris, Kaldor and Lucas, 1970; Hawkes, 1970), 33 percent in the U.S.A. (Prince, 1971), 60 percent in
Uganda (Maynard, Sadikali, Anthony and Barker, 1970) and Ghana (Morrow, Sai and Barker, 1971), and 15 percent in London (Farrow, Lamb, Coghill, Lindon, Preece, Zuckerman and Stewart, 1974). From the data currently available, it appears that HBsAg may be detectable shortly before the onset of acute illness. As the antigen disappears, antibody to the Dane particle core becomes detectable and persists for a relatively short time. Antibody to the surface antigen specificities may not appear for many months, and then possibly only as a result of subsequent challenge (see figure 1(b)]. Barker, Peterson, Shulman and Murray (1973) have described primary and secondary antibody responses in adults receiving material known to transmit type B hepatitis. Some of those showing secondary responses subsequently developed disease. It was concluded that primary responses were common after exposure to HBsAg and that protective immunity frequently but not invariably follows initial exposure.

II. Chronic Liver Disease

Gitnick, Gleich, Schoenfield, Baggenstoss, Sutnick, Blumberg, London and Sommerskill (1969) studied 31 patients with chronic active liver disease and found HBsAg in 3, all of whom had cirrhosis. These findings led to the speculation that in some patients, a chronic viral infection existed, even in the presence of cirrhosis. Maynard et al. (1970) found HBsAg in 31 percent of patients in Uganda, as compared with 2 percent in normal controls, and suggested that infection with HBsAg may be of pathogenic importance in the development of cirrhosis. In 1972, Nowaslowski, Krawczynski, Brzosko and Madalinski suggested a correlation between hepatocellular damage in cirrhosis and the presence of HBsAg/HBsAb immune complexes. Brzosko,
Figure 1(1).3.1. Serologic responses in a moderately severe case of type B hepatitis. Antibody to hepatitis B core antigen measured by complement fixation (C.F.), antibody to hepatitis B surface antigen measured by passive haemagglutination (PHA), hepatitis B antigen measured by radioimmunoassay (RIA).
Mikulka, Biedrzycka, Roszkowska, Rudkowski, Rabenda, Oziemska-Losinska and Debaki (1973) identified HBsAg in 69 percent of children with chronic hepatitis, and antibody to core antigen (HBcAg) in all of 50 cases studied. More recently, Van Waes, Segers, Van Eegmond, Van Nimmen, Barbier, Wieme and Demeulenaere (1974) have reported on a long-term follow-up of 45 patients with chronic hepatitis and 41 patients with cirrhosis. Antigen was present in 42 percent and 49 percent respectively. The clinical, biochemical and histological findings were similar in antigen positive and antigen negative patients. They concluded that when no irreversible lesions existed, the disappearance of antigen was taken as an indication that the disease would resolve.

III. Hepatoma

Reports from many laboratories indicate a higher incidence of HBsAg in patients with hepatoma than among the general population (Sherlock, Fox, Niazi and Scheuer, 1970; Hadziyannis, Merikas and Afroudakis, 1970; Vogel, Anthony, Natu, Mody and Barker, 1970; Dennison, Peters and Reynolds, 1971; Specht and Noto, 1972), suggesting that hepatoma could be the last stage in a process initiated by acute hepatitis, leading to chronic hepatitis and post-necrotic cirrhosis. Simons (1972) presented evidence to suggest that the use of sensitive techniques will result in a more significant correlation between HBsAg and hepatoma. Using radio-immunoassay (RIA), Reed, Riddleston, Steen, Williams, Zuckerman, Bowes and Earl (1973) found 23 percent of 38 patients to be HBsAg positive, the frequency being higher in those born outside Great Britain (66 percent compared with 15 percent). In the absence of conclusive proof of the oncogenic properties of HBsAg,
it may be that hepatitis B virus is merely a passenger.
Nowasowski (67) reported that the only cells supporting viral replication within the region of a liver cancer were normal hepatocytes, although this does not exclude the possibility of transformation by the HB Ag genome.

IV. Other Clinical Conditions

A number of symptoms have been described which do not involve the liver and are most probably due to circulating immune complexes. McKenna et al. (1971) have identified HB Ag in synovial fluid, while joint symptoms have occasionally been reported in acute and chronic hepatitis (Alpert, Isselbacher and Schur, 1971; Buckley, Heiser, Goldfinger and Isselbacher, 1970), and antigen has been found in patients exhibiting polyarteritis nodosa (Baker, Sidal and Kalan, 1971). Similar findings led McCarty and Ormiste (1973) to suggest that an arthritic strain of HB Ag may exist. The antigen has also been associated with glomerulonephritis and nephrotic syndrome (Coombes, Shory, Borrera, Stastney, Eigenbrodt, Hull and Carter, 1971) and various skin rashes (Alpert et al., 1971). Trepo, Zuckerman, Bird and Prince (1974) have suggested that circulating immune complexes may be responsible for vasculitis or polyarteritis, but do not appear to be pathogenic for the liver. Vos, Grobbelaar and Milner (1973) have presented evidence that the HB Ag carrier state may predispose some patients to develop renal disease, their evidence being based on the observation that many carriers showed depressed C3-complement levels and that a relatively high percentage of patients with renal disease carried HB Ag.
HB$_s$ Ag Subtypes and Liver Disease

One of the earliest reports implicating a particular antigenic subtype with disease was a publication by Wenzel, Le Bouvier and Beam (1972) relating drug abuse and hepatitis in American marines. All positive sera possessed antigenic specificity "ay". Subsequent reports have shown a predominance of this subtype among drug abusers (Le Bouvier et al., 1972; Iwarson et al., 1973) and in association with serum hepatitis outbreaks in a number of renal dialysis units, including the exceptionally severe outbreak in Edinburgh (Marmion and Tonkin, 1972; Moseley, Edward, Meihaus, Gordon and Redecker, 1972). Gordon, Berberian and Stevenson (1972) were unable to find a correlation between the forms of hepatic injury and the antigenic subtype isolated from both acute and chronic hepatitis patients, while Feinman et al. (1973) found abnormalities of liver function and histology to be equally common in asymptomatic HB$_s$ Ag carriers of both 'ad' and 'ay' subtypes in Canada. A number of other studies, however, show that the 'y' specificity is frequently associated with clinical illness, while 'd' specificity is often associated with chronic asymptomatic carriage (Schmidt, Roberto and Lennette, 1972; Holland et al., 1972; Perry and Chaudhary, 1973; Iwarson et al., 1973; Sama, Krishnamurthy and Gurdeep, 1974). In a study relating the degree of liver damage in acute viral hepatitis to the HB$_s$ Ag subtype, Nielsen and Le Bouvier (1973) found the 'y' specificity in 79 percent of cases. However, the acute disease was more severe in patients possessing the 'd' specificity as judged by histological and biochemical criteria. A global pattern of subtype distribution seems to be emerging (see chapter 1, II). In America and most Western European countries
where the 'd' specificity predominates among asymptomatic carriers, 
the 'y' specificity is most frequently identified in clinically 
apparent hepatitis. It remains to be seen whether the reverse is 
true in those parts of the world where 'y' is the common specificity 
among asymptomatic (blood donor) carriers.
CHAPTER 4

TRANSMISSION OF HB$_3$Ag

For many years, it was assumed that serum hepatitis was only transmitted by parenteral routes (Proprct, 1938; Beeson, 1943; Turner, Snively, Grassman, Buchanan and Foster, 1944; Neefe, Stokes, Henhold and Lucke, 1944; Grassman, Stuart and Stokes, 1945; MacFarlane and Chesnay, 1944). The discovery of HB$_3$Ag led to renewed interest in the epidemiology of the disease.

I. Parenteral Transmission

In 1967, Ringertz and Zetterberg reported a number of cases among Swedish track-finders. It was suggested that spread of HB$_3$Ag was via bushes contaminated with blood and the communal wash basins shared by competitors, since only eight secondary cases developed from over six hundred index cases, suggesting that transmission was by parenteral means rather than the faecal-oral transmission associated with hepatitis A (infectious hepatitis). The increasing drug abuse among young adults, coupled with a tendency to share syringes, has led to a corresponding increase in the number of cases of infection among such communities (Cherubin, Hargrove and Prince, 1969;Bienicke and Nordenfelt, 1970; Nelson et al., 1971; Szmuness Bennett and Prince, 1971; Grady/Gulhane, Forrest, Iber and the Boston Inter-Hospital Liver Group, 1972), which provide a growing reservoir of potential infection to the community at large. Tattooing has been implicated as a parenteral route for transmission of HB$_3$Ag (Robertson, 1951; Sterner, Agell, Gerzen and Berg, 1971; Goatling, 1971; Scutt, 1972) as have dentists and barbers (Seowalka, Gnan, Krasanitski and Passendorfer, 1970; Kohn, 1970; Levin, Maddrey,
Wands and Mendeloff, 1974) and menstrual blood (Massur, 1973). Typing and control sera used as standard laboratory reagents have been shown to contain HB$_3$Ag (Ginsberg and Conrad, 1972; Welti, Heil and Miale, 1973).

Attention has been focussed on the possible role of insect vectors in the transmission of HB$_3$Ag. The high prevalence in the tropics (Blumberg, Gerstley, Sutnick, Hillman and London, 1970) may depend upon blood-sucking arthropods to ensure a high frequency of exposure during the early years of life when the risk of developing chronic HB$_3$Ag carriage is greatest (Samanessa, Pick and Prince, 1970). Prince, Metselaar, Kafuko, Mukwaya, Ling and Overby (1972) found HB$_3$Ag in 28 out of 187 pools of wild-caught mosquitoes from Kenya and Uganda. In controlled experiments with mosquitoes fed from a carrier, they were able to show that HB$_3$Ag disappeared in parallel with blood meal digestion, being present for at least 90 hours. They could find no evidence of replication inside the mosquito. These findings were subsequently confirmed by Byrom, Davidson, Draper and Zuckerman (1973). A recent report from Greece provides further evidence that mosquitoes may act as vectors for HB$_3$Ag (Papaevangelou and Kourea-Krematouinou, 1974).

There is only one, as yet unconfirmed, report that some species of mosquito may serve as biological vectors for HB$_3$Ag (Smith, Ogunba and Francis, 1972).

Zebe, Samwald and Rits (1972) have reported that transmission of HB$_3$Ag in a dialysis unit outbreak (Barker, Shulman, Murray, Hirschman, Ratner, Diefenbach and Geller, 1970) may have occurred via cockroaches. This possibility was tested experimentally by infecting cockroaches with HB$_3$Ag-positive serum. Antigen remained
detectable for long periods (10-15 days) in both vomitus and
haemolymph, suggesting a carrier state rather than just mechanical
contamination. It seems likely that other orders of blood-
sucking anthropods (ticks, lice, sand-flies, tse-tse flies etc.)
may play a similar role, their relative importance depending upon
the frequency with which they bite man. This may explain the higher
incidence of HB$_3$Ag in the lower socio-economic groups where over-
crowding is more likely to occur (Morrow et al., 1971).

II. Non(parenteral) Parenteral Transmission

It seems unlikely that a viral species should require modern
artificial mechanisms of transmission such as blood transfusion
and injections for its survival and propagation in nature. The
possibility of non-parenteral transmission of HB$_3$Ag is at present
being studied in many laboratories. Krugman et al. (1967) were
the first to demonstrate direct oral transmission of HB$_3$Ag using the
Willowbrook MS2 agent, while other reports indicate that laboratory
technicians have become infected by swallowing blood or serum while
pipetting specimens from HB$_3$Ag positive dialysis patients (Westwood,
Chaudhary and Perry, 1973). Infection with serum by mouth is,
however, not equivalent to faecal-oral transmission, and early work
with volunteers failed to show infection by the oral route with
extracts from the faeces of serum hepatitis patients (Neefe, 1946).
This finding may be explained in part by the presence of a "faecal
inhibitory factor" present in the human intestine (Piazza et al.,
1971), capable of neutralising the antigenicity of HB$_3$Ag. This
factor is neither an antibody nor interferon, and appears to be
species specific. In 1970, Prince, Hargrove, Samuness, Cherubin,
Fontana and Jeffries presented epidemiologic evidence suggesting
that the major proportion of HBsAg positive cases of acute hepatitis among urban adults were transmitted non-parenterally. Essentially similar conclusions were drawn by Szmuness and Prince (1971) on the basis of epidemiological analysis of viral hepatitis in Eastern Europe. Subsequently, a number of family studies have suggested that non-parenteral transmission may be a major factor in the home environment (Van, Spence and Gilmore, 1973; Garibaldi, Hatch, Bianco, Hatch and Gregg, 1972; Dietzmann, Madden, Seuer and Dunlop, 1973; Ricci, De Bac and Caramia, 1973; Bruguera, Bosch and Rodes, 1973; Bruguera, Bosch, Rodes and Pedrera, 1974), although parenteral transmission can never entirely be ruled out.

Marmion and Tonkin (1972) and Chalmers (1973) have reviewed possible routes of transmission specific for dialysis units. Some of these, e.g. blood transfusion, blood contaminated equipment, "needle sticks" and other types of tissue penetration seem quite obvious. Others such as contamination of conjunctiva or other mucosa, spillage of blood over intact skin, aerosol formation and exposure to contaminated linen have not yet been sufficiently documented. However, the findings of a point prevalence study in 15 American haemodialysis centres indicate that although HBsAg can be introduced into the dialysis centre by blood transfusion, the role of transfusion in its perpetuation may have been exaggerated (Szmuness, Prince, Grady, Mann, Levine, Freidman, Jacobs, Josephson, Kigot, Shapiro, Stenzel, Suki and Vyas, 1974). The uniformity of antigenic subtypes detectable during outbreaks of dialysis-associated hepatitis would also seem to argue against the predominant role of blood transfusion (Moseley et al., 1972). It appears that other, presumably non-parenteral, routes must exist,
and that they probably outweigh transfusions in relative importance. Data from this study also suggests that the parenteral route is not always responsible for clinical and asymptomatic hepatitis among dialysis centre staff, since after the first six months, the prevalence of hepatitis did not appear to increase with increasing length of employment. Nor did the cumulative presence of HB₃Ag and HB₃Ab differ among physicians, nurses, technicians and auxiliary personnel, suggesting that all personnel are heavily exposed to infection from the very start of employment. Non-parenteral mechanisms of virus transmission among both patients and staff are supported by the results of an environmental surface surveillance study using a swab-rinse assay in conjunction with radioimmunoassay (Favero, Maynard, Petersen, Boyer, Bond, Berquist and Szmuness, 1973). HB₃Ag was recovered from such surfaces as gloved hands, needle-clippers, furniture and external parts of dialysis equipment with and without traces of blood contamination, although it is disputable whether such material is infectious in practice.

Venereal transmission of HB₃Ag would seem to be a distinct possibility (Vahrman, 1973; Jeffries et al., 1973). Heaphre et al. (1974) found evidence of HB₃Ag transmission from antigen positive males to their intimate female contacts. Heathcote and Sherlock (1973) studied the spread of serum hepatitis in London, and concluded that sexual contact with an HB₃Ag carrier emerged as the single most important factor. In a later publication, HB₃Ag was identified in semen (Heathcote et al., 1974), a finding subsequently confirmed by Linneman and Goldberg (1974). Heathcote, Gateau and Sherlock (1974) presented evidence that exposure to HB₃Ag was more common among sexual partners than among blood relatives. They also found that contacts of carriers
with evidence of liver dysfunction are at greater risk of presumed non-parenteral exposure than are contacts of asymptomatic HBsAg carriers. Henigst (1973) found a significantly higher incidence of HBsAg (3 percent) among a group with frequently changing sexual partners, as compared with the normal population (0.4 percent) in Munich. Fulford, Dane, Catterall, Woolf and Denning (1973) have suggested that HBsAg may be primarily a sexually transmitted agent, and that such well recognized means of transmission as transfusion, the shared needles of drug users and tattooing, could be incidental and may not contribute significantly to the maintenance of the virus in the community. These parenteral routes may, however, carry a greater risk of clinical illness. Conversely, in a study of 449 family contacts from 197 households containing HBsAg carriers, Szmuness, Prince, Hirsch and Brotman (1973) concluded that sexual transmission does not appear to be of primary importance.

Data emerging from a number of laboratories suggest that non-parenteral transmission may account for the spread of HBsAg particularly in closed institutions (Kliman et al., 1971; Nelson et al., 1971; Szmuness, Prince, Etling and Pick, 1973; Bryan, Carr and Gregg, 1973; Medhurst, Madhavan and Quinn, 1973). Villarejos, Visona, Gutierrez and Rodriguez (1974) have reported HBsAg in the saliva of 76 percent of antigen positive acute hepatitis patients during the first three weeks after clinical onset, and in 86 percent of chronic carriers intermittently. The incidence of HBsAg positive "sneeze samples" from antigenaemic patients were found to be 35 percent. Antibody was found in saliva early in the clinical disease, a feature which may limit infectiousness via this route.
III. Transmission in Pregnancy

In 1954, Stokes, Berk, Malasut, Drake, Barondeas, Bashe, Wolman, Farquhar, Bevan, Drummond, Maycock, Capps and Bennett postulated that hepatitis could be transmitted from mother to foetus through the placenta. At that time, however, this concept could not be evaluated due to the lack of a suitable test for hepatitis virus identification. With the discovery of HBsAg, techniques became available with which to study transmission from mother to neonate.

Does transmission occur before, during or after birth? Krech and Sonnabend (1970) reported a 6 percent incidence of hepatitis in circumstances which suggested transmission during the foetal period or shortly after delivery. Prior to this, London, Di Figlia and Rodgers (1969) were unable to find evidence of transplacental transmission in the case of a young woman who became HBsAg positive six months before giving birth to an HBsAg negative baby. Smethick and Go (1970) were also unable to find evidence of transplacental transmission from a study of 2,225 cord sera. Keys, Nobel, Ritman, Oh, Gitnick and Hewitt (1971) suggested that transmission may also occur during passage through the birth canal or by maternal contact in the external environment. More recently, Buchholz, Frosner and Ziegler (1974) found HBsAg in the cord blood of an infant delivered by Caesarian section, thereby establishing that foetal infection can occur, probably after infection of the placenta. In 1972, Cossart, Hargreaves and March followed the pregnancies of 5 HBsAg-positive hepatitis patients and three asymptomatic carriers. All babies were HBsAg negative, suggesting that transplacental transmission did not occur, but two became positive at a later date. The mothers
of both these children remained HB\textsubscript{s}Ag positive after delivery, while the other mothers had either become HB\textsubscript{s}Ag negative or had not subsequently looked after their child. It is possible that infection occurred as a result of breast feeding, but these workers were unable to detect HB\textsubscript{s}Ag in milk, a finding confirmed by London \textit{et al.} (1969). However, Linneman and Goldberg (1974) have recently detected HB\textsubscript{s}Ag in the milk of an antigen positive mother. Schwietzer, Wing, McPeak and Spears (1972) found that of 26 women who developed HB\textsubscript{s}Ag-positive viral hepatitis during late pregnancy, or up to six months after delivery, 19 had HB\textsubscript{s}Ag positive cord blood, and 10 of their babies became HB\textsubscript{s}Ag positive. The antigen appeared to cross the placenta in three cases, and neonatal contamination at birth probably occurred in one. The following year, Schwietzer, Moseley, Ashcavi, Edwards and Overby (1973) published results of a transmission study involving infected symptomatic mothers and their offspring. It was concluded that the developmental period when exposure occurred, rather than the clinical status of the mother or the HB\textsubscript{s}Ag subtype, was the most important factor in determining whether the infant became chronically infected. The highest risk occurred when the antigen was present during the last three months before, or the first two months after delivery. Children of asymptomatic carriers are less likely to become chronically infected than those of mothers with clinical hepatitis. Transfer of some maternal factor such as antibody or anti-viral proteins, in the early gestational period, may lead to resolution of what otherwise could become a persistent infection. These findings have recently been confirmed in Britain by Dr. Yvonne Cossart (1974). Paulson, Rotini and Tomaro (1973) have reported transmission of HB\textsubscript{s}Ag and HB\textsubscript{s}Ab from mother to neonate during the later stages of gestation,
resulting in precipitating immune complexes as evidenced by a drop in the immunoglobulin level of the neonate. This passive antibody failed to protect. A follow-up study of 31 infants whose mothers had HB Ag positive acute hepatitis, while pregnant or in the first two months post partum, revealed that the most common response of the neonate was chronic HB Ag carriage coupled with histological features of unresolved hepatitis. Occasionally, acute hepatitis developed, but this was followed by rapid healing with removal of the antigen and the appearance of antibody. In a recent study, however, Dupuy, Frommel and Alogille (1975) have shown that fatal HB Ag hepatitis can result from maternal contamination.

Transfer of HB Ag from mother to offspring appears to be commonplace and provides a means of perpetuating and expanding the reservoir of infection. Post-natal rather than transplacental (which may occur rarely via a transplacental lesion) or parenteral transmission, would seem to represent the major hazard.

IV. Susceptibility

A genetic susceptibility to infection has been proposed by Blumberg, Friedlander, Woodside, Sutnick and London (1969) in which susceptibility to HB Ag is inherited as an autosomal recessive trait (see chapter 5). Petrakis (1972) has pointed out that in the above report there is a critical mating between HB Ag-positive parents which does not support the simple autosomal recessive hypothesis, since only two of seven offspring are HB Ag positive. It may be that the other five were not exposed. However, Vyas (1974) has provided the first discrete evidence against Blumberg's genetic hypothesis. In a family where both parents were HB Ag carriers, two of the three children were HB Ag and HB Ab negative, while the
remaining child possessed \( \text{HB}_a \text{Ab} \), indicating past exposure. If the recessive hypothesis had been correct, this child would have become a chronic \( \text{HB}_a \text{Ag} \) carrier. Results of a recent study in Greenland (Skindt, MoNeir and Anderson, 1974) suggest that variation in genetic susceptibility appears to be of secondary importance since the prevalence of \( \text{HB}_a \text{Ag} \) was found to vary 10-fold between different towns despite the existence of very uniform genetic, cultural and climatic conditions. The incidence of 7.1 percent among the normal population is similar to figures obtained from tropical countries and institutions for the mentally retarded (Prince et al., 1970; Blumberg et al., 1970). The common feature of both polar and tropical populations being a poor socio-economic environment.
MECHANISMS OF PATHOGENICITY

When an individual is exposed to HB$_s$Ag, a variety of responses may occur. He may develop antigen in his blood which may persist for a short or long time. He may develop specific antibody or immune complexes. These types of response may or may not be accompanied by clinical evidence of hepatitis. The manner in which a particular individual responds will be the result of an interaction between the host and the infecting agent. The nature of the interaction will be largely determined by the genetic make-up of the host and that of the agent. Blumberg et al. (1967) suggested that the HB$_s$Ag carrier state was the result of an inadequate immune response. He later proposed the following genetic hypothesis (Blumberg et al., 1969); there is a gene HB$_s$Ag$^1$ which when present in double dose confers susceptibility to persistence of the antigen. That is, individuals homozygous for this gene, when exposed to HB$_s$Ag, become chronic carriers, whereas persons with the alternative genotypes HB$_s$Ag$^1$/HB$_s$Ag$^0$ or HB$_s$Ag$^0$/HB$_s$Ag$^0$, when exposed are infected only for a short time. Dudley, Fox and Sherlock (1972) and Dudley, O'Shea and Sherlock (1973) put forward the theory that the outcome of infection with HB$_s$Ag may be determined by the competence of the cell-mediated (T-lymphocyte dependent) immune response. They used a leucocyte-migration-inhibition technique to measure cellular immunity to HB$_s$Ag in HB$_s$Ag-positive patients, patients with HB$_s$Ab, and normal controls (Dudley, Guistino and Sherlock, 1972). Cell-mediated immunity, specific for HB$_s$Ag, was only found in patients with a previous history of HB$_s$Ag infection.
These findings together with those of Laiwah (1971), Almeida and Waterson (1969) and Popper and MacKay (1972) are consistent with the view that liver cell injury is not a direct cytopathic effect of HBsAg but rather a result of the interaction of virus-infected liver cells and the products of the immune response, either antibodies or sensitised lymphocytes. Such a theory would seem to be in conflict with the findings of Keel, Eddleston, Cullens, Williams, Zuckerman, Peters, Williams and Maycock (1973) who infused high-titre antibody-containing plasma into chronic HBsAg carriers, with no resultant liver function test abnormalities, thus indicating that immune complexes do not, in this instance at least, play a part in hepatocellular damage. Sutnick, Bugbee, London, Loeb, Peyretti, Litwin and Blumberg (1973) showed there to be no difference between the lymphocyte response to phytohaemagglutinin between HBsAg carriers and normal donors. This led them to suggest that a general immunodeficiency state is not a prerequisite for developing persistent antigenaemia, and that T-lymphocyte abnormality, when present, may be a result of the underlying liver disease rather than a cause.

One obvious way that the virus may damage the liver cell is by causing sufficient functional disturbances to kill it. Alternatively, it might produce chronic hepatitis through some pathogenic immune mechanism:

1. Cellular material released as a result of viral infection may be immunogenic.

2. A virus which buds at the cell surface may incorporate cell membrane components which may then become immunogenic by virtue of their association with the virus.
(3) The cell membrane may be altered by incorporation of viral components which might be recognised as foreign.

(4) The continued release of virus particles from the cell surface might lead to a cell-mediated immune reaction or antigen-antibody complexes at the cell surface.

Alberti, Realdi, Tremolada and Cadrobi (1979) have demonstrated HBsAg on the cell surface in acute but not in chronic hepatitis. Chronic hepatitis is associated principally with lymphocyte infiltration, and as lymphocytes are mediators of the cellular type of immune response, the outcome of infection with HBsAg may be partially determined by the vigour of the cell-mediated response to the infection. Those with a vigorous cell-mediated response develop acute hepatitis. A few will be overwhelmed by the infection, but the majority recover and clear the antigen. Where there is little or no cell-mediated response, due either to antigen overload or immunosuppression, the virus will continue to proliferate causing little or no damage, and circulating antigen will persist. In the intermediate situations a partial cell-mediated response may occur which is sufficient to cause some liver damage but insufficient to control the growth of the virus. Continuous cycles of virus growth and host response would result in chronic active hepatitis. It is possible that a combination of immune pathogenic responses are in operation, providing an interesting theory which requires further study. Gudat, Bianchi, Sonnabend, Thiel, Aenishaemalin and Stalder (1975) have investigated the appearance of HBsAg in liver cell nuclei, and HBsAg in cytoplasm
in order to identify expression patterns which, together with histologic parameters, could be integrated into four reaction types of diagnostic and prognostic importance.

In 1969, Almeida et al. reported the presence of massive immune complexes in the serum of a fatal case of fulminant hepatitis, and suggested a similarity to serum sickness, a condition where severity depends upon the balance between antigen and antibody. This theory appeared to be supported by the finding of HB$_s$Ab or immune complexes in 90 percent of cases of acute serum hepatitis and 100 percent of chronic serum hepatitis patients (Brzosko, Madalinski, Krawczynski, Skwarska and Nowasowski, 1971). Recently, Madalinski, Sztachelska-Budkowska and Brzosko (1974) dissociated immune complexes from patients with acute and chronic hepatitis, using DEAE-cellulose chromatography, and showed that the antibodies belonged to the three major classes of immunoglobulins: IgG, IgM and IgA. The deposition of immune complexes in certain sites is probably responsible for non-hepatic complications. Grob, Jemelka and Muller (1971) found that HB$_s$Ag-positive patients with active chronic hepatitis had a decreased component of C3 complement more often than HB$_s$Ag-negative patients. Nydegger, Lambert, Gerber and Miescher (1974) used $^{125}$I-labelled C1q to detect the presence of immune complexes in HB$_s$Ag carriers. No increased binding of label was found among 18 asymptomatic carriers, but 20 percent to 60 percent of sera from acute transient and chronic persistent hepatitis patients were found to bind C1q, suggesting the presence of immune complexes. In a recent letter to Lancet, Kater, Schmitz-du Moulin and Borst-Eilers (1974) presented evidence that IgG deposited in the sinusoids of HB$_s$Ag-positive hepatitis patients was either HB$_s$Ab or
HBcAb, thereby adding support to the hypothesis that immune complexes play a decisive role in the course of serum hepatitis. However, Prince and Trepo (1971) found only a weak correlation between the presence of immune complexes in the serum and liver damage, while Moulias et al. (1975) were unable to find any such association. In the few instances where high titre HBsAb plasma has been infused into HBsAg-positive patients or carriers, there has been no evidence of hepatocellular damage directly related to the formation of immune complexes (Lepore, McKenna, Martinez, Stutman, Bonanno and Conklin, 1972; Read et al., 1973).
CHAPTER 6

CONTROL MEASURES

I. Introduction

Control of serum hepatitis at present relies largely on the observance of safety precautions by hospital and laboratory personnel, screening of blood donations and the education of certain sections of the public, particularly drug abusers. Following outbreaks of severe liver disease among hospital and laboratory personnel, stringent safety precautions have been set out (Sutnick, London, Millman, Gerstley and Blumberg, 1971; Percy-Robb, Proffitt and Whitby, 1970). The most important of these is the provision of adequate facilities for the handling and disposal of potentially infective material, the observance of strict hygiene among staff members, with prohibition of mouth pipetting and the liberal use of effective antiseptics. Renal dialysis units may be classified according to whether they are HBsAg positive or negative, with appropriate restrictions on patients and staff.

II. Blood Donor Testing

There appears to be disagreement over the effectiveness of blood donor testing in reducing the incidence of posttransfusion hepatitis. A significant reduction has been reported in the East of Scotland (Cameron, 1973, personal communication) and North London (Cleghorn, 1973, personal communication) Regional Transfusion Centres. The most dramatic effect was reported at the Philadelphia General Hospital (U.S.A.) (Senior, Sutnick, Goeser, London, Dahlke and Blumberg, 1974) where the incidence was reduced from 17.9 percent to 5.6 percent by the use of I.D. for testing donor blood. On the other hand, Rienicke,
Banke and Dybkjaer (1973) and Arnt-Hanser and Pyke (1973) both report that systematic removal of HBsAg-positive blood detected by I.D. and C.I.E.O.P. failed to reduce the incidence of post-transfusion hepatitis. It may be that more sensitive techniques are required before this complication is significantly reduced in all recipient populations. Haemagglutination (HA) and radio-immunoassay (RIA) are capable of detecting HBsAg below the sensitivity of I.D. and C.I.E.O.P. (Shaffer et al., 1972; Ginsberg, Conrad, Bancroft, Ling and Overby, 1972). Despite the improved sensitivity, Hollinger, Asch, Gitnick, Roche and Melnick (1973) have suggested that the large scale use of RIA would not result in a major reduction in cases of posttransfusion hepatitis, a suggestion which would seem to be borne out by the findings of Jennings, De Pratti, Monroe, Pollock, Watson-Williams and Claus (1973) who compared RIA and C.I.E.O.P. for detection of HBsAg among blood donors in California, and concluded that RIA would be of little value to Blood Transfusion Services in reducing the incidence of clinical hepatitis following transfusion.

III. Passive Immunisation

Normal gamma globulin (prepared by the fractionation of pooled plasma) does not appear to be effective in the prevention of post-transfusion hepatitis. A field trial conducted in 1945 (Crossman, et al.) and a second trial reported by Mirick, Ward and McGollum (1965) suggested that normal gamma globulin could prevent or modify posttransfusion hepatitis. However, volunteer studies (Drake, Barondess, Bashe, Henle, Henle, Stokes and Fennell, 1953) and a small clinical study (Holland, Robinson, Morrow and Schmidt, 1966) did not support this conclusion. The results of a
recent co-operative study (Grossman et al., 1970) have indicated that normal gamma globulin is entirely without value in preventing or modifying posttransfusion hepatitis. Preliminary results using gamma globulin prepared by fractionation of plasma known to contain specific HBsAb demonstrated passive though not necessarily protective immunity in man (Prince, Szmuness, Woods and Grady, 1971; Gocke, 1971; Conrad, Young, Park, Dinger, Gerling, Bickley, Boykins, Tidwell, Petty and Knowles, 1971; Krugman, Giles and Hammond, 1971). Soulies, Blatix, Benamon, Courouce, Amouch and Drouet (1972) at the National Transfusion Centre in Paris have prepared hepatitis B immune globulin and administered it to 27 subjects, 19 of whom had received a transfusion later found to contain HBsAg, and 8 of whom had been accidentally exposed (needle-sticks) to antigen-containing blood. The hyper-immune globulin was administered between three hours and seven days after exposure. None of the 27 recipients developed jaundice. Follow-up of 18 subjects showed significant transaminase elevations in only 2 subjects and HBsAg was not detected in any of the 18 cases. It was concluded that passive immunisation appeared to be both effective and innocuous. Despite these encouraging reports, the use of hyper-immune HBsAb globulin, whether administered to the patient or to the blood prior to transfusion (Katz, Rodrigues and Ward, 1971), must proceed with caution, since in theory its administration could attenuate the disease and lead to an increased risk of producing a chronic carrier state with associated liver disease. Lepore et al. (1972) infused high titre HBsAb plasma (220-350 mls per day) for three days into a woman who developed HBsAg-positive fulminant hepatitis with coma shortly after giving birth. A dramatic clinical
response ensued during which the patient's blood became HBAg negative and she continued to make a steady and ultimately complete recovery. The following year, Read et al. (1973) investigated the effect of infusing an immunoglobulin preparation with a high titre of HBAb. Six patients with active chronic hepatitis received antibody intravenously. The infusions were well tolerated and only two patients showed evidence of minor and transient immune complex reactions. One patient was HBAg negative at the start of the investigation and he was the only one to show antibody, detectable by I.D., after infusion. Where the initial HBAg titres were high, little change in titre was noticed, while in two patients with initially low titres, HBAg was cleared from the circulation for up to 9 days. No patient showed abnormal liver function test results, suggesting that immune complexes play only a minor role, if any, in causing hepatocellular damage.

IV. Active Immunisation

Krugman and Giles (1973) investigated the effect of active as well as passive immunisation, and reported that both were associated with a protective effect, greater attenuation of the infecting agent and a decrease in the chronic carrier rate. As early as 1971, Krugman, Giles and Hammond produced relative protection by active immunisation with a boiled MS-2 virus preparation. De Gast, Houwen and Nieweg (1973) have shown specific lymphocyte stimulation by heat inactivated HBAg, suggesting that vaccination with such a preparation might induce not only humoral but also cell-mediated immunity. Dreesman et al. (1973) have isolated a small polypeptide by acid treatment of reduced non-alkylated HBAg. This polypeptide retained antigenic activity and may provide a source of material for development
of a hepatitis B vaccine (see General Discussion). The preparation of active subunits of HB₃Ag has also been described by Rao and Vyas (1973) using ultra-centrifugation and chromatography. Such lines of research may lead to the preparation of a synthetic material containing the immunogenic reagent, since there is little doubt that polypeptides and other moieties such as lipoproteins can be attached to a macro-molecular carrier and used for immunisation (Sela, 1966).

V. Transfer Factor

Vyas, Ibrahim, Rao and Likhite (1974) claim to have achieved in vitro transfer of cell-mediated immunity to non-immune cells, as measured by inhibition of leucocyte migration in the presence of HB₃Ag, using RNA extracted from the lymphoid tissue of immune guinea pigs. Bearing in mind the immune RNA-mediated transfer of delayed skin reactivity to tuberculin, varidase and monilia antigens (Han, 1973), these findings hold promise for clinical application of systemic transfer of immunity to HB₃Ag in man.

VI. Miscellaneous

The work of Tullis, Himaan, Sproul and Nickerson (1970) suggests that the incidence of posttransfusion hepatitis can be greatly reduced by the use of frozen blood. This work has been confirmed by the results of Hayashi, Nakamura and Giorgi (1971) and Carr, De Quesada and Shires (1973). However, Werch, Grey, Hersh and Melnick (1971) found that HB₃Ag could still be detected in frozen reconstituted cells if the donor had an unusually high HB₃Ag titre.

Lo Grippo (1969) has shown that biological products may be effectively sterilised by treatment with beta-propiolactone, particularly if this is combined with exposure to ultraviolet light.
Clinical trials including both active and passive immunisation are still in progress, and until such data have been analysed, prevention of serum hepatitis must depend largely upon the observance of measures that limit the transfer of \( \text{HB}_3 \text{Ag} \) from one individual to another by any of the proven or implied routes.
I. Hepatitis B

A number of workers have found HB\textsubscript{Ag} and HB\textsubscript{Ab} among apparently naturally infected chimpanzees and gibbons (Maynard, Hartnell and Berquist, 1971; Lichter, 1969), and several subsequent studies have demonstrated the susceptibility of chimpanzees to type B hepatitis (Maynard, Berquist and Krushak, 1972; Barker, Chisari, McGrath, Dalgard, Kirschstein, Almeida, Edgington, Sharpe and Peterson, 1973; Desmyter, Liu, Somer and Mortelmans, 1973). Disease in these animals is generally mild, accompanied by enzyme elevations and with an absence of icterus or significant clinical signs (Maynard et al., 1972; Barker et al., 1973), while serologic events are remarkably similar to those seen in man (Barker, Maynard, Purcell, Hoofnagle, Berquist, London, Gerety and Krushak, 1975).

Areas in which the chimpanzee will prove a useful animal model include preliminary evaluation of the safety and effectiveness of HB\textsubscript{IgG} and vaccines, assessment of methods of eliminating HB\textsubscript{Ag} from blood and blood products, and in the critical evaluation of infectious material for subsequent animal challenge studies.

Barker, Almeida, Hoofnagle, Gerety, Jackson and McGrath (1974) have isolated and purified HB\textsubscript{Ag} from the liver of an immunosuppressed chimpanzee which died of pneumonia following inoculation with HB\textsubscript{Ag}. Chimpanzees may, therefore, become an important if somewhat expensive source of HB\textsubscript{Ag}.
II. Hepatitis A

Although transmission of hepatitis A to Fatas monkeys was documented by Beazeroft in 1968 and 1969, the most convincing evidence was obtained by Deinhardt, Holmes, Capps and Popper (1966) who first reported the development of biochemically and histologically typical hepatitis A in Saguinus fuscicollis, Saguinus nigricollis and Saguinus adipus marmosets. In subsequent experiments Holmes, Wolfe, Deinhardt and Conrad (1971) were able to repeatedly produce hepatitis in marmosets inoculated with acute phase serum from volunteers infected with the MS-1 strain of hepatitis A, but not with normal serum. Mascoli, Ittensohn, Villetayos, Arguedas, Provost and Hilleman (1973) infected marmosets using specimens from patients acutely ill with epidemiologically proven hepatitis A in Costa Rica. They were able to produce hepatitis and transmit the agent serially. Convalescent serum (but not pre-exposure or acute phase serum) from patients involved in the original outbreak was found to be protective.

Purcell, Feinstone and Kapikian (1974) used immune electron microscopy to study hepatitis A. Stool extracts from patients infected with the MS-1 strain were reacted with convalescent serum from one of the volunteers. Small 27 nm virus-like particles coated with antibody were visualised in acute phase specimens but not in pre-infection specimens from five out of eight volunteers.
SECTION TWO

TECHNIQUES CURRENTLY AVAILABLE FOR HBsAg TESTING

Introduction

The need for a simple but sensitive technique for testing large numbers of blood donors on one hand, and a very sensitive yet specific research tool on the other, has led to the development of a number of tests that differ greatly in sensitivity, specificity, simplicity and cost; each possessing its own characteristic advantages and disadvantages. Each of the techniques to be considered has been shown to be sufficiently specific, provided appropriate controls are included. It is proposed to group the various techniques into chapters according to the type of reaction involved.
CHAPTER 1
PRECIPITATION REACTIONS IN AGAROSE

I. **Immunodiffusion (I.D.)**

This is the technique by which HB\textsubscript{s}Ag was first detected (Blumberg, 1964), and is still employed in many laboratories because it is simple and specific and provides a useful means of establishing identity. The reactants diffuse through a medium of approximately 1 percent agarose to produce visible precipitation lines where HB\textsubscript{s}Ag and HB\textsubscript{s}Ab meet in optimal proportions. Reagents are dispensed, via capillary tubes, into wells cut in the medium. Wells may be replaced by filter paper discs soaked in reagent and placed on the agarose surface (Dequessney and Becker, 1970).

Quantitation of HB\textsubscript{s}Ag is possible by allowing diffusion to occur through a medium into which HB\textsubscript{s}Ab is incorporated. The distance from the centre of the antigen well to the ring of precipitation is proportional to the antigen concentration. The sensitivity of I.D. may be increased in a number of ways:

1. Pre-filling or topping-up wells (Kim and Tilles, 1971).
2. Concentrating reagents e.g. with lyphogel (Ashoovhi and Peters, 1971).
3. Use of an enhancement or reinforcement pattern (testing each serum adjacent to a positive serum) (Schmidt and Bennette, 1972).
4. Use of a closed system of hexagonal wells in conjunction with 8 percent polyethylene glycol has been claimed to increase sensitivity by a factor of 8 to 16-fold (Traavik, Siebke and Kjoldsberg, 1972).
5. Controlled evaporation from the reagent surface, a process termed 'rheophoresis' (Holper and Jambasian, 1972).

Despite the many possible modifications, I.D. remains a slow, insensitive technique uneconomic in terms of reagents. However, because of its ability to produce reactions of identity, it remains a popular method of subtyping both HB$_s$Ag and HB$_s$Ab, as indicated by precipitation 'spurs' (Levane et al., 1969; Gust, 1971; Le Bouvier, 1971; Boenisch and Katz, 1971; Kaplan and Grady, 1971; Mazzur, Falkor and Blumberg, 1972; Le Bouvier, 1972; Le Bouvier et al., 1972; Gordon et al., 1972; Magnus and Espmark, 1972; Wensel et al., 1972; Bancroft et al., 1972).

II. Counter-electrophoresis (C.I.E.O.P.)

This technique replaced I.D. as the most widely used in large scale testing for HB$_s$Ag. Pairs of wells, usually 2 mm. in diameter and 3 mm. apart, are cut in agarose. Test serum (cathodic well) is caused to migrate in an electric field through a suitable medium of diffusion against a stream of HB$_s$Ab (anodic well) migrating in the opposite direction as a result of endosmotic flow. This technique was first applied successfully to the detection of HB$_s$Ag and HB$_s$Ab by Bedarida, Trinchieri and Carbonara (1969). Since then a number of modifications have been described (Gocks and Howe, 1970; Passendorfer, Krasnitsky and Wewalka, 1970; Prince and Burke, 1970; Vergani, 1971). The technique has also been modified for simultaneous detection of HB$_s$Ag and HB$_s$Ab by interposing the test sample between an HB$_s$Ab-containing well on one side and an HB$_s$Ag-containing well on the other (Suckerman and Taylor, 1970). However, this may lead to the crossing-over of one of the reactants, resulting
in the formation of a false positive. A discontinuous buffer system in which buffer in the gel is at a lower ionic strength than that in the electrode vessel is reputed to improve sensitivity (Combridge and Shaw, 1970). Sensitivity may also be improved by diluting reagents in homologous species normal serum (Dreesman, Hollinger and Melnick, 1972) and by staining plates after testing with tannic acid (Hopkins and Das, 1972). Rearrangement of the wells allows counter-electrophoresis to produce reactions of identity and so be applicable to subtyping (Das, Hopkins, Cash and Cuming, 1971). The principle advantage of C.I.E.O.P. over I.D. is speed, since results are available within two hours. Both techniques suffer from the disadvantage of requiring a fixed HB Ag-HB Ab ratio and consequently do not take into account the possibility of false negative reactions due to either HB Ag or HB Ab in excess (Kohn, 1970). This source of potential errors may be eliminated by using an inhibition reaction, which is also claimed to increase sensitivity (Milner, Dobie and Grobbelaar, 1972). False positive reactions may occur due to the reaction of other antigen–antibody precipitating systems such as anti-ruminant antibody (Alter, Polesky and Holland, 1972; Lama, M., Krakur, Greenwalt and Levin, 1974) and lipoprotein isoprecipitins (Verrucci, Blumberg and Morganti, 1970; Blumberg, Hann, London and Yin, 1974). All positive reactions should be confirmed by tests of identity. Holland et al. (1972) have used C.I.E.O.P. for subtyping, using antiserum rendered monospecific by absorption.
CHAPTER 2
AGGLUTINATION REACTIONS

I. Latex Agglutination

Latex agglutination is now an accepted test for the presence of rheumatoid arthritis factor (Singer and Plotz, 1956) and is widely used as a test for human chorionic gonadotrophin in the urine of pregnant women (Graham and Kauah, 1967). In 1971, Leach and Ruck evaluated a commercial latex agglutination test developed by Pfizer Limited for the detection of HB\textsubscript{s}Ag. Antibody raised in guinea pigs by immunisation with purified HB\textsubscript{s}Ag was used to coat an 0.5 percent suspension of 0.8 μ, polystyrene latex spheres. The latex was stabilised with bovine serum albumin and preserved with 0.1 percent sodium ascorbate. The preparation appeared to remain stable at 4°C for at least three months. Test procedure involved 25 ul of test serum being mixed with 25 μl of normal guinea pig serum on a glass slide, before the addition of 25 μl of the antibody-coated latex reagent. The slide was then rocked to and fro for 5 minutes, by which time an HB\textsubscript{s}Ag-positive serum showed definite agglutination.

Hopkins and Das (1974) have evaluated latex reagents from two commercial sources (Pfizer Limited and Hoechst Limited (rabbit HB\textsubscript{s}Ab)) and compared them with G.I.E.O.P. for sensitivity and specificity, using sera from blood donors, patients and plasma fractions. Good agreement was obtained when testing blood donations; however, false reactions were observed with both latex preparations among hepatitis patients' sera. The small number of false negative reactions encountered were probably due to the subtype of the
antibody on the latex particles, rather than a lack of sensitivity relative to C.I.E.O.P. False positive reactions were encountered with both kits, but were more frequent with the Hoechst reagent. Such reactions may be caused by a number of factors, such as incomplete clotting of plasma or the presence of rheumatoid factor (Burrell, Dickson, Gerber, McCormick and Marmon, 1972; Ziegenfuss, 1972). Heating the test serum at 60°C for 10 minutes is claimed to reduce the false positive rate to about 3 percent (Perkins, Perkins and Vyas, 1974). The main advantage of latex agglutination is speed. In their present state of development, however, commercial latex agglutination tests for HBsAg would seem to be unsuitable for use on patient's sera due to lack of specificity. This drawback may be partially overcome by use of a 'control' latex preparation coated with normal immunoglobulin, although the simultaneous presence of HBsAg and non-specific agglutinins could not be ruled out.

Malin and Edwards (1972) claim to have improved specificity by coating 0.5 µm diameter latex spheres with antibody of human origin. These reagents retained sensitivity for up to eight weeks when stored at 4°C. Stevens, Ziegler and Kelly (1972) described a charcoal particle agglutination-inhibition technique in which antigen was absorbed to particulate charcoal. As with latex agglutination, the HBsAg-HBsAb complexes caused macroscopic agglutination. The test appeared capable of detecting 1.5 µg of HBsAg protein per ml, making it about 10 times more sensitive than C.I.E.O.P. False positive reactions did occur.

Kachani and Gocke (1973) described an agglutination-flocculation test (AFT) for detection of HBsAg. This test involved the
simultaneous agglutination and flocculation of HBsAg by HBsAb in the presence of 0.5 μm diameter acrylic particles. Preparation of reagents required the sensitisation of acrylic particles with varying concentrations of hyperimmune HBsAb gamma globulin in borate buffer. Sensitisation was carried out at room temperature and was followed, after 1 hour, by the addition of 5 percent bovine serum albumin. The antibody-coated particles were not washed, as the presence of excess antibody in the fluid phase produced simultaneous flocculation, leading to improved sensitivity. Test sera were heated at 56°C for 10 minutes, then 25 μl. placed into each of six wells in a standard agglutination tray, and 25 μl. of foetal calf serum added to prevent weak non-specific reactions. 25 μl. of bromophenol blue was added to each well before mixing with an applicator stick. Finally, 25 μl. of each of the antibody-coated particle preparations was added to the appropriate wells and the plate mixed on a rotostat for 30 minutes before being read.

The authors claimed the technique to be 3-fold more sensitive than RIA and 1000-fold more sensitive than C.I.E.O.P. There are, however, numerous disadvantages which make this test unsuitable for blood donor testing:

1. Each sample must be tested a number of times, against different concentrations of antibody, to overcome prozone reactions.
2. High-titre rheumatoid sera are a source of false positive reactions.
3. Hyperimmune antibody of high specific activity is crucial for coating the acrylic particles. This would necessitate the immunisation of animals with pure HBsAg, and require fractionation of the resultant antibody.
4. Sera need to be heated prior to testing to remove a thermolabile inhibitor of agglutination-flocculation which is present in most normal sera.

II. Haemagglutination (HA) and Haemagglutination-Inhibition (HAI).

In 1969, Juji and Yokahi published details of an HA technique in which formalinised erythrocytes were sensitised with purified HB$_s$Ab and used to detect HB$_s$Ag by direct haemagglutination*. The purified HB$_s$Ab was prepared by a complex procedure involving the formation of immune complexes, which were then dissociated and the HB$_s$Ab isolated by centrifugation and chromatography. Formalinised group 'O' Rh positive human erythrocytes were tanned and coated with the purified HB$_s$Ab. The sensitised cells were resuspended to 0.5 percent in phosphate buffered saline (P.B.S.), pH 7.2, containing 1 percent normal rabbit serum. Tests were conducted in disposable plates with 'U'-bottomed wells, reagents being added in 25 µl volumes. After mixing the plates were left overnight at room temperature. This technique was found to be more sensitive than I.D., but unfortunately it was not evaluated for use in mass donor testing due to a high incidence of false positive reactions among the negative controls. It was also found that the sensitised cells deteriorated rapidly if stored at 4°C.

The following year, Vyas and Shulman (1970) described an HAI test in which fresh human erythrocytes were sensitised with highly purified HB$_s$Ag. These cells were used for measuring HB$_s$Ab

* sometimes called reverse passive haemagglutination (RPHA).
by direct HA and HB _Ag_ by an inhibition reaction. Plasma was collected from asymptomatic HB _Ag_ carriers and the antigen isolated by ultra-centrifugation using density gradients. The purified antigen gave a single band in agarose and, when concentrated 10-fold, gave no precipitation reaction with rabbit antiserum against whole human plasma. Human group '0' erythrocytes, collected in anticoagulant and washed in saline, were made up to a 40 percent suspension and incubated with purified antigen and chronic chloride, the latter acting as a coupling reagent. A factor affecting sensitivity of the coated cells was found to be the temperature at which sensitization occurred, this being optimum between 22°C and 41°C (Hawkes, 1973). The sensitised cells were made up to a 0.2 percent suspension in FBS pH 7.3 containing 0.5 percent bovine serum albumin, 0.0025 percent polyvinylpyrolidine (PVP) and Tween 80 at a concentration of 1 in 20,000. The tests were conducted in microtitre plates with 'V'-bottomed wells, using 25 µl volumes of reagents. The inhibition reaction, used to test for HB _Ag,_ used 10 units of HB _Ab_ activity. Sensitivity for HB _Ab_ detection was approximately 3000 times greater than I.D., while sensitivity for HB _Ag_ was approximately 120 times that of C.I.E.O.P. The technique initially gained acceptance in many laboratories since its sensitivity compared favourably with C.I.E.O.P. (Shaffer et al., 1972). To achieve reproducible results, Prince et al. (1974) found it necessary to store the sensitised cells in liquid nitrogen until required. Reesink, Duijvel and Brusselmans (1973) also found it necessary to modify the technique in order to obtain a degree of reproducibility. They recommend that the following conditions should be observed:
1. Careful selection of donor cells, as only about 5 percent of donor blood is suitable for sensitisation.

2. Improvement of the chronic chloride coupling, allowing a less pure HB Ag preparation to be used. Their proposed method of antigen purification involved Freon extraction, absorption and elution from Aerosil, gel filtration and extensive dialysis followed by concentration.

3. The use of a special preserving fluid which allowed the sensitised cells to be stored at 4°C for up to six weeks.

Karak, Sturgeon and Gitnick (1973) have successfully automated the test using a sampling rate of 60 tests per hours, results being available within 30 minutes. Gold et al. (1974) have applied the test to subtyping HB Ag in terms of anti-d and anti-y specificity by using a blocking antigen of known subtype.

In an attempt to circumvent the need for density gradient centrifugation, Nelson, Phipps, Watson, Watts and Zwolenski (1973) have extracted HB Ag from serum with Aerosil 200 and subsequently purified it by column fractionation, pepsin digestion and filtration through a 0.22 μm millipore filter. Prior to sensitisation the HB Ag was inactivated by heating at 95°C for one minute. The sensitised cells were found to be unstable unless stored in liquid nitrogen. A cell suspension of 2.5 percent was used in a Technicon autoanalyzer, the agglutination pattern being recorded on absorbant paper. Sensitivity for HB Ag detection was only 4 times greater than C.I.E.O.P., while the sensitivity for HB Ab detection was 32 times better.

Since publication of the work of Juji and Yokochi (1969), the literature has, until recently, contained no reports concerning the
use of HB$_3$Ab-coated cells for the detection of HB$_3$Ag. In 1973, however, Hirata, Emerick and Bolesy published details of such a technique using purified guinea pig antibody coupled to double-aldehyde-treated cells. Human type '0' erythrocytes were stabilised by treatment with pyruvic aldehyde followed by formaldehyde. The stabilised cells were then incubated with a relatively low ionic strength buffer (0.1M phosphate, pH 7.2) containing antibody. Test sera were diluted in phosphate buffer containing gelatin, and tests conducted in 'v'-bottomed microtitre plates. Sensitivity was reported to be considerably greater than G.I.E.O.P. The test has been modified to subtype HB$_3$Ag by coating cells with monospecific antibody prepared with the aid of an immunoabsorbant (Hollinger, Wabi, Dresman and Melnick, 1973).

At the present time, three commercial companies are offering HB$_3$Ag test kits based on this principle. Table 1(2)2.1 compares the characteristics of each of the products.

Addendum.

In 1969 Avrameas, Taudou and Chillon (Imunochemistry, 5, 67) published data concerning different methods for coupling antigens to erythrocytes.
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CHAPTER 3

RADIOIMMUNOASSAY (RIA)

I. Introduction

A number of techniques have been described, most of which use a radioactive marker, usually HBsAg labelled with $^{125}$Iodine. HBsAg is purified by ultra-centrifugation and gel chromatography before being radiolabelled by the chloramine-T method of Hunter and Greenwood (1962). After labelling, it is necessary to separate free label and labelled serum proteins from labelled HBsAg. This is usually achieved by column chromatography. Most radioimmunoassays are based upon competition for antibody binding sites between standard labelled antigen and any unlabelled antigen which may be present in the serum being tested. The major difference between techniques is the manner in which the immune precipitate of antibody-bound HBsAg is separated from free HBsAg after incubation.

II. Double Antibody Precipitation (D.A.-RIA)

The commonest means of separation is the double antibody technique (Aach, Grisham and Parker, 1971; Coller, Millman, Halbnerr and Blumberg, 1971; Hollinger, Vornan and Breessman, 1971; Lander, Alter and Purcell, 1971). The serum to be tested is incubated with serum containing HBsAb and labelled HBsAg. This is followed by the addition of an anti-gamma-globulin to co-precipitate the HBsAg bound to the first antibody. After incubation the co-precipitate is isolated by centrifugation and counted in an automatic gamma counter. If HBsAg is present in the test serum it will compete with the labelled HBsAg for HBsAb combining sites, thereby reducing the amount of radioactivity in the co-precipitate as compared to simultaneously run negative sera. Detection of circulating HBsAb involves
incubating the test serum with labelled HB$_s$Ag and then adding an anti-human-IgG. Immune complexes are isolated and counted as for HB$_s$Ag detection. If HB$_s$Ab is present in the test sample, it binds labelled HB$_s$Ag causing a marked increase in radioactivity in the co-precipitate in comparison to control sera. Walsh, Yalow and Berson (1970) used paper chromatoelectrophoresis for the separation of free and antibody bound HB$_s$Ag. This technique takes advantage of the differences in mobility of free and bound HB$_s$Ag in an electric field.

A major disadvantage of D.A.-RIA is the time taken to complete the test, since the standard procedure for detection of the HB$_s$Ag requires 2 to 4 days.

III. **Solid Phase (S.P.-RIA)**

Attempts to reduce the time factor have led to the development of solid phase techniques (Ling and Overby, 1972; Ginsberg, Bancroft and Conrad, 1972). One such test (marketed by Abbott Laboratories Ltd., under the name AUSRIA-I) involves placing 0.1 ml. of test sample on the bottom of a polypropylene tube coated with purified hyperimmune HB$_s$Ab (usually raised in guinea pigs). The tubes are then capped and incubated for 3 to 16 hours, depending on temperature. The serum sample is aspirated and the bottom of the tube rinsed 5 times with tris-HCl buffer before the addition of 0.1 ml. of $^{125}$I-labelled HB$_s$Ab. The tube is then capped and incubated for a further 90 minutes, after which it is once again rinsed 5 times and counted in an automatic gamma counter for 1 minute. For each group of tests 10 control tubes containing normal human serum are included and the standard deviation of the counts of the negative control tubes computed. HB$_s$Ag present in the test serum should complex with the
specific HBsAb in the tube and not be removed during washing. The labelled antibody should then complex with another site on the bound HBsAg particle and remain in the tube during subsequent washing, thus raising the radioactivity count above that of the negative control tubes. In theory this technique should be capable of detecting HBsAg having only two HBsAb-binding sites available. This may be somewhat optimistic, although in practice the test has proved to be considerably more sensitive than C.I.E.O.P. (Hansson and Johnsson, 1974). Count rates for the negative control tubes are generally less than 0.3 percent of the radioactivity used. A factor of 5 standard deviations from the negative control mean is used to determine whether a serum is positive or negative, i.e. sera having count rates higher than the mean of the negative controls plus 5 standard deviations are considered positive. AUSRIA-1 has recently been superseded by an improved kit (AUSRIA-2) in which the unlabelled HBsAb is absorbed on to polystyrene beads resulting in an increased surface area and a theoretically improved sensitivity. The solid phase system can be employed for the detection of HBsAb by using HBsAg-coated beads in conjunction with labelled HBsAg (Ginsberg et al., 1972). Purcell, Wong, Alter and Holland (1972) adapted the S.P.-RIA for use with microtitre serological equipment. Polyvinyl 'V'-bottomed microtitre plates serve as the solid phase into each well of which 75 µl. of an appropriate dilution of HBsAb was added and the plate incubated for 4 hours at 4°C. Residual HBsAb was aspirated by washing twice with saline and 0.2 ml. of bovine serum albumin added. After a further overnight incubation at 4°C the plates were again washed with saline and the residual fluid aspirated from the wells. The test was performed by adding 25 µl. of sample to the bottom of each well, followed by an overnight incubation at 4°C. The following
day the wells were washed 5 times with saline, and 50 μl. of $^{125}$I-labelled HB$_s$Ag added. The plate was then incubated by rocking gently at 37°C for 4 hours. The wells were once again washed with saline before being cut out and placed in the tube of an automatic gamma counter and counted for 4 to 20 minutes each. Results were expressed as the ratio of residual counts in the sample well to the mean residual count of wells that received a sample known to be HB$_s$Ag negative. Radio-labelled HB$_s$Ab was prepared from both human and guinea pig sera. The most sensitive system was obtained using HB$_s$Ab of guinea pig origin, although this system also exhibited many false positive results. When human HB$_s$Ab was used specificity improved but at the expense of sensitivity.

Figenschau and Ulstrup (1974) have described a technique in which Protein-A-carrying Staphylococcus aureus is used as a solid phase anti-gamma globulin reagent. The test is easy to perform, being complete in two hours. The competition principle is used in the test for HB$_s$Ag, while HB$_s$Ab is tested by the direct binding of labelled HB$_s$Ag.

IV. Radioelectrocomplexing

Simons (1973) described a simple, sensitive, rapid and relatively inexpensive radioelectrocomplexing technique which involved complexing radio-labelled HB$_s$Ag or HB$_s$Ab with unlabelled HB$_s$Ag or HB$_s$Ab as a result of counter-directional movement produced during electrophoresis. Microscope slides were covered with 4 ml. of 1 percent agar in 0.05M barbital buffer pH 8.6, and two wells 3 mm. diameter and 4 mm. apart were punched as 4 parallel pairs in the central area of each slide. To detect HB$_s$Ab, 5 μl. of undiluted test serum and labelled HB$_s$Ag were pipetted into the anodic and
cathodic wells respectively. After electrophoresis (10 mA, 200 volts per slide for 90 minutes), the agar was cut into three zones (HB$_s$Ag well zone, inter-well zone and HB$_s$Ab well zone) and transferred to gamma spectrometer tubes. The percentage distribution of label in the three zones was calculated from these values and the radioelectrocomplexing index determined. In the absence of HB$_s$Ab, labelled HB$_s$Ag moved through the inter-well zone and into the HB$_s$Ab well zone. When HB$_s$Ab was present, the labelled HB$_s$Ag was confined to the inter-well zone and the HB$_s$Ag well zone.
CHAPTER 4

MICROSCOPY

I. Electron Microscopy (E.M.)

The technique of negative staining has been used by many workers to study the fine structure of HB$_3$Ag (Bayer et al., 1968; Dane et al., 1970). Direct examination of negatively stained preparations is particularly useful for the demonstration of naturally occurring immune complexes that may prove difficult to identify by routine serological procedures. The addition of HB$_3$Ab (Immune Electron Microscopy, I.E.M.) is, however, preferable for diagnostic identification of samples in which the three characteristic morphological forms of the HB$_3$Ag are not readily visible. A technique described by Kelen, Hathaway and McCleod (1971) offers a rapid and relatively simple procedure for this purpose. Specimens were diluted 1 in 2 or 1 in 4 and incubated with an equal volume of HB$_3$Ab, then placed on an agar surface. A formvar grid was floated on the surface of the drop. The fluid phase diffused into the agar causing any immune complexes present to be concentrated onto the surface of the grid, which was subsequently stained and viewed. Mayerick and Smith (1973) have described a similar technique using agarose and polyethylene glycol to concentrate the test serum. They claim a sensitivity similar to complement fixation.

While E.M. is essential for studying the morphological characteristics of HB$_3$Ag, it has certain disadvantages, being an elaborate technique requiring specialised personnel and expensive equipment. It has limited application and is time consuming since each sample should be scanned for at least 15 minutes before being recorded as negative. A recent publication by Sama, Benz, Aach,
Hacker and Kaplan (1973) reported 8 false positive samples among patients suffering from primary biliary cirrhosis, and implied that E.M. alone is an unreliable test for HBAg.

II. Immunofluorescence (I.F.)

The technique of immunofluorescence has been used to identify HBAg in cells from liver, spleen, bone marrow, testis and mesentery (Millman, Zavatone, Gerstley and Blumberg, 1969) from patients with HBAg-positive hepatitis. Cells were stained directly with specific HBAb conjugated with fluorescein isothiocyanate and the specificity determined by blocking with unconjugated HBAb. Similar methods have been used to detect HBAg in sections of liver from patients with circulating HBAg (Noweslowski, Brzosko, Madalinski and Krawczynski, 1970) and in cultured human liver cells inoculated with HBAg-positive serum (Brighton, Taylor and Zuckerman, 1971). The technique is difficult to master, particularly when looking for HBAg in mammalian haemopoietic tissues due to their 'sticky' nature. It has the advantage of being able to detect cell-localised HBAg, which would be otherwise undetectable except by thin section E.M. or autoradiography. Immunofluorescence is a complex technique involving much washing and is not readily applicable to testing large numbers within a short space of time. Brighton et al. (1971) used the technique to demonstrate HBAg in cultured hepatocytes following inoculation, and were able to demonstrate progressive changes spreading from cytoplasm to nucleoli.
CHAPTER 5
MEASUREMENT OF CELL-MEDIATED IMMUNE RESPONSE

I. Lymphocyte Transformation

This test provides a sensitive means of detecting previous infection when specific antibodies are not detectable in the circulation by such sensitive tests as HA and RIA. Lymphocyte transformation was first applied to HBsAg by Laiwah (1971) using phytohaemagglutinin (PHA), purified protein derivative (PPD), and serum rich in HBsAg, as transforming agents. Partially purified lymphocytes from 20 ml. of venous blood were resuspended in Eagles medium containing 20 percent normal human serum to give an approximate cell concentration of $6 \times 10^6$ cells per ml. All lymphocyte cultures were set up in duplicate, each tube containing lymphocyte suspension, Eagles medium, penicillin and streptomycin. Nothing further was added to the control cultures. Each test was stimulated with (a) PHA, (b) PPD, (c) HBsAg. All tubes were incubated for 120 hours at 37°C. Assessment of transformation was measured by uptake of $^{14}$C-labelled thymidine, added 18 hours before harvesting (i.e. after 102 hours incubation), determined in a scintillation counter. In a recent publication, Laiwah, Chaudhari and Anderson (1973) have shown that purified HBsAg is equally as good a stimulant as HBsAg-rich serum in both lymphocyte transformation and leucocyte migration inhibition. Pettigrew, Goudie, Russell and Chaudhari (1972) were able to confirm the findings of Laiwah (1971), but noted that all HBsAg-rich sera were not equally effective in stimulating the transformation of sensitised lymphocytes. They found evidence of cell-mediated immunity in all eleven cases of chronic alcoholic liver disease studied; this leads them to suggest that HBsAg may be an aetiological factor in the development of alcoholic cirrhosis.
II. Leucocyte Migration Inhibition

This technique may, in common with lymphocyte transformation, be used to assay the state of cell-mediated immune responsiveness of an individual to HB$_s$Ag. The test described by Laiwah et al. (1973) is a modification of that reported earlier by Sorborg and Bendixen (1967). Leucocytes at a concentration of approximately $1.2 \times 10^6$ cells per ml. were mixed with either normal (control) serum or HB$_s$Ag-rich serum and incubated at $37^\circ$C for 90 minutes. Capillary tubes of 50 µl. capacity were then filled with the incubated cell suspension and one end sealed. They were spun at 900 g. for 5 minutes and the packed cell layers separated by breaking the capillary tubes just below the cell-fluid interphase. The stubs containing the cells were immediately laid on the floor of migration chambers containing Eagles medium. After incubation at $37^\circ$C for 18 hours the area of cell migration was magnified by a projection microscope and measured by planimetry. The effect of the stimulating agent on cell migration was expressed as the Migration Index (M.I.), where

$$M.I. = \frac{\text{mean migration area of stimulated cultures}}{\text{mean migration area of control cultures}}$$

Ibrahim, Vyas and Perkins (1975) have used this technique in conjunction with HA and RIA to study the immune response to HB$_s$Ag in normal persons, HB$_s$Ag carriers, acute HB$_s$Ag-positive hepatitis patients and convalescent hepatitis B patients. Their findings suggest that HB$_s$Ag is eliminated rapidly by a cell-mediated immune response, detectable for a limited period, followed by an HB$_s$Ab response in relatively few patients more than 3 months after the clearance of circulating HB$_s$Ag.
I. Complement Fixation (C,F.)

The serum to be tested is mixed with complement and HB⁺Ab (or HB⁺Ag). If an antigen-antibody reaction occurs complement will be 'fixed' as demonstrated by testing for residual complement. A number of procedures are available, although the micro-technique has been most widely used for HB⁺Ag testing (Purcell, Holland, Walsh, Wong, Morrow and Chanock, 1969; Shulman and Barker, 1969; Taylor, 1970). Overnight incubation at 4°C provides a more sensitive but often less specific test, while incubation at 37°C for only 60 minutes results in a less sensitive but more specific reaction. The test for HB⁺Ag has been automated (Sturgeon, Kwak, and Gitnick, 1971; Kwak, Gitnick and Sturgeon, 1973). Sera containing high concentrations of HB⁺Ag may not react at low dilutions due to a prozone phenomenon, therefore test sera should be assayed over a range of dilutions. HB⁺Ag and HB⁺Ab may be present in the same specimen as immune complexes (Almeida et al., 1969), a situation which may result in anti-complementary activity (Shulman and Barker, 1969). Ross and Pringle (1971) have found that heating the test serum at 85°C for 1 hour may liberate HB⁺Ag, HB⁺Ab being destroyed at this temperature. C,F, is slightly more sensitive than C,I,E,O,P. for detection of HB⁺Ag, but approximately equivalent for the detection of HB⁺Ab.

II. Immune Adherence Haemagglutination (I.A.H.)

The phenomenon of immune adherence was first described by Laveran and Mesnie (1901) and was based on the observation that complexes of antigen, antibody and complement adhere to primate
erythrocytes. An I.A.NI. assay for \( HB_\text{B} \),Ag has been described by Mayumi, Okochi and Nishioka (1971), and is reputed to be 100 times more sensitive than C.F., but only 10 times more sensitive for the detection of \( HB_\text{B} \),Ab (Nishioka, 1972). The major disadvantage of this technique is that large proxones are observed when the \( HB_\text{B} \),Ag titre is high, and for this reason it is necessary to test sera over a wide range of dilutions. Conversely, if the \( HB_\text{B} \),Ag concentration is low the presence of normal serum complement \( C^3 \) inactivator may inhibit the reaction, causing a false negative result.

III. Platelet Aggregation

The interaction of \( HB_\text{B} \),Ag-\( HB_\text{B} \),Ab immune complexes on the surface of blood platelets results in their aggregation under certain conditions, with a consequent change in their settling patterns (Melartin, Myllyla and Penttinen, 1970). Unfortunately, individual batches of platelets vary in their sensitivity to immune complexes, thus application of this principle for large scale \( HB_\text{B} \),Ag testing is not recommended. False reactions due to non-immunological factors may also occur.
SECTION THREE
AIMS OF PROJECT

I. Introduction

While 'third generation' assay techniques, such as R.P.H.A., HAI and RIA, are beginning to be seriously evaluated by some Blood Transfusion Centres, many still rely on C.I.E.O.P. to detect HB\textsubscript{s}Ag carriers among their populations. The shortcomings of C.I.E.O.P. have already been mentioned (Part One, Section Two, Chapter 1, II), and the advantages and disadvantages of R.P.H.A., HAI and RIA are discussed in Part One (Section Two, Chapter 3). It is hoped that this project will provide a simple, sensitive, rapid and economic test for HB\textsubscript{s}Ag suitable for adoption by any Blood Transfusion Centre.

II. General Aim

To improve HB\textsubscript{s}Ag testing facilities within the Blood Transfusion Service, and at the same time avoid the necessity of purchasing large quantities of expensive commercial reagents.

III. Specific Aims

1. Development of a simple, sensitive and economic HB\textsubscript{s}Ag test suitable for assaying large numbers of blood donations in a relatively short time, thereby causing the minimum of delay to fresh blood required for transfusion and/or processing.

2. Evaluation of the above test in terms of sensitivity and specificity by (a) comparison with R.P.H.A. and RIA using established HB\textsubscript{s}Ag panels, and (b) comparison with C.I.E.O.P. in determining the incidence of HB\textsubscript{s}Ag and HB\textsubscript{s}Ab in the blood donor population of the South-East Region of Scotland.
3. Application of the test to HB₃Ag subtyping, thereby providing essential confirmatory and useful epidemiological information.

4. Modification of the test for detection of Hepatitis B core antibody (HB₃Ab).

5. Re-investigation of C₁,E₀,P₀-negative donations implicated in posttransfusion hepatitis.

6. Follow-up of recipients of hyperimmune HB₃Ab gamma globulin given as a preventative measure in cases of accidental (usually needle-stick) exposure for HB₃Ag.
PART TWO

Basic developments.
I. Introduction

The association of HB<sub>5</sub>Ag with posttransfusion hepatitis stimulated demand for a rapid means of testing blood donations prior to use. At one time, C.I.E.O.P. fulfilled the requirement admirably since it used inexpensive equipment, was easy to perform and allowed fresh blood to be used within hours of donation, an important consideration concerning the availability of labile products such as cryoprecipitate and platelets. As the technology of HB<sub>5</sub>Ag testing became more sophisticated and sensitive, the inadequacies of C.I.E.O.P. became apparent. Commercial R.P.H.A. and RIA tests are available but at a price which may prove prohibitive to some centres. Direct haemagglutination tests suffer nonspecific false positive reactions, while RIA introduces the additional hazard of isotope handling, is time-consuming and requires expensive counting equipment.

The decision to undertake the development of a haemagglutination test designed specifically for the requirements of the Blood Transfusion Service was guided by the following considerations:

1. Haemagglutination (HA) and haemagglutination-inhibition (HAI) are sensitive techniques which have been in use in one form or another for many years within the Blood Transfusion Service. They are, on the whole, simple to perform and easy to read, requiring no sophisticated or expensive equipment.

2. Basic reagents (erythrocytes, HB<sub>5</sub>Ag, HB<sub>5</sub>Ab, normal serum) are in abundant supply at most centres as a result of current C.I.E.O.P. testing.
3. The stable nature of 'fixed' erythrocytes could lead to standardisation of testing within and between centres if reagents are prepared in bulk. This would be much more difficult with a test such as RIA where labelling and standardisation must be performed at relatively short intervals.

4. There is every possibility of eventual automation, since many Blood Transfusion Centres are gaining experience in the use of auto analysers for various routine blood bank assays.

A basic concept for tanning and sensitisation of erythrocytes will be outlined and followed by a series of experiments designed to determine the optimal conditions of a number of variables. The resultant modified procedure will then be described in detail and compared for sensitivity and specificity with current techniques.

II. Brief Background to Erythrocyte Sensitisation

Red blood cells are convenient, passive carriers of antigen (Hirata and Stashak, 1965; Sindo and Wakakura, 1952), acting as markers which allow agglutination by specific antibody to be observed with the naked eye. If the cells are tanned before being sensitised with antigen they are more readily agglutinated by specific antibody (Shiari, 1964). Treatment with tannic acid increases the instability of the cells causing a normally non-agglutinating reaction to result in agglutination (Pirofsky, Cordova and Imel, 1962). Once treated with tannic acid at the right concentration the cells will exhibit agglutination settling patterns. The tendency to agglutinate may be balanced by adding a stabilising agent such as normal serum so that autoagglutination is just cancelled out. The cells are thus in a very sensitive state, ready to agglutinate in the presence of a very small amount of specific antibody. This form of passive
haemagglutination is quite distinct from the active haemagglutination
seen with Influenza Virus (Francis, 1947), where the virus alone is
responsible for causing certain types of erythrocytes to agglutinate,
since it actively absorbs onto receptor sites on the erythrocyte
surface.

Felton, Francis and Scott (1961) used passive haemagglutination
to study Herpes Simplex Virus. They showed that pH and tannic acid
concentration were critical, while the age of the antigen and the
temperature and time of sensitisation were more flexible, allowing
some diversity of performance and application. A few years earlier,
Scott, Felton and Barney (1957) had shown that the concentration of
antigen used for sensitisation had a direct effect upon the HA titre
of specific antibody, the titre being reduced when lesser concentrations
of antigen were used.

III. Initial (Starting) Procedure for Preparation of HB$_3$Ag-coated
Human Erythrocytes

1. HB$_3$Ag (C, I, E, O$^+$-positive) serum was treated with beta-
propiolactone and ultra-violet light in an attempt at inactivation.

2. The antigen was pelleted by centrifugation (120,000 g, av.
for 5 hours at $20^\circ$C), washed and resuspended in 1/20th of the
original volume, heated at $60^\circ$C for 30 minutes and stored at $-20^\circ$C
until required.

3. Ten mls. of human group 0 rhesus negative blood was
collected in anticoagulant and allowed to stand overnight at $4^\circ$C.

4. The blood was washed three times in physiological saline
and made up to a 6.6 percent suspension in phosphate buffered saline
(P.B.S.) pH 7.2 and an equal volume of 1 in 10,000 tannic acid was
added before incubating at $37^\circ$C for 15 minutes.
5. The tanned cells were washed in P.B.S. pH 6.4 and re-suspended to a 4 percent suspension in the same buffer prior to adding HB\textsubscript{Ag} in a ratio of 4:1 (v/v; cells; HB\textsubscript{Ag}). The mixture was then incubated at room temperature (approx. 20°C) for 60 minutes.

6. The coated cells were washed three times in stabilising buffer (P.B.S. pH 7.2 containing 2 percent normal human serum) and allowed to stand overnight at 4°C as a 1 percent cell suspension.

IV. Initial Test Procedures

HB\textsubscript{Ab} was tested by direct haemagglutination of antigen coated cells, while HB\textsubscript{Ag} was tested by an inhibition reaction. Both reactions were performed in disposable u-bottomed microtitre plates using 25 μl. volumes of reagent. Positive reactions suggestive of HB\textsubscript{Ab} were confirmed by (a) retesting with pooled-plasma-coated cells and (b) demonstrating that activity could be inhibited by HB\textsubscript{Ag} but not normal serum. Positive reactions suggestive of HB\textsubscript{Ag} were confirmed by testing the inhibitory effect of specific antibody compared with normal serum.

V. Experiments to Determine the Optimum Conditions for Tanning, Sensitisation and Performance of the Test

1. Effect of tannic acid concentration on sensitivity

Different concentrations of freshly prepared tannic acid (M & B batch no. 06743) in P.B.S. (Appendix A) pH 7.2 were used to tan erythrocytes in a 37°C water bath for 15 minutes. The effect upon sensitivity of the HA test is shown in table 2.V.1. At a 1 in 5,000 concentration cell lysis occurred, while a 1 in 50,000 concentration proved too weak as evidenced by a decline in the ability to detect HB\textsubscript{Ab}. Tannic acid at a concentration of 1 in 10,000 proved optimal. It is suggested that this type of experiment be
repeated for different brands of tannic acid, and indeed each batch, since experience has shown that considerable variation exists (the B.D.H. product was satisfactory at 1 in 5,000, while MERC tannic acid appeared to be unsatisfactory at any concentration).

Table 2.4.1. Effect of tannic acid concentration on sensitivity

<table>
<thead>
<tr>
<th>Tannic acid concentration</th>
<th>Reciprocal of HB Ab titre s(H,M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 in 5,000</td>
<td>cell lysis</td>
</tr>
<tr>
<td>1 in 10,000</td>
<td>4,096</td>
</tr>
<tr>
<td>1 in 20,000</td>
<td>1,024</td>
</tr>
<tr>
<td>1 in 50,000</td>
<td>8</td>
</tr>
</tbody>
</table>

2. Effect of HB₅Ag concentration (for cell coating) on sensitivity

Partially purified HB₅Ag was prepared as previously described. The final pellet was resuspended in 1/20th its original volume and serially diluted in PBS pH 6.4. Each dilution was titrated using latex agglutination (Pfizer batch no. L13/3) and C.I.E.O.P. (using antiserum T2160C supplied by Hoechst Ltd.) Each HB₅Ag dilution served to sensitize an aliquot of cells, the results of which are given in table 2.4.2. Optimal sensitivity was observed using the dilution of partially purified antigen containing 128-256 latex units (4-8 C.I.E.O.P. units) of HB₅Ag activity. If more HB₅Ag was coated onto the cells autoagglutination ensued, while reduction of HB₅Ag on the cell surface reduced sensitivity.
Table 2.V.2 Effect of HB Ag concentration (for cell coating) on sensitivity

<table>
<thead>
<tr>
<th>Reciprocal of HB Ag dilution</th>
<th>Reciprocal of HB Ag titre (latex units)</th>
<th>Reciprocal of HB Ag titre (CIEOP units)</th>
<th>Reciprocal of HB Ab titre (T2160C)</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>512</td>
<td>16</td>
<td>20,480</td>
<td>auto-agglutination</td>
</tr>
<tr>
<td>2</td>
<td>256</td>
<td>8</td>
<td>20,480</td>
<td>good</td>
</tr>
<tr>
<td>4</td>
<td>128</td>
<td>4</td>
<td>5,120</td>
<td>good</td>
</tr>
<tr>
<td>8</td>
<td>64</td>
<td>2</td>
<td>1,280</td>
<td>good</td>
</tr>
<tr>
<td>16</td>
<td>32</td>
<td>-</td>
<td>40</td>
<td>good</td>
</tr>
<tr>
<td>32</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>good</td>
</tr>
<tr>
<td>64</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>good</td>
</tr>
<tr>
<td>128</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>good</td>
</tr>
</tbody>
</table>

3. Effect of temperature and time of sensitisation on sensitivity

Aliquots of tanned cells were incubated with optimal concentrations of HB Ag at four different temperatures each for a period of one hour. Further aliquots of cells were then sensitised for varying times at optimum temperature. Tables 2.V.3a and 2.V.3b present data indicating that sensitisation is most efficient over the temperature range 20°C-37°C, while at that temperature maximum HB Ag coating of the cells was obtained after only 60 minutes. Longer sensitisation times did not improve sensitivity.
Table 2.4.3a Effect of temperature of sensitisation on sensitivity

<table>
<thead>
<tr>
<th>Temperature °C for 1 hour</th>
<th>Reciprocal of HB Ab titre (H.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>400</td>
</tr>
<tr>
<td>20</td>
<td>4,000</td>
</tr>
<tr>
<td>37</td>
<td>4,000</td>
</tr>
<tr>
<td>60</td>
<td>1,000</td>
</tr>
</tbody>
</table>

Table 2.4.3b Effect of time of sensitisation on sensitivity

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Reciprocal of HB Ab titre (H.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>200</td>
</tr>
<tr>
<td>15</td>
<td>400</td>
</tr>
<tr>
<td>30</td>
<td>1,000</td>
</tr>
<tr>
<td>60</td>
<td>4,000</td>
</tr>
<tr>
<td>90</td>
<td>4,000</td>
</tr>
<tr>
<td>120</td>
<td>4,000</td>
</tr>
</tbody>
</table>

4. Effect of purity of HB Ag used for cell coating on sensitivity

Four aliquots of HB Ag were prepared and adjusted to contain optimal concentrations of antigen.

(a) HB Ag in normal serum.

(b) Partially purified HB Ag prepared as just described.

(c) Partially purified HB Ag prepared by polyethylene glycol extraction and filtration through Sephadex G200 (Appendix B).
Purified HB_s Ag prepared by density gradient centrifugation (Appendix C) and normally used for radiolabelling.

HB_s Ag from each preparation was used to coat tanned cells. Coating unpurified HB_s Ag resulted in autoagglutination of the sensitised cells (Table 2.V.4) possibly due to the excess of protein resulting from the presence of normal serum. Preparations (b) and (c) showed optimal sensitisation, while the highly purified HB_s Ag exhibited a much reduced sensitivity. An interesting observation was that within a few days of sensitisation, cells coated with (c) and (d) had lost most of their activity, presumably as a result of HB_s Ag eluting from the cell surface, suggesting that over-purification is to be avoided. It may be that a certain amount of non-specific protein is required to allow a stable attachment between antigen and cell, excess leading to autoagglutination.

Table 2.V.4. Effect of purity of HB_s Ag used for cell sensitisation on sensitivity

<table>
<thead>
<tr>
<th>HB_s Ag preparation</th>
<th>Reciprocal of HB Ab titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) unpurified</td>
<td>autoagglutination</td>
</tr>
<tr>
<td>(b) pelleted/washed</td>
<td>20,680</td>
</tr>
<tr>
<td>(c) polyethylene glycol/6200</td>
<td>20,680</td>
</tr>
<tr>
<td>(d) density gradient centrifugation</td>
<td>320</td>
</tr>
</tbody>
</table>
5. Effect of sensitised cell concentration on sensitivity

Sensitised cells were made up to different concentrations in stabilising buffer and used to titrate an HB_{Ab}-positive serum. Table 2.V.5 shows that the 0.75 percent cell suspension combined good sensitivity with ease of reading. Increasing the cell concentration reduced sensitivity, while using fewer cells resulted in poor readability.

Table 2.V.5. Effect of sensitised cell concentration on sensitivity

<table>
<thead>
<tr>
<th>Cell concentration (percent)</th>
<th>Reciprocal of HB_{Ab} titre (\text{T2160C})</th>
<th>Readability</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5,120</td>
<td>good</td>
</tr>
<tr>
<td>1</td>
<td>20,480</td>
<td>good</td>
</tr>
<tr>
<td>0.75</td>
<td>40,960</td>
<td>good</td>
</tr>
<tr>
<td>0.5</td>
<td>40,960</td>
<td>good</td>
</tr>
<tr>
<td>0.25</td>
<td>unreadable</td>
<td>unreadable</td>
</tr>
</tbody>
</table>

6. Effect of using serum or plasma as test sample

Two blood samples were taken from each of five accredited donors (i.e. donors of long standing, where previous donations have not been implicated in posttransfusion hepatitis). One sample was allowed to clot in order to obtain natural serum, while the second sample was collected in anticoagulant in order to obtain plasma. Each serum and plasma sample was then serially diluted and tested against coated cells. The results in table 2.V.6 indicate that even in the absence of HB_{Ab} the use of plasma could lead to non-specific false positive reactions. Such reactions were not evident even with high concentrations of serum.
Table 2.V.6. Effect of using plasma or serum as test sample

<table>
<thead>
<tr>
<th>Accredited donors</th>
<th>Reciprocal of serum/plasma dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>1. plasma serum</td>
<td>+</td>
</tr>
<tr>
<td>2. plasma serum</td>
<td>+</td>
</tr>
<tr>
<td>3. plasma serum</td>
<td>+</td>
</tr>
<tr>
<td>4. plasma serum</td>
<td>+</td>
</tr>
<tr>
<td>5. plasma serum</td>
<td>+</td>
</tr>
</tbody>
</table>

+ positive agglutination
- negative agglutination
‡ weak agglutination

7. Effect of collecting fresh blood in different anticoagulants on the suitability of cells for sensitisation

Fresh group 'O' rhesus negative blood from the same donor was collected in four different anticoagulants, stored at 4°C overnight, tanned and sensitised with HB Ag. Only blood collected in lithium-heparin was found to be unsuitable as evidenced by autoagglutination of the sensitised cells (table 2.V.7).
Table 2.V.7. Effect of collecting fresh blood in different anticoagulants on suitability of cells for sensitisation

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>Reciprocal of HB$_a$Ab titre (T2160)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>32,000</td>
</tr>
<tr>
<td>A.C.D.</td>
<td>32,000</td>
</tr>
<tr>
<td>E.D.T.A.</td>
<td>32,000</td>
</tr>
<tr>
<td>Lithium-heparin</td>
<td>autoagglutination</td>
</tr>
</tbody>
</table>

A.C.D. = acid citrate dextrose  
E.D.T.A. = ethylene diamine tetra acetic acid

5. Variation among different batches of HB$_a$Ag sensitised cells

Serum from 10 different HB$_a$Ag carriers (8 adw, 2 ayw) were prepared for cell sensitisation and each preparation used to sensitise an aliquot of cells from each of three cell donors. The data in Table 2.V.8 suggests that the techniques for HB$_a$Ag preparation, cell tanning and sensitisation are sufficiently reproducible to be able to produce a standardised reagent. The prosone type of phenomenon observed with preparation 5 remains unexplained. However, this could be overcome by mixing with any of the other preparations.
Table 2.V.8. Variation among different batches of HB Ag sensitised cells

<table>
<thead>
<tr>
<th>Batch no.</th>
<th>HB Ag source</th>
<th>HB Ag subtype</th>
<th>Donor cell batch</th>
<th>Reciprocal of HB Ab titre (H.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Patient</td>
<td>ayw</td>
<td>a</td>
<td>10 20 40 80 160 320 640 1280 2560 5120 10240</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Donor</td>
<td>ayw</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Donor</td>
<td>adw</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Donor</td>
<td>adw</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Donor</td>
<td>adw</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Donor</td>
<td>adw</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Donor</td>
<td>adw</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Donor</td>
<td>adw</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Donor</td>
<td>adw</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Donor</td>
<td>adw</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b</td>
<td></td>
</tr>
</tbody>
</table>

+ = positive agglutination
- = negative agglutination
* = weak agglutination
9. Effect of HB\textsubscript{s}Ab concentration on HAI sensitivity

An HB\textsubscript{s} Ab-positive serum was titrated and the last dilution to show complete agglutination was considered to represent 1 HA unit of HB\textsubscript{s} Ab activity (i.e. a doubling dilution back would contain 2 HA units of activity etc.) The endpoint of an HB\textsubscript{s}Ag titration was determined using 2, 4, 8 and 16 units of HA activity. Table 2.V.9 indicates that the sensitivity of the inhibition reaction is increased as the amount of HB\textsubscript{s} Ab decreases. However, there comes a point at which the dilution effect of adding test serum and cells results in premature collapsing of the agglutination pattern leading to false HB\textsubscript{s}Ag positive results.

Table 2.V.9. Effect of HB\textsubscript{s}Ab concentration on HAI sensitivity

<table>
<thead>
<tr>
<th>HA units of HB\textsubscript{s}Ab activity</th>
<th>Reciprocal of HB\textsubscript{s}Ag titre (B.D.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>premature collapsing</td>
</tr>
<tr>
<td>4</td>
<td>1,000</td>
</tr>
<tr>
<td>8</td>
<td>800</td>
</tr>
<tr>
<td>16</td>
<td>200</td>
</tr>
</tbody>
</table>

10. Effect of pre-incubation time on HAI sensitivity

HB\textsubscript{s}Ag positive serum was titrated by pre-incubating with 4 HA units of HB\textsubscript{s} Ab activity at 37\(^\circ\)C for varying time intervals prior to the addition of sensitised cells. Table 2.V.10 shows that maximum inhibition of HB\textsubscript{s}Ab under these conditions occurs after 30 minutes. Further pre-incubation did not enhance sensitivity.
Table 2.V.12. Effect of pre-incubation time on HAI sensitivity

<table>
<thead>
<tr>
<th>Pre-incubation time</th>
<th>Reciprocal of HB Ag titre ($B_D_{4.5}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>15 mins.</td>
<td>300</td>
</tr>
<tr>
<td>30 mins.</td>
<td>2,000</td>
</tr>
<tr>
<td>60 mins.</td>
<td>20,000</td>
</tr>
<tr>
<td>120 mins.</td>
<td>2,000</td>
</tr>
<tr>
<td>240 mins. (RT)</td>
<td>2,000</td>
</tr>
<tr>
<td>overnight (4°C)</td>
<td>2,000</td>
</tr>
</tbody>
</table>

R.T. = room temperature

11. **Effect of storage (at 4°C) on sensitivity**

Freshly prepared sensitised cells were kept at 4°C until cell lysis was apparent (approximately 7.5 weeks). At intervals during storage the cells were titrated against an HB$_a$Ab-positive serum. Some cells were frozen in liquid nitrogen in the presence of dimethylsulphoxide (DMSO) to prevent the formation of disruptive ice crystals. At 4°C the cells retained sensitivity for approximately 6 weeks after which rapid deterioration was observed due to cell lysis caused by ageing (Fig.2.V.11). Storage in liquid nitrogen proved unsuitable as approximately 80 percent of the cells showed lysis during subsequent washing to remove DMSO.
VI. Final (Detailed) Procedure for Preparation of HB$_s$Ag-sensitised Human Erythrocytes

In the light of data obtained from the preceding experiments, the following procedure was adopted for preparation of HB$_s$Ag, tanning and sensitisation of cells.

1. Preparation of HB$_s$Ag

HB$_s$Ag was obtained by bleeding a carrier into a dry bottle and allowing the blood to clot. HB$_s$Ag-positive serum was recovered and treated with betapropiolactone and ultra-violet light.
95. (Appendix D) according to the method of Lo Grippo and Hartmen (1958) and Lo Grippo (1973). The treated serum was then spun at 120,000 g. (av) for 5 hours on an M.S.E. Super-speed 75 centrifuge using an 8 x 25 ml. titanium angle head rotor. The resultant pellet was resuspended overnight in 2 mls. physiological saline followed by gentle mixing with a Pasteur pipette before being transferred to a new centrifuge tube and made up to 16 mls. with saline. The suspension was then re-pelleted as before and the second pellet resuspended in 0.5 mls. FBS pH 6.4. The concentration of HBsAg was then adjusted to 128-256 latex units by addition of FBS pH 6.4, whereupon the preparation was heated to 60°C for 30 minutes, then divided into 0.5 ml. aliquots and stored at -20°C until required.

2. Tanning and sensitisation

Human group O rhesus negative venous blood was collected in A.C.D. and allowed to stand overnight at 4°C. The following day 3 mls. were washed three times in 12 mls. of sterile physiological saline (M.S.E. Minor bench centrifuge no. 3 for 5 minutes.) 0.08 mls. of packed, washed cells were resuspended in 1.25 mls. PBS pH 7.2 (6.6 percent cell suspension) and incubated with an equal volume of freshly prepared 1 in 10,000 tannic acid (M & B batch no. 06743) prepared in the same buffer. The mixture was incubated in a 37°C water bath for 15 minutes, shaking gently every few minutes to ensure thorough mixing. The tanned cells were pelleted (M.S.E. Minor bench centrifuge no. 3 for 1 minute) and washed once in 2.5 mls. PBS pH 6.4 before being resuspended in 2 mls. PBS pH 6.4 (i.e. 4 percent cell suspension). 0.5 mls. prepared HBsAg was added to the tanned cells (i.e. 4 volumes cells:1 volume HBsAg) and the sensitising
mixture incubated for 60 minutes at room temperature (approximately 22°C) using either a Luckham rotostat or a Matlurn mixer to ensure thorough mixing. The sensitised cells were washed three times in 3 ml. of cell diluent (PBS pH 7.2 containing 2 percent normal human serum), made up to a 1 percent cell suspension and left at 4°C overnight. Next morning, the supernatant was replaced with fresh cell diluent and the cells stored at 4°C until required. Photograph 2.4 shows HBsAg absorbed onto the surface of a red cell. Cells from the same source were sensitised with a 1 in 125 dilution of pooled human plasma and used as 'control' reagents.

VII. Final Test Procedure

Tests were performed in disposable u-bottomed microtitre plates (Flow Laboratories) into which reagents were added in 25 μl. volumes using a pasteur pipette. Serum specimens were diluted to 1 in 10 in PBS pH 7.2 prior to testing.

(a) Direct haemagglutination test for HBsAb

25 μl. of test serum dilution (1 in 10) was pipetted into a well and 25 μl. of 0.75 percent HBsAg-sensitised cells added. Each plate contained a positive control (antisera + sensitised cells) and a negative control (normal serum + sensitised cells). The plate was gently shaken to ensure thorough mixing and incubated for 60 minutes in a 37°C incubator. The presence of detectable HBsAb was characterised by a smooth matt of agglutinated cells covering the bottom of the well, while a compact button of cells indicated that no HBsAb could be detected (photograph 2.4d). Positive reactions were confirmed by retesting with plasma-sensitised 'control' cells, and by showing that activity could be inhibited by HBsAg but not normal serum.
Photograph 2.VI.1. HBAg adsorbed onto the surface of tanned human erythrocyte. Mag. = 35,000

Photograph 2.VII.1. Haemagglutination settling patterns in u-bottomed wells.
(b) Haemagglutination-inhibition test for HB$_s$Ag

An HB$_s$Ab-positive serum was serially diluted in PBS pH 7.2 containing 10 percent normal human serum and the end-point (i.e. the lowest concentration producing complete agglutination) determined. This was defined as one haemagglutinating unit (1 HA unit). To test for HB$_s$Ag, 25 µl. of test serum dilution were mixed with 25 µl. of HB$_s$Ab containing 4 HA units of activity for 30 minutes at 37°C. 25 µl. of HB$_s$Ag-sensitised cells were then added and after gentle mixing, the plate was re-incubated for a further 50 minutes. HB$_s$Ag in the test sample would neutralise the 4 HA units of antibody activity and cause the sensitised cells to fall as a negative button. Absence of HB$_s$Ag in the test serum would leave the agglutinating ability of the 4 HA units intact, leading to a positive agglutination pattern. A positive control (antiserum + HB$_s$Ag + sensitised cells) and a negative control (antiserum + normal serum + sensitised cells) were included in each plate. Positive reactions were confirmed by demonstrating the ability of the sample to reduce the titre of HB$_s$Ab as compared with normal serum.

VIII. Sensitivity of HA and HAI

The ability of the inhibition reaction to detect HB$_s$Ag (pool of subtypes adw/ayw) was compared with I.D., C.I.E.O.P. (Hopkins and Das, 1972), latex agglutination (Leach and Ruck, 1971), using two commercial reagents (Pfizer Limited; Hoechst Limited), I.E.M. (Kelen et al., 1971), RIA-SP (Austria-I) using reagents from Abbott Laboratories Limited (Kent) and RIA-DA based on the test described by Lander et al. (1971) and performed by the Virus Diagnostic Laboratory at Edinburgh University Medical School.
The sensitivity of the direct haemagglutination test for HBsAb was assessed by comparing the 'end-point titre' of a known positive serum as determined by HA and C.I.E.O.P.

Figure 2.VIII.a indicates that HAI exhibits a degree of sensitivity similar to both HA techniques, being more sensitive than I.D., C.I.E.O.P., latex agglutination and I.E.M. The direct HA test is capable of detecting very low levels of HBsAb compared with C.I.E.O.P. (figure 2.VIII.b).

**Figure 2.VIII.a. Relative sensitivity of HAI**
IX. Specificity of HAI and HA

The specificity of HAI was tested by coding HB₅Ag-positive and HB₅Ag-negative sera and distributing them randomly among the test sera.

Figure 2.IX.a shows the results obtained when HB₅Ag-positive sera were coded, mixed with HB₅Ag-negative sera and tested by a variety of techniques. HAI detected all positive samples as did C.I.E.O.P. and RIA, and gave no false positive reactions, while one batch of latex, I.D., and I.E.M. failed to detect several of the positive sera, and latex reagents from both commercial sources gave false positive reactions.
Table 2.IX.b confirms the specificity of HA and HAI by showing complete agreement with C.I.E.O.P., when sera from selected blood donors and dialysis patients were tested for antigen and antibody. In two patients, low levels of antibody capable of agglutinating HB$_{Ag}$-sensitised cells were in fact due to reactions against serum protein(s) as demonstrated by the agglutination of plasma-sensitised cells. This antibody did not inhibit the reactivity of HB$_{Ag}$.

Figure 2.IX.a. Results obtained when HB$_{Ag}$-positive and HB$_{Ag}$-negative sera were coded and tested by a variety of techniques.
Table 2.IX.d. Results obtained when sera from selected blood donors and dialysis patients were tested for HB$_s$Ag and HB$_s$Ab by HAI, HA and C$_s$I,$E_o$P$_s$.

<table>
<thead>
<tr>
<th></th>
<th>No. tested</th>
<th>C$_s$I,$E_o$P$_s$</th>
<th>HAI/HA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HB$_s$Ab</td>
<td>HB$_s$Ag</td>
</tr>
<tr>
<td>Donors</td>
<td>76</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Dialysis patients</td>
<td>14</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

* Different samples from those possessing HB$_s$Ab
X. Discussion

The agglutination of particulate antigen by its specific antibody is probably the simplest way of estimating the quantity of that antibody in serum. Some soluble antigens can be coated onto inert materials which then react as if they themselves possessed the specificity of the antigen. Red blood cells are convenient passive carriers of antigens since it is possible to coat almost any antigen onto their complex surface, thus providing a sensitive indicator of specific antibody activity. Correctly sensitised cells lend themselves to the observation of settling patterns, e.g. in the wells of perspex trays or in glass tubes.

Many antigens may be effectively coated onto red cells merely by exposing the cells to antigen for a period of time, but sensitivity of the resultant cell is greatly improved if their agglutinability has been increased by previous tanning (Boyden, 1951). To avoid difficulties due to the presence in the test sera of heterophile agglutinins, it is sensible to use homologous species cells wherever possible. The haemagglutination test described in this thesis was designed to test human serum for HBsAb and HBsAg, and therefore uses human group O rhesus negative erythrocytes, partly because they are easily obtainable, but mainly because there is little chance of a test serum possessing antibody to either the blood group or rhesus determinants.

There is, of course, the possibility that if antigen coated cells are kell positive, the presence of anti-kell in the test serum could lead to haemagglutination in the absence of an HBsAg-HBsAb reaction. This possibility is, however, remote since a saline-reacting anti-kell is uncommon and tannic acid treatment would tend to reduce such activity by altering the cell surface. The use of
fixed cells would further reduce the likelihood of such misleading reactions. It is also unlikely that antibodies to the HL-A system would lead to misinterpretation in hepatitis B testing since such antibodies, even if present are not haemagglutinating.

It is generally assumed that tannic acid acts upon cells in such a way as to cause them to take up protein antigens. However, this may not be its main function since cells will adsorb antigen and become agglutinable without the aid of tannic acid (Hirata and Stashak, 1965; Sindo and Wakakura, 1952) though the sensitivity of such preparations is usually low. The quantity of protein antigen attached to the cell increases after tannic acid treatment (Shioiri, 1964) but its chief function appears to be to increase the instability of the cells so making a normally non-agglutinating reaction result in agglutination. In this respect, the role of tannic acid may be analogous to that of some enzymes, for it has been observed that the red blood cells of some cold-blooded animals may absorb bacterial antigens but are not agglutinated by the homologous antibody unless they have been altered in some way by the action of proteolytic enzymes (Neter, Cohen, Westphal and Lüderitz, 1959). Once treated with tannic acid at the right concentration, red cells will show agglutination settling patterns. This tendency to agglutinate may be balanced by adding normal serum as a stabiliser (Atkin, 1909) so that it is just cancelled out. The coated cells are thus in a very sensitive state ready to agglutinate in the presence of a very small amount of antibody, hence the great sensitivity of the test. The greater the concentration of tannic acid used, the greater the agglutinability of the cells. However, they must not be made so agglutinable that they cannot be stabilised. Some batches of cells may be abnormally sensitive to the action of tannic acid, and to avoid autoagglutination or cell lysis,
it is advisable to carry out tests using different concentrations of tannic acid for each 'source' of cells. Tanning is usually carried out at 37°C (George and Vaughan, 1962; Stavitsky, 1954) but appears to be effective in the cold (Shioiri, 1964) or at room temperature (Boyden and Sorkin, 1955). A period of approximately 15 minutes is considered sufficient for the reaction. There is evidence that it is completed very rapidly, increased time of exposure having little effect on the sensitivity of the cells produced (Herbert, 1967). After tanning a single wash was found sufficient to remove excess reagent.

In order to successfully coat HB₂Ag onto tanned human red cells, it was necessary to adhere closely to predetermined criteria relating to antigen concentration and degree of purity. Of particular interest was the finding that optimal coating required partially purified (pelleted, washed) HB₂Ag. The autoagglutination observed when neat HB₂Ag-containing serum was used probably reflects the influence of excess non-specific protein. At the other extreme, the poor sensitivity and rapid deterioration in activity of cells coated with highly purified HB₂Ag suggests that a certain amount of non-specific protein is required to facilitate and stabilise the adsorption of HB₂Ag onto the tanned red cell surface.

The apparent absence of marked variation among different sensitised cell batches avoids dependence upon a select group of red cell donors and reflects the inherent stability of the technique. As is the case with many haemagglutination tests, serum as opposed to plasma is the preferred test material. In this particular technique, blood collected in anticoagulant may be converted to a usable serum by the addition of commercial bovine thrombin, while serum prepared by recalcification invariably leads to autoagglutination of the HB₂Ag-coated cells.
The relatively high sensitivity of the HA and HAI tests should improve efficiency of HBsAb and HBsAg detectability and lead to a greater economy of specific antiserum for antigen testing. Despite the inherent specificity of an inhibition reaction, experience in a variety of other biological systems, including hormone estimation where the tanned cell haemagglutination system has been used extensively, has shown that non-specific inhibition can occur despite the use of specific antibody (Stavitsky and Ingraham, 1964). However, the specificity of the present HAI test seems to be acceptable.

It is probable that a proportion of the normal, apparently healthy population may show positive reactions with HBsAg-coated cells due to low levels of antibody directed against or cross-reacting with plasma proteins. Bruselhuis (1971, personal communication) found 13 such reactions in 30,000 samples tested by the Netherlands Red Cross Transfusion Service using I.D. Multi-transfused patients are likely to produce such reactions due to the development of iso-precipitins (Blumberg, 1964; Langenhuyzen, 1971). The use of highly purified HBsAg for cell coating may overcome this problem, but as has already been shown, this is not practicable. Therefore, sera showing agglutination with HBsAg-coated cells should be retested against 'control' cells coated with pooled human plasma, when a specific HBsAb will show no agglutination.

Antigen may also be attached to red cells by means of a chemical bond. Jandl and Simmons (1957) reported a method of coupling proteins to red cells by the use of metallic cations. They found the most useful of them to be chromium. Vyas and Shulman (1970) adopted this principle to couple HBsAg to red cells using chromic chloride. The logistics of application of this technique for large scale testing are not practicable due to the high degree of purity of HBsAg required (Vyas et al., 1972) and the great variability observed between different
batches of cells (Reesink and Duimiel, 1973). Furthermore, it is desirable to use treated (inactivated?) HB$_3$Ag for coating cells which will subsequently be used as a routine test reagent. In our experience, the outcome of coupling betaprone-ultra-violet light-irradiated HB$_3$Ag to human erythrocytes by chromic chloride has proved unreliable.

It is hoped that the tanned cell HA/HAI technique described will be a step toward fulfilling the current HB$_3$Ag/HB$_3$Ab testing requirements of the Blood Transfusion Service.
PART THREE

MODIFICATIONS OF BASIC TEST
CHAPTER 1

SUBTYPING BY HAI

I. Introduction

The foregoing has resulted in a simple, efficient and economic test for HB\textsubscript{Ag} and HB\textsubscript{Ab}. Despite the apparent specificity of the inhibition principle, it is of utmost importance to obtain confirmatory data on any sample giving an HB\textsubscript{Ag} positive result.

One could recommend that a sample be shown to be positive by at least one other technique. Such a principle may have been acceptable when the commonly used tests were I.D. and C.I.E.O.P, where it was a relatively simple matter to show identity with known positive controls (Das et al., 1971). With 'third generation' methodology, however, a situation may arise where a sample is positive only by HAI, RPHA or EIA, the latter two being particularly prone to false positive reactions. In such circumstances, the ability to identify a positively reacting sample as belonging to one of the established subtypes would provide valuable confirmatory information.

II. Preparation of Monospecific Antisera

A number of HB\textsubscript{Ab} positive sera were tested by C.I.E.O.P. against HB\textsubscript{Ag} previously subtyped by Dr. G.L. Le Bouvier (Yale University, U.S.A.) One antiserum reacted only with HB\textsubscript{Ag} possessing the 'd' antigenic determinant, suggesting the presence of anti-d (and presumably anti-a) in the antibody positive serum. Anti-a and anti-w were neutralised by concentrating the antiserum five times with lyphogel (Gelman) and incubating with different concentrations of HB\textsubscript{Ag} (ay) at room temperature for one hour followed by 4\textsuperscript{o}C overnight. Most of the resulting immune complex was removed by centrifugation at 20,000 r.p.m. (Sorval RC 2B) for one hour. The supernatant was tested
for the presence of anti-d by direct haemagglutination using HB₃Ag (ad) coated cells (figure 3.1.II.a). The absence of anti-ay activity was demonstrated by the absence of agglutination of HB₃Ag (ay) coated cells (table 3.1.II.b).

Figure 3.1.II.a. Titration of HB₃Ab (anti-d) following adsorption with different concentrations of HB₃Ag (ay).

<table>
<thead>
<tr>
<th>Reciprocal of HB₃Ag(ay) used for adsorption</th>
<th>Reciprocal of HB₃Ab titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEAT</td>
<td>2 4 8 16 32 64 128 256 512 1024 2048 4096</td>
</tr>
<tr>
<td>5</td>
<td>agglutination due to anti-d</td>
</tr>
<tr>
<td>10</td>
<td>agglutination due to anti-ad</td>
</tr>
<tr>
<td>100</td>
<td>d a</td>
</tr>
<tr>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>NIL</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1.II.b. Confirmation of monospecific HB₃Ab (anti-d) following adsorption with HB₃Ag (ay).

<table>
<thead>
<tr>
<th>HB₃Ag coated cells</th>
<th>ad</th>
<th>ay</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB₃Ab (anti-ad) adsorbed with HB₃Ag (ay)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Unabsorbed</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ agglutination - no agglutination

Anti-γ was prepared in the same way by adsorbing HB₃Ab (anti-ay) with HB₃Ag (ad).
III. Procedure for Subtyping HB Ag for Determinants d and y

Monospecific antibody was used in conjunction with cells coated with the appropriate HB Ag subtype. For example, anti-d was used in conjunction with HB Ag (ad) coated cells. Absence of agglutination was indicative of 'd' in the test sample, while the presence of agglutination suggested that the anti-d had not been neutralised and the test sample did not, therefore, possess the d determinant. As d and y are mutually exclusive, confirmation was obtained by using anti-y and HB Ag (ay) coated cells to test for the y determinant, which if present, neutralised the anti-y and caused the HB Ag (ay) coated cells not to be agglutinated.

The reliability of subtyping by HAI was investigated by typing HB Ag positive sera from the following different sources: (i) National Institutes of Health, Bethesda, U.S.A., (ii) American Red Cross and (iii) Local (blood donor) carriers, and patients involved in the dialysis-associated hepatitis outbreak in Edinburgh in 1969. Sera from (i) and (ii) had been subtyped at source by I.D., while sera from (iii) had been subtyped by Dr. G.L. Le Bouvier (Yale University, Connecticut, U.S.A.), also using I.D. All sera were double-coded and interspersed with negative sera prior to subtyping.

IV. Results

Complete agreement was found between the subtyping data obtained by HAI and I.D. (table 3.1.1V).
### Table 3.1.IV. Comparison of HB_Ag subtyping by HAI and I.D.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Origin</th>
<th>I.D.</th>
<th>HAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>206</td>
<td></td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>209</td>
<td></td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>213</td>
<td>National Institutes</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>217</td>
<td>of Health</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>228</td>
<td></td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>1001</td>
<td></td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>1008</td>
<td></td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>1006</td>
<td>American Red Cross</td>
<td>d</td>
<td>y</td>
</tr>
<tr>
<td>2002</td>
<td></td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>2005</td>
<td></td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>BD-2-Edin</td>
<td>Local(donor)carrier</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>BD-7-Edin</td>
<td>&quot;</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>BD-9-Edin</td>
<td>&quot;</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>H226</td>
<td>Dialysis patient</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>O.G.</td>
<td></td>
<td>y</td>
<td>y</td>
</tr>
</tbody>
</table>

### V. Discussion

At the time of writing, most HB_Ag subtyping is performed by I.D. or C.I.E.O.P. Unfortunately, both techniques are wasteful of precious monospecific typing antisera and are relatively insensitive, being unable to subtype sera found positive only by the more sensitive tests such as RPHA, RIA and HAI.

Application of HAI to HB_Ag subtyping permits rapid (within 2 hours) identification of the d and y determinants, with a sensitivity similar to that of other 'third generation' tests, and therefore provides essential confirmatory information relatively quickly.
The discovery of at least two mutually exclusive but relatively common determinants of the HB\textsubscript{a}Ag system clearly implies that techniques and reagents used for blood donor testing should be capable of detecting both subtypes with more or less equal sensitivity.

Subtyping of HB\textsubscript{a}Ag and HB\textsubscript{a}Ab provides an important epidemiological tool to probe the relationship between subtype and disease.

By adopting the same approach used for d/y subtyping, the HAI test has been applied successfully to the w/r system. Furthermore, the HAI test may be used to subtype HB\textsubscript{a}Ab by using HB\textsubscript{a}Ag positive sera of known subtype.
CHAPTER 2

PREPARATION OF HB$_{Ag}$-COATED, GLUTERALDEHYDE-FIXED SPHEROCYTES

I. Introduction

Since the shelf life of fresh HB$_{Ag}$-coated erythrocytes is only four to six weeks routine blood donor testing (for example, 500 tests per day) would necessitate reagent preparation at least once per month. The interval between preparation of subsequent batches of reagent could be increased if fixed erythrocytes were used. Furthermore, if large scale production were possible, reagent standardisation would also be improved.

Fixation of erythrocytes renders them mechanically more robust as evidenced by the absence of lysis in distilled water, and by an increased shelf life. Das (1970) reported that the storage life of fibrinogen-coated sheep erythrocytes was extended from one month to more than five months as a result of prior fixation in formaldehyde. In the same year, Hoq and Das (1970) published details of a study concerning the preparation of human erythrocytes for the assay of human serum fibrinogen degradation products. Part of this study involved a comparison of formaldehyde, pyruvic aldehyde and glutaraldehyde as cell fixatives. Results indicated a high incidence of autoagglutination among formaldehyde and pyruvic aldehyde-fixed cell batches, while none of seventeen batches of gluteraldehyde-fixed cells were so affected.

It seemed logical, therefore, to investigate the use of gluteraldehyde for the fixation of human group 0 rhesus negative erythrocytes prior to tanning and coating with HB$_{Ag}$. Unfortunately, three out of five cell batches fixed in gluteraldehyde according to the method
of Hoq and Das (1970) and subsequently tanned and sensitised with HBsAg resulted in autoagglutination. Such a high incidence of unusable reagent was clearly unacceptable. Tanning and sensitisation of 'fresh' cell batches seldom resulted in autoagglutination, so it was decided to modify the method of glutaraldehyde fixation by using only as much fixative as would provide an acceptably robust cell.

As standardisation of reagents should theoretically be enhanced by a high degree of cell uniformity it was also decided to introduce a form of 'selection' by washing the fresh erythrocytes in a controlled hypotonic medium to 'select' the more robust among them.

II(a). Preparation of Spherocytes

One unit (approximately 450 ml.) of human group 0 rhesus negative blood not more than three weeks old and collected in anticoagulant (citrate phosphate dextrose) was obtained from the blood bank.

The molarity of phosphate buffer pH 7.2 (Appendix E) required for selection of the appropriate cells was determined by serially diluting isotonic (0.2M) buffer in deionised water. 0.1 ml. of fresh, well-mixed blood was added to 2 ml. of each dilution of buffer in a test tube. The cells were evenly suspended and allowed to stand at room temperature for 60 minutes. The suspension showing at least 50 percent haemolysis was arbitrarily considered optimum. This usually occurred within the range 0.045M to 0.030M but was determined for each unit of blood being spherocyted. The remainder of the blood unit was then mixed with the optimum hypotonic concentration of buffer in the ratio one volume of blood to four volumes of hypotonic buffer. The bulk volume was divided into 200 ml. aliquots for ease
of handling. After 60 minutes at room temperature the aliquots were centrifuged at 1500 r.p.m. for 15 minutes at 10°C on an M.S.E. 6L centrifuge and the supernatant (showing considerable haemolysis) was replaced with fresh buffer. This procedure was repeated until only a minimum of haemolysis could be observed. At this point the remaining cells accounted for approximately half of those originally present. These erythrocytes were considered to be uniform since they represented the most robust of the original cell population. Wet film microscopy showed them to have lost their characteristic biconcave appearance and become rounded or 'spherocyted'. They were thus subsequently referred to as spherocytes.

II(b). Fixation of Spherocytes

One aliquot of spherocytes was divided into smaller quantities (approximately 25 ml.) to each of which glutaraldehyde (Koch-Light, 25 percent) was added to give the following glutaraldehyde concentrations: 1 in 100, 1 in 200, 1 in 400, 1 in 800, 1 in 1600, 1 in 3200 and 1 in 6400. After thorough mixing the spherocyte aliquots containing fixative were left at room temperature overnight. The following morning spherocytes from each aliquot were examined microscopically and subjected to a 'water resistance' test which involved adding 25 μl. of 'settled' glutaraldehyde-treated spherocytes to 2 ml. deionised water, when properly fixed spherocytes resisted haemolysis. Dilutions of glutaraldehyde up to 1 in 1600 produced acceptable fixation, although dilutions up to 1 in 400 tended to distort the shape of the spherocytes, causing them to revert to biconcave discs.

The remaining aliquots of fresh spherocytes were fixed in the appropriate concentration of glutaraldehyde (approximately 0.02 per cent).
as above and stored at 4°C in a total volume of 200 ml. in deionised water containing 0.1 percent sodium azide.

III. Tanning and Sensitisation of Glutaraldehyde-fixed Spherocytes

The procedure was exactly that used for fresh cells, except that the concentration of tannic acid was halved.

IV. Performance and Storage of HB_s Ag-coated, Glutaraldehyde-fixed Spherocytes

Two batches of glutaraldehyde-fixed spherocytes were each divided into five aliquots and coated with HB_s Ag (adyw) as previously described. After stabilisation, the sensitivity of the sensitised fixed spherocytes was determined by titration of a standard HB_s Ab positive control. This titration was repeated at approximately one month intervals for one year, during which time each of the ten aliquots was stored at 4°C.

Table 3.2.IV.a shows that spherocytting and subsequent fixation in weak glutaraldehyde solution has no adverse effect on HB_s Ag coating. Furthermore, table 3.2.IV.b shows that glutaraldehyde-fixed HB_s Ag-coated spherocytes may be stored at 4°C for up to one year without appreciable loss of sensitivity.
Table 3.2.IV.a. Comparative sensitivity of HB Ag-coated fresh human erythrocytes and HB Ag-coated glutaraldehyde fixed spherocytes

<table>
<thead>
<tr>
<th>HB Ag coated cells</th>
<th>Reciprocal of HB Ag (GSA-2-73) titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>fresh erythrocytes</td>
<td>8000</td>
</tr>
<tr>
<td>fixed spherocytes 1a</td>
<td>16000</td>
</tr>
<tr>
<td>fixed spherocytes 1b</td>
<td>16000</td>
</tr>
<tr>
<td>fixed spherocytes 1c</td>
<td>8000</td>
</tr>
<tr>
<td>fixed spherocytes 1d</td>
<td>16000</td>
</tr>
<tr>
<td>fixed spherocytes 1e</td>
<td>8000</td>
</tr>
<tr>
<td>fixed spherocytes 2a</td>
<td>16000</td>
</tr>
<tr>
<td>fixed spherocytes 2b</td>
<td>16000</td>
</tr>
<tr>
<td>fixed spherocytes 2c</td>
<td>16000</td>
</tr>
<tr>
<td>fixed spherocytes 2d</td>
<td>16000</td>
</tr>
<tr>
<td>fixed spherocytes 2e</td>
<td>8000</td>
</tr>
</tbody>
</table>

Table 3.2.IV.b. Comparative storage life (at 4°C) of HB Ag coated fresh human erythrocytes and HB Ag coated glutaraldehyde fixed spherocytes

<table>
<thead>
<tr>
<th>HB Ag coated cells</th>
<th>Time (in months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fresh erythrocytes</td>
<td>0 1 2 3 4 5 6 7 8 9 10 11 12</td>
</tr>
<tr>
<td>fixed spherocytes</td>
<td>+ + - (lysis)</td>
</tr>
</tbody>
</table>

+ denotes acceptable sensitivity
V. Discussion

Glutaraldehyde-fixed spherocytes prepared from reasonably uniform human group 0 rhesus negative erythrocytes appear to be stable carriers of Hb\textsubscript{Ag} that provide a sensitive indicator of specific antibody. Osmosis provides a useful means of selecting those cells most suited to fixation and subsequent Hb\textsubscript{Ag} coating. The desired level of osmotic effect will vary somewhat according to the particular sample of erythrocytes being treated, but a medium having an osmolality between 100 m.mol. and 200 m.mol. should be suitable. This is conveniently produced in an aqueous solution of sodium and potassium phosphate with a molarity between 0.07M and 0.15M. Under such conditions, the less physically robust cells will undergo haemolysis while the more physically robust are converted to spherocytes. While considerable variation is possible, a recovery of spherocytes of the order of 20 percent to 50 percent of the original erythrocyte population is preferable. A plurality of treatments may be required to obtain a suitable level of spherocyte conversion, the number of treatments depending to some extent upon the ratio of erythrocytes to medium. Using the 1 to 4 ratio previously described, it is usual to perform at least four treatments.

Fixation of spherocytes may be possible with agents such as alcohol or acetic acid. However, the class of fixatives comprising the aldehydes were of general interest in this project, and in particular those derived from dicarboxylic acids (for example, gluteraldehyde). A suitable concentration of gluteraldehyde, leading to fixation in 12 to 72 hours, was between 0.06 percent to 0.08 percent of the stock solution (Koch Light, 25 percent). The resultant fixed spherocytes were conveniently stored at 4°C in
deionised water containing an antibacterial agent (0.1 percent sodium azide) until required for coating with HB$_3$Ag.

The finding that glutaraldehyde-fixed spherocytes may be stably coated with HB$_3$Ag opened the way for large scale preparation of a highly standardised HB$_3$Ag test reagent, particularly as the test procedure may be modified to provide maximum economy of reagent utilisation.
CHAPTER 3

MINITURISATION OF TEST PROCEDURE

I. Introduction

In its present form the HAI test for HBsAg is simple to set up and to read. Its reliability is such that it may be used with confidence for large scale blood donor testing. The South-East of Scotland Regional Transfusion Centre (at Edinburgh Royal Infirmary) currently tests about 5,000 blood donations per month for HBsAb and HBsAg. This is equivalent in terms of reagent usage to 10,000 tests per month. The monthly requirement of HBsAg-coated, glutaraldehyde-fixed spherocytes equals 10,000 x 25 μl. = 250,000 μl. or 250 mls. of working suspension (0.75 percent). If 50 mls. of neat HBsAg positive serum yields approximately 3 ml. of HBsAg suitable for spherocyte coating, and 0.5 mls. of such an HBsAg preparation results in sufficient coated spherocytes for 400 tests (therefore 50 mls. neat HBsAg positive serum provides 2,400 tests), then 10,000 tests would require \( \frac{10,000}{2,400} \times 50 = 200 \) ml. HBsAg positive serum to be processed. For the purpose of reagent standardisation, it is advisable to prepare larger quantities of reagent, say sufficient for one million tests, and correspondingly more HBsAg positive plasma would require to be processed. One million tests would necessitate the processing of \( \frac{1,000,000}{10,000} \times 200 = 20 \) litres of strongly HBsAg positive sera, clearly a mammoth undertaking for any Regional Blood Transfusion laboratory quite apart from the hazards involved in the handling of so much potentially dangerous material. Clearly, there is a case for investigating the possibility of a more economic usage of reagents. It is the purpose of this chapter
to investigate the possibility of reducing the volume and concentration of reagents required for HBAg testing by HAI.

II. Materials, Methods and Results

Terasaki microtest tissue culture trays (Bio-cult) are admirably suited for use in a miniturised version of the HAI test. Each tray measures 82 mm. by 57 mm. and contains 60 x 15 μl. wells which have a bevelled side and a flat base. Diagram 3.3.II.a shows the cross-sectional view of a Terasaki tray as compared to a 'U' bottomed microlitre plate.

Diagram 3.3.II.a. Diagramatic comparison of Terasaki tray well and microlitre plate (u-bottomed) well.
Test procedure is essentially the same as previously described for the 'u' bottomed microlitre plate except that 5 μl. volumes of reagents are added using an Eppendorf 5 μl. pipette with disposable tip. During incubation (30 minutes at 37°C) the cells settle to form a continuous circle around the periphery of the base of the well, at which point positive and negative agglutination patterns are indistinguishable. The tray is tilted, carefully to avoid undue vibration, to an angle of 40° and left for approximately 15 minutes. In those wells where no agglutination has occurred, the cells gradually slide down the sides of the bottom of the well, while in those wells where agglutination is present, they remain as a complete circle of agglutination around the base of the well (diagram 3,3,II,b).

Diagram 3,3,II,b. Cell settling patterns in Terasaki tray
The distinction between positive and negative may be enhanced by centrifuging the plates (Griffin & George bench centrifuge with serological head, 60 seconds at No. 2 setting) prior to placing them at an angle.

Further economies are possible if the concentration of sensitised cells is reduced. The optimum cell concentration for use in the miniturised (Terasaki tray) system was found by titrating an HB$_3$Ab positive serum using different concentrations of sensitised cells. The results shown in table 3.3.II.c. indicate that a 0.25 percent cell suspension provides the most acceptable results in terms of sensitivity and ease of reading.

**Table 3.3.II.c. Effect of HB$_3$Ag coated spherocyte concentration on sensitivity and readability of the Terasaki tray HAI test system.**

<table>
<thead>
<tr>
<th>HB$_3$Ag coated cell concentration</th>
<th>Reciprocal of HB$_3$Ab titre ($\times 100$)</th>
<th>Readability</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 percent</td>
<td>-</td>
<td>too many cells</td>
</tr>
<tr>
<td>1 percent</td>
<td>-</td>
<td>too many cells</td>
</tr>
<tr>
<td>0.5 percent</td>
<td>20,480</td>
<td>good</td>
</tr>
<tr>
<td>0.25 percent</td>
<td>40,960</td>
<td>good</td>
</tr>
<tr>
<td>0.125 percent</td>
<td>40,960</td>
<td>poor</td>
</tr>
<tr>
<td>0.05 percent</td>
<td>-</td>
<td>not visible</td>
</tr>
</tbody>
</table>

### III. Discussion

As a test vehicle the Terasaki tray allows a reduction in the volume of each reagent from 25 ul to 5 ul. This is an important economy not only in terms of HB$_3$Ag-coated gluteraldehyde-fixed spherocytes but also of test serum which may not be readily available.
in the quantities necessary for confirmatory and/or subtyping tests using other techniques. Reduction in volume combined with the use of only a 0.25 percent spherocyte suspension leads to an overall economy of the order of fifteen-fold. In practical terms, this means that if, as has already been stated, 20 litres of neat HBsAg positive serum requires processing to provide sufficient reagents for one million tests by the microlitre plate method, the same number of tests could be performed by the miniturised (Terasaki tray) method as a result of processing only \(\frac{20}{15} = 1.3\) litres of neat serum.

Logistics such as these place bulk reagent preparation easily within the capability of many Blood Transfusion Laboratories.

Economics of this magnitude are seldom achieved without sacrifice, and in this instance the robustness of the test appears to suffer. The miniturised method is particularly vulnerable to rough handling and/or vibration once it has been placed at the 40° angle. Fortunately, this problem is easily overcome by using an angled shelf bracketed to a solid vibration-free wall (diagr. 3.3.III). This arrangement also has the advantage of introducing a degree of uniformity to each test since the angle is kept constant. If the angle is much greater than 40° agglutinated cells will collapse prematurely, while a smaller angle will cause slower cell settling.

It was also found that a 1 in 5 dilution of test serum could be used.
Diagram 3.4. Wall-mounted shelf recommended for Terasaki tray system

- Empty well
- Well containing reagents prior to incubation
- Settled cells after incubation
- Solid wall
- Tray
- Shelf
- Plate at 40° angle

Positive agglutination (cells remain as circle)
Negative agglutination (cells slide to lower edge of base of well)
CHAPTER 4

BULK PREPARATION OF HBsAg-COATED, GLUTERALDEHYDE-FIXED SPHEROCYTES

I. Introduction

Large-scale preparation and storage of a reagent used for serological testing is very important for standardisation and economy, particularly if that reagent is sufficiently stable to survive transportation to and subsequent storage in other laboratories. Unfortunately, the highly sensitive 'third generation' HBsAg test reagents are only available to the majority of Blood Transfusion Centres on a commercial basis, the cost ranging from 10 new pence per test (Hepatest- R.P.H.A., Wellcome Reagents, if bought in bulk) to 50 new pence per test or more, apparently depending upon geographical location (Austria-II, KIA Abbott Laboratories). Experimental data suggest that HBsAg-coated, glutaraldehyde-fixed spherocytes are a stable, sensitive and specific reagent well suited to testing large numbers of blood donors.

This chapter describes the bulk preparation of HBsAg-coated, glutaraldehyde-fixed spherocytes in a quantity sufficient for approximately one million tests (if the miniaturised test system is employed).

II. Tanning, Sensitisation and Stabilisation of Sufficient Reagent for One Million Tests

Sixteen mls. of packed glutaraldehyde-fixed spherocytes were washed twice with 500 ml. volumes of physiological saline (MRC bottle spun at 1,000 r.p.m. for 5 minutes on MSE 6L centrifuge at room temperature). The cells were resuspended in 500 ml. of tannic acid (M. & B., 1 in 60,000 dilution in PBS pH 7.2 previously heated to 37°C)
and left in a 37°C waterbath for 15 minutes with occasional mixing. The tanned cells were spun down as before, washed twice with 500 ml PBS pH 6.4 and finally resuspended in 400 ml of the same buffer. Twenty ml HBsAg, prepared as previously described and containing both the 'd' and 'y' determinants, was added and the spherocyte-antigen suspension mixed continuously at room temperature (see discussion). After 20 hours, a 1 ml aliquot was removed, stabilised (as previously described) and used to titrate a known standard HBsAb-containing serum. Sampling was repeated at approximately 20 hour intervals until a satisfactory sensitivity was achieved, when the bulk of the reagent was removed from the mixer and the spherocytes recovered by centrifugation as before. The sensitised spherocytes were then stabilised by resuspension and mixing for 4 hours at room temperature in 500 ml PBS pH 7.2 containing 2 percent normal human serum to give a cell suspension of approximately 3.0 percent (or ten times working concentration). Storage of these cells overnight at 4°C was followed by a repetition of the stabilising procedure. Thereafter, the 3.0 percent suspension was dispersed into 50 ml aliquots and stored at 4°C until required, when each aliquot (or part thereof) could be diluted ten-fold with PBS pH 7.2 containing 2 percent normal human serum to achieve working concentration.

III. Discussion

The ability to prepare a large batch of stable HBsAg test reagents in a Blood Transfusion Centre provides the basis for uniformity of donor HBsAg testing and represents a considerable saving in both cash (one million Hepatests = £60,000, while one million AUSKIA - II = £500,000) and time, and allows third generation
testing efficiency to be made available at a fraction of the cost of purchasing commercial reagents.

Bulk preparation seems to result in reagents which are every bit as sensitive and stable as those produced in much smaller 'experimental' quantities, since transportation over thousands of miles via air freight appears to have no adverse effect even when cells are maintained in the liquid state.

If sampling of the sensitising mixture suggests that a satisfactory sensitivity is not being achieved, it is possible to add more HB$_3$Ag. However, care must be taken in this respect since an excess of antigen will lead to autoagglutination, presumably resulting from overcoating of the fixed spherocytes. It is well to bear in mind that stabilisation of 'samples' requires overnight at 4°C, during which time the bulk reagent is still sensitising. Experience of bulk reagent preparation suggests that sensitivity plateaus after about 48 hours if no further HB$_3$Ag is added.

An interesting observation was that cell sensitisation proved successful when mixing with a mechanical turntable, but unsuccessful when mixing with a magnetic stirrer and plastic-coated follower. Adsorption of HB$_3$Ag onto the follower has been eliminated, but the electrochemical or electrophysical possibilities for such a failure have yet to be investigated.

**Addendum**

The work reported in this chapter and in chapter 2 (IIa and IIb) was undertaken in collaboration with Mr. A.D. Watt (Chief Technician, Microbiology Unit, Blood Transfusion Service, Royal Infirmary, Edinburgh) and has been included in this thesis to emphasise the potential of the technique developed in Part Two.
CHAPTER 5
REAGENT SAFETY

I. Introduction

Reagents used routinely should be made as safe as possible for the protection of staff members who are in daily contact with them. This is particularly important when a known pathogen, such as HB Ag, is involved. General viral inactivating procedures, such as treatment with betapropiolactone and ultra-violet irradiation (La Grippo, Hayashi and Saeed, 1971), are necessary precautionary measures, but until a valid test of infectivity becomes available, the effectiveness of such measures for the inactivation of HB Ag must remain questionable.

Gamma irradiation from a cobalt source is a recognised method of virus inactivation for which some data is available (Ginosa, 1968). Unfortunately, the reagent to be irradiated should be in the lyophilised state to prevent the generation of excess heat.

Pasteurisation (60°C for 10 hours) is considered by some to be an effective means of inactivating HB Ag (Mozen, Schroeder and Cabasso, 1972) based upon the hepatitis-free history of human albumin prepared by Cohn fractionation.

II. Pasteurisation of HB Ag-coated, Glutaraldehyde-fixed Spherocytes

A 3 percent suspension of HB Ag-coated glutaraldehyde-fixed spherocytes was divided into 8 x 1 ml. aliquots which were allowed to stand in a 60°C waterbath for periods ranging from 0 to 24 hours. At the appropriate times, an aliquot was removed and stored at 4°C. After removal of the final aliquot (24 hours), each was diluted ten
times (with PBS pH 7.2 containing 2 percent normal human serum) to a working concentration of 0.5 percent and used to titrate a standard HB$_2$Ab-positive serum. Table 3.5.II indicates that this reagent may be maintained at a temperature of 60°C for at least 16 hours before significant reduction in sensitivity occurs.

Table 3.5.II. Effect of heating HB$_2$Ag-coated glutaraldehyde-fixed spherocytes at 60°C for various times

<table>
<thead>
<tr>
<th>Time (hours) at 60°C</th>
<th>Reciprocal of HB$_2$Ab titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40,960</td>
</tr>
<tr>
<td>2</td>
<td>40,960</td>
</tr>
<tr>
<td>4</td>
<td>40,960</td>
</tr>
<tr>
<td>8</td>
<td>40,960</td>
</tr>
<tr>
<td>10</td>
<td>40,960</td>
</tr>
<tr>
<td>16</td>
<td>40,960</td>
</tr>
<tr>
<td>20</td>
<td>40,960</td>
</tr>
<tr>
<td>24</td>
<td>5,120</td>
</tr>
</tbody>
</table>

III. Gamma Irradiation of Pasteurised HB$_2$Ag-coated, Glutaraldehyde-fixed Spherocytes

HB$_2$Ag-coated, glutaraldehyde-fixed spherocytes were adjusted to a 5 percent suspension in PBS pH 7.2 and divided into 1 ml aliquots in clean glass screw cap tubes (Flow Laboratories). The contents of each tube was 'shell frozen' in liquid nitrogen and lyophilised on a Virtis dryer. The lyophilised reagent was stored at room temperature. Twelve tubes containing lyophilised reagent were exposed to 2.5 megarads of gamma irradiation from a cobalt source (by kind permission of Ethicon Limited).
Lyophilised and lyophilised-irradiated reagents were reconstituted by adding 1 ml. PBS pH 7.2 containing 2 percent normal human serum to the tube, allowing to stand for 5 minutes and adding a further 9 ml., resulting in 10 ml. of working concentration. Titration of a standard HBsAb positive serum showed the sensitivity of both lyophilised and lyophilised-irradiated reagents had been reduced by one or two doubling dilutions.

IV. Discussion

BPHA tests for HBsAg have to some extent allayed fears concerning reagent infectivity by using cells coated with specific antibody presumed to be free of HBsAg. Unfortunately, such reagents are expensive to produce, unstable once reconstituted and subject to non-specific agglutination due to the presence of impurities or unwanted specificities among the immunoglobulins used for coating. The HAI test, on the other hand, exhibits a high degree of specificity and is capable of equal or greater sensitivity than BPHA. Reagents are relatively inexpensive to produce in large quantities and are extremely stable, as further evidenced by their ability to withstand pasteurisation, lyophilisation and gamma irradiation. HBsAg-coated reagents do, however, carry an increased infectivity risk and for this reason reagents were submitted to the procedures detailed above.

Pasteurisation of 'bulk' reagents presented no additional problems and appeared to leave sensitivity as unaffected as in the pilot experiments. The reduced titre obtained after 24 hours at 60°C could have resulted from elution of HBsAg from the spherocye surface or deterioration of HBsAg antigenic sites. These possibilities were not investigated further. Subsequent batches of reagents were treated at 60°C for 16-18 hours (conveniently
overnight). Experience has shown that their stability remains unimpaired over the following months of storage at 4°C. Similarly, gamma irradiation appears to have little effect upon subsequent reagent stability whether stored in the lyophilised or liquid state. The slight loss of sensitivity following reconstitution is disappointing, more so since the best settling patterns were obtained using a 1 in 10 dilution of test sera (pre-lyophilisation reagents could be used with a 1 in 5 dilution of test sera). Lyophilisation appears to be responsible as the sensitivity of pre- and post-irradiated lyophilised reagents was identical. It may be that the forces acting upon the 'frozen' reagents during 'drying' caused the less tightly bound antigen particles to be dislodged from the spherocyte surface.
CHAPTER 6
COMPLEXED REVERSE PASSIVE HAEMAGGLUTINATION (C.R.P.H.A.)

I. Introduction

In a recent publication (Technical Report Series No. 570, 1975), the World Health Organisation have recommended that RPHA be used for routine blood donor (HB$_{5}$Ag) testing. Three such tests are commercially available and were briefly compared in Part One (section 2, chapter 2) of this thesis. RPHA has the advantage of being a simple one-step test, with a sensitivity between C.I.E.O.P. and HLA, which may be read within 1 to 3 hours. Unfortunately, the commercial product is expensive and unstable once reconstituted, and the purified animal hyperimmune globulin used leads to occasional false positive reactions. The long and complex procedure of raising antisera in animals, purifying it and finally adsorbing it onto a suitable carrier places RPHA reagent preparation beyond the scope of most Blood Transfusion Service laboratories.

The addition of one simple step to the procedure for preparation of HB$_{5}$Ag-coated, gluteraldehyde-fixed spherocytes enables these reagents to be used in a one-stop RPHA test for HB$_{5}$Ag.

II. Antibody Overcoating of HB$_{5}$Ag-coated, Gluteraldehyde-fixed Spherocytes

An aliquot (5 mls. of 3 percent suspension) of HB$_{5}$Ag (adyw)-coated gluteraldehyde-fixed, stabilised spherocytes was spun gently on a bench centrifuge and the supernatant removed. The packed cells were resuspended in 20 mls. of neat human serum known to contain a high (HA titre $> \frac{1}{1000}$) level of HB$_{5}$Ab (it is preferable that the antiserum be active against all the HB$_{5}$Ag determinants
coated onto the spherocyte surface, and it may be necessary to mix antisera in order to achieve such broad-spectrum activity), and an equal volume of PBS pH 7.2 added. The container was then attached to a mechanical (Mattburn mixer) mixer and the HB₃Ag-coated spherocyte-antiserum suspension mixed at room temperature for approximately 72 hours. (It is essential that the mixing be continuous in order to prevent the formation of immune aggregates).

The antigen-antibody coated spherocytes were then washed three times in PBS pH 7.2, adjusted to working strength (approximately 0.3 percent), and allowed to stand at 4°C for a period of 4 to 5 days, after which they were ready for use. The presence of HB₃Ag-HB₃Ab immune complexes on the spherocyte surface led to the adoption of the term complexed reverse passive haemagglutination (C.R.P.H.A.)

III. C.R.P.H.A. Test for HB₃Ag

Test sera were diluted 1 in 5 in PBS pH 7.2 as for HAI. 10 μl. of the test serum dilution was placed into the well of a Terasaki tray and 5 μl. of C.R.P.H.A. reagent (0.3 percent suspension) added. The tray was gently shaken to enhance mixing, and floated on the surface of a 37°C waterbath for 1 hour, by which time the spherocytes had 'settled' to form a circle round the base of the well. The tray was then gently placed at an angle of 40 to 50° as for HAI and read after 5 to 15 minutes or when the negative controls (10 μl. 1 in 5 normal serum + 5 μl. C.R.P.H.A. reagent) were down. The presence of detectable HB₃Ag in the test sera was evidenced by a complete circle of agglutination, while in the absence of an antigen-antibody reaction, the spherocytes slide down to the lower part of the base of the well.
IV. Comparison of C.R.F.H.A. Sensitivity with other Third Generation Tests for HB Ag

(a) Titration sensitivity

Doubling dilutions of two HBAg positive sera (one adw and one ayw) tested by C.R.F.H.A., HAI, RPHA (Hepatest), and RIA (Ausria-2).

(b) Panel studies

C.R.F.H.A., HAI and RIA were used to test two well documented panels (B and C) supplied by the Central Public Health Laboratory (C.P.H.L.) and two lesser known panels provided by the West of Scotland Regional Transfusion Centre (Law Hospital) and the Natal Blood Transfusion Centre (Durban, South Africa).

V. Results

Titration study results (table 3.V.a) indicate that C.R.F.H.A. performed as described is as sensitive as the commercial RPHA (Hepatest), but less sensitive than HAI or RIA (Ausria-2).

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<tr>
<th>Test</th>
<th>Reciprocal of HBAg(adw) titre</th>
<th>Reciprocal of HBAg/ayw titre</th>
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</thead>
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<tr>
<td>C.R.F.H.A.</td>
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<td>HAI</td>
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<td>16,000</td>
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<td>RPHA (Hepatest)</td>
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<td>RIA (Ausria-2)</td>
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</table>
Results obtained from C.R.P.H.A. panels B and C (tables 3.6.V.b and 3.6.V.o) suggest that the sensitivity of C.R.P.H.A. implied by the titration studies is borne out when sera containing various concentrations of HB$_s$Ag are tested under code. Results obtained from testing the Natal B.T.S. panel (table 3.6.V.d) and the West of Scotland B.T.S. panel (table 3.6.V.e) further confirm that the sensitivity of C.R.P.H.A. is comparable with established 'third generation' tests, although results obtained with samples No. 20 and 27 in the Natal panel and samples No. 21 and 26 in the West of Scotland panel suggest that the C.R.P.H.A. reagents are vulnerable to occasional false positive as well as false negative (sample 15, table 3.6.V.e) reactions as is the case with commercial RPHA reagents. This aspect of their performance requires further investigation.

VI. Discussion

Continuous mechanical mixing of HB$_s$Ag-coated, gluteraldehyde-fixed, stabilised spherocytes in an environment of neat serum containing an excess of specific antibody results, after 2 to 4 days, in a suspension of HB$_s$Ag-coated spherocytes which are 'overcoated' with specific antibody but are not agglutinated (presumably because no HB$_s$ antigenic sites remain exposed to react with antibody overcoated onto other antigen-coated spherocytes). However, these antibody overcoated spherocytes will agglutinate if introduced into a test medium possessing free antigen.

Preliminary observations suggest that C.R.P.H.A. reagents are extremely stable, presumably because they utilise the HB$_s$Ag-coated spherocyte as a carrier to which antibody is linked by a stable antigen-antibody interaction, a feature which removes the necessity
of preparing and passively coating purified HB\textsubscript{3}Ab which results in a rather unstable cell-antibody bond.

Provided a reasonable balance of anti-d and anti-y is maintained in the overcoating procedure, sensitivity of C.R.P.H.A. is equivalent to any of the commercial RPHA tests, while pilot experiments suggest that certain modifications of test procedure may further increase sensitivity.

The simplicity and economy inherent in the preparation of HAI reagents applies equally to C.R.P.H.A., since antibody overcoating requires only one simple additional step. If after the first overcoating, free antigenic sites remain, the overcoating procedure may be repeated as often as is necessary (provided the specificity of HB\textsubscript{3}Ab and HB\textsubscript{3}Ag correspond).

C.R.P.H.A. reagents also possess the potential for HB\textsubscript{3}Ag and HB\textsubscript{3}Ab subtyping, although this has not been investigated in any detail. For example, if ady coated spherocytes are overcoated with anti-ad, then they will be agglutinated by a and d antigenic determinants in test serum, but will also be agglutinated if anti-y is present, and vice versa.
Table 3.6 V. 1 Results of testing C.R.P.H.L. Panel B by C.R.P.H.A.

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### Table 3.6.V.c: Result of testing C.P.H.L. Panel C by C.R.P.H.A., HAI and EIA

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Table 3.6. V.v. Results of testing panel provided by the West of Scotland Blood Transfusion Service using C.R.P.H.A., HAI and RIA

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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>32</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>33</td>
<td>-</td>
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<tr>
<td>34</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>35</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>36</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
PART FOUR

APPLICATIONS
CHAPTER 1

EVALUATION OF HAI FOR DETECTION OF HB s Ag AMONG BLOOD DONORS

I. Introduction

Despite the rejection of blood donations found to possess HB s Ag by C.I.E.O.P., hepatitis continues to complicate the therapeutic use of blood and blood products (Reinicke et al., 1973). More sensitive serological tests for HB s Ag may further reduce the incidence of posttransfusion hepatitis but are unlikely to eliminate it completely because of the possible infectivity of blood containing antibody to the Dane particle core in the absence of detectable HB s Ag (Hoofnagle, Gerety, Li and Barker, 1974) and the possible existence of a hepatitis C virus (Prince, Brtoman, Grady, Kuhne, Hassi, Levine and Millman, 1974) - quite apart from other possible causes such as hepatitis A virus and other microbial agents (Zuckerman, 1970).

'Third generation' tests for HB s Ag using the principles of haemagglutination and radioimmunoassay are now commercially available and reports suggest that these kits can detect carriers of HB s Ag too weak to be positive by C.I.E.O.P. (Ling and Overby, 1972; Cayzer, Dane, Cameron and Denning, 1974). Unfortunately, the effective use of such commercial reagents in the blood transfusion service will prove expensive and only time will tell whether such expense is justified.

Data reported in Part Two (VIII and IX) of this thesis suggests that the HAI test possesses the characteristics of accepted "third generation" technology. The potential of HAI for use in mass blood donor testing was investigated by conducting a multi-centre evaluation involving over 70,000 donations.
II. Materials and Methods

Three Scottish Regional Transfusion Centres were involved in the evaluation study: the South-East of Scotland Regional Transfusion Centre, Edinburgh, the East of Scotland Regional Transfusion Centre, Dundee, and the North of Scotland Regional Transfusion Centre, Inverness. The initial part of the study involved the use of fresh human erythrocytes coated with HBsAg. Antigen was prepared centrally at Edinburgh, but cell sensitisation and subsequent reagent standardisation were performed at each centre independently. However, with the implementation of bulk production of fixed spherocytes, it became possible to supply each centre with reagents from the same batch, thereby improving standardisation considerably. The miniturised (Terasaki tray) version of HAI was performed at each centre in parallel with C.I.E.O.P. 4086 of the 44,053 donations tested at Edinburgh were also tested with the 'Hepatest' (Wellcome Reagents) K.P.H.A. kit. Positive HAI reactions were confirmed by subtyping.

III. Results

The overall incidence of HBsAg among donors at the three centres taking part was 0.04 percent. HAI detected seven HBsAg carriers missed by C.I.E.O.P. (table 4.1.III). Five of these were 'new' donors and two were donors whose previous donation had been implicated, among others, in posttransfusion hepatitis. Each centre found at least one additional positive by HAI. Among the 4,086 donations tested using 'Hepatest', three HBsAg carriers were found, but these were positive by HAI and C.I.E.O.P. In addition, the commercial RPbA tests gave 20 positive reactions which were subsequently shown to be nonspecific.
Table 4.1.III. Incidence of HB$_{Ag}$ among blood donors tested at three centres

<table>
<thead>
<tr>
<th></th>
<th>No. donations tested</th>
<th>No. HAI pos.</th>
<th>No. CIEOP pos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edinburgh</td>
<td>44,053</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>Inverness</td>
<td>16,371</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Dundee</td>
<td>9,800</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>70,224</td>
<td>26 (0.04%)</td>
<td>19 (0.03%)</td>
</tr>
</tbody>
</table>

IV. Discussion

Recent reports have highlighted the inadequacies of C.I.E.O.P. for detecting HB$_{Ag}$ in blood donations (Reinicke et al., 1973; Ling and Overby, 1972; Cayzar et al., 1974). The American Red Cross seem to have opted for RIA, while Regional Transfusion Centres in the U.K. appear to favour RPHA. Three commercial RPHA assays are currently available and have been reviewed in Part One (section two, chapter 2) of this thesis. The cheapest of these retails at approximately 10 pence per test if bought in bulk. All three tests are susceptible to a small number of false positive reactions, these being resolved by absorption-titration experiments.

HAI has a sensitivity for HB$_{Ag}$ detection comparable with other third generation techniques and, being an inhibition reaction, has an inherent specificity not found in RPHA tests. The use of monospecific antibody (prepared by absorption) allows HAI to be used to subtype antigen-positive sera, providing an essential confirmatory step.
The cost of HAI is negligible due to the availability of raw material, the considerable expertise in haemagglutination technology within the blood transfusion service and the simplicity of reagent preparation combined with the small quantities (5 µl.) needed for testing.

The prime criterion in evaluating a new screening test such as this must be the number of additional antigen carriers detected in comparison with current techniques. The results indicate that HAI is capable of detecting donations containing HBAg in concentrations below the sensitivity of C.I.E.O.P.*

HAI may prove an acceptable alternative to commercial third generation HBAg tests for use in large scale blood donor testing. It seems that with appropriate training, the technique may be readily introduced into most regional transfusion centres, thereby keeping reagent costs to a minimum.

* HAI detects 50-100 ngm HBAg protein per ml.
CIEOP detects approximately 5000 ngm HBAg protein per ml.
CHAPTER 2

PREVALENCE OF HB$_{Ag}$ SUBTYPES AMONG (BLOOD DONOR) CARRIERS IN SOUTH-EAST SCOTLAND AND THEIR ASSOCIATION WITH ABNORMAL LIVER FUNCTION

I. Introduction

The 'd' determinant of HB$_{Ag}$ appears to predominate among apparently healthy blood donors in Western Europe and the U.S.A. (Mazzur et al., 1974), while the 'y' determinant is frequently associated with clinical evidence of HB$_{Ag}$ positive hepatitis in these regions (Wenzel et al., 1972; Le Bouvier et al., 1972; Moseley et al., 1972). Some workers have been unable to find such a distinct association between the HB$_{Ag}$ subtype and liver damage (Gordon et al., 1972; Feinman et al., 1973). On the basis of subtype distribution among acute and chronic cases of type B hepatitis, it has been suggested that infection with the d determinant is more likely to result in mild illness progressing to the chronic HB$_{Ag}$ carrier state, whereas infection with the y determinant seems likely to result in a severe, acute, but self-limiting illness (Holland et al., 1972; Dodd et al., 1973; Suckerman, Hacker and Ash, 1974).

HB$_{Ag}$ (blood donor) carriers were subtyped for determinants a, d, y, w and r by HAI and liver function data (serum glutamic pyruvic transaminase, SGPT; serum glutamic oxaloacetic transaminase, SGOT) was obtained, where possible, in an attempt to correlate HB$_{Ag}$ subtype with abnormal liver function in the South-East of Scotland.

II. Materials and Methods

HAI subtyping was performed as described in Part Three (chapter 1) of this thesis. Liver function tests (SGPT and SGOT) were performed.
in the Department of Clinical Chemistry at the Edinburgh Royal Infirmary. Eighty apparently healthy blood donors found to carry HB$_3$Ag were subtyped. Thirty gave regular (three-monthly) blood samples for HB$_3$Ag and liver function determination.

III. Results

As expected, the 'd' determinant was found to predominate among HB$_3$Ag (blood donor) carriers in the South-East of Scotland (table 4.2.III.a). All samples possessed both the 'a' and 'w' determinants in addition to either 'd' or 'y'.

Table 4.2.III.a. Incidence of HB$_3$Ag subtypes among apparently healthy HB$_3$Ag carriers in South-East Scotland

<table>
<thead>
<tr>
<th>No. subtyped</th>
<th>No.adw</th>
<th>No.ayw</th>
<th>No.adr</th>
<th>No.ayr</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>64 (80%)</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Of the thirty carriers who presented regularly for follow-up study, only four exhibited persistent abnormal liver function, while two more showed occasional slight SGPT and SGOT elevations (table 4.2.III.b). Of these 6, one carried HB$_3$Ag of subtype ayw.
Table 4.a.III.b. Incidence of abnormal liver function among apparently healthy HB^Ag carriers in South-East Scotland

<table>
<thead>
<tr>
<th>Liver Function</th>
<th>Normal</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>adw (26)</td>
<td>21</td>
<td>5</td>
</tr>
<tr>
<td>ayw (4)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Total (30)</td>
<td>24</td>
<td>6</td>
</tr>
</tbody>
</table>

IV. Statistical Analysis

In order to test the (Null) hypothesis that there is no significant difference between the adw and ayw groups in relation to liver function other than would be expected to occur by chance, Fisher's exact test was applied as follows (nomenclature according to Geigy Scientific Tables):

<table>
<thead>
<tr>
<th>Normal</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>adw</td>
<td>x₁</td>
</tr>
<tr>
<td>ayw</td>
<td>x₂</td>
</tr>
<tr>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

From significance limits for four-fold tables; the first table was rearranged so that N₁, N₂ and x₁ (N₁ - x₁), that is:

<table>
<thead>
<tr>
<th>Normal</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>adw</td>
<td>1</td>
</tr>
<tr>
<td>ayw</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>
Therefore the Null hypothesis cannot be rejected, that is, there is no significant relationship between adw and ayw subtypes and liver function.

Fisher’s exact test was applied because the figures in certain boxes were too small to apply $\chi^2$ analysis.

V. Discussion

The predominance of the adw configuration of HB\textsubscript{Ag} determinants (80 percent) among apparently healthy blood donors in the South-East of Scotland is in agreement with previous studies covering Western Europe and the U.S.A. (Mazzur \textit{et al.}, 1974). If the thirty HB\textsubscript{Ag} carriers followed for liver function studies are representative of other apparently healthy HB\textsubscript{Ag} carriers in this region, it would appear that approximately one in five of such carriers are experiencing a mild chronic liver disorder. Statistical evaluation of the present study suggests that among apparently healthy carriers of HB\textsubscript{Ag} persistent liver dysfunction is not significantly associated with either the d or y antigenic determinant. This is in conflict with the frequent association of the y determinant in more severe 'clinical' cases of HB\textsubscript{Ag} positive hepatitis (Wenzel \textit{et al.}, 1972; Le Bouvier \textit{et al.}, 1972; Roseley \textit{et al.}, 1972).

Following the discovery of the 'e' antigen system of HB\textsubscript{Ag} by Magnus and Espmark (1972) and its possible association with chronic liver dysfunction (Feinman, Berris, Sinclair, Krobel, Murphy and Maynard, 1975), the incidence of e and anti-e among apparently
healthy HB$_3$Ag (blood donor) carriers in this region was investigated using the I.D. technique. At the time of writing, 22 HB$_3$Ag carriers have been tested. Three were e-antigen positive, 16 possessed e-antibody, while neither e-antigen nor e-antibody could be found in 6. The three e-antigen positive carriers were the only ones to exhibit persistent elevations of SGOT and SGPT, suggesting that in apparently healthy HB$_3$Ag carriers, the e antigen may provide a better marker of possible chronic hepatitis than the HB$_3$Ag subtype. There was no correlation between presence of e-antigen and the d or y determinant.
CHAPTER 3
RE-INVESTIGATION OF BLOOD DONATIONS IMPLICATED IN POSTTRANSFUSION HEPATITIS

I. Introduction, Materials and Methods

Data presented in Chapter 1 of this part of the thesis clearly indicates that HAI is capable of detecting HBsAg carriers who would be missed by C.I.E.O.P. Since not all recipients of HBsAg positive blood develop antigenemia and/or hepatitis (Gocks and Kavey, 1969), it is important to establish whether or not those carriers found to be HAI positive but C.I.E.O.P. negative are likely to be infective. From mid-1973 the South-East of Scotland Regional Blood Transfusion Service has stored 0.5 ml. aliquots of serum at -20°C from all donations for a period of six months. Since that time eight patients with suspected posttransfusion (viral) hepatitis have been reported to the Blood Transfusion Centre, and it has been possible to investigate each case by retesting the original C.I.E.O.P. negative donations using HAI, EIA (Ausria I) and RPHA (Hepatest). In addition, two suspected posttransfusion hepatitis cases were referred from Manchester Blood Transfusion Centre for similar investigation, although in this case the donations actually tested were obtained at a return visit of the donors and were not therefore truly representative of the original donations.

II. Results and Comments

The retesting of more than 100 donations implicated in eight cases of posttransfusion hepatitis in Edinburgh showed that in two cases an HBsAg positive unit of blood had been transfused which was not detected during initial testing by C.I.E.O.P. (table 4.3.11).
Table 5.3.11. Re-investigation of donors implicated in posttransfusion hepatitis

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. of donations involved</th>
<th>Counter-electrophoresis</th>
<th>Ausria 1</th>
<th>EHA1</th>
<th>Turkey cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vei. E+</td>
<td>8</td>
<td>All neg.</td>
<td>All neg.</td>
<td>All neg.</td>
<td>All neg.</td>
</tr>
<tr>
<td>McG. E+</td>
<td>14</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Moo. E-</td>
<td>18</td>
<td>&quot;</td>
<td>1 Pos.</td>
<td>1 Pos.</td>
<td>1 Pos.</td>
</tr>
<tr>
<td>Lil. E+</td>
<td>18</td>
<td>&quot;</td>
<td>All neg.</td>
<td>All neg.</td>
<td>All neg.</td>
</tr>
<tr>
<td>New. E+</td>
<td>32</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Lid. E+</td>
<td>14</td>
<td>&quot;</td>
<td>1 Pos.</td>
<td>1 Pos.</td>
<td>1 Pos.</td>
</tr>
<tr>
<td>Gow. E-</td>
<td>3</td>
<td>&quot;</td>
<td>All neg.</td>
<td>All neg.</td>
<td>All neg.</td>
</tr>
<tr>
<td>Psa. E-</td>
<td>10</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Far. M+</td>
<td>4</td>
<td>&quot;</td>
<td>1 Pos.</td>
<td>1 Pos.</td>
<td>&quot;</td>
</tr>
<tr>
<td>Ali. n.t.</td>
<td>17</td>
<td>&quot;</td>
<td>All neg.</td>
<td>1 Pos.</td>
<td>1 Pos.</td>
</tr>
</tbody>
</table>

E = Edinburgh
M = Manchester
+ = HB Ag Positive
- = HB Ag Negative
n.t. = not tested

Re-investigation of the two cases from Manchester revealed a positive HAI result from each though, interestingly, one was missed by MPHA and the other by MIA. The positive unit transfused in case 'Far.' was subtyped by HAI as adw, while that transfused in case 'Ali.' was found to be ayw, suggesting that the commercial MIA test used may be more sensitive for the detection of adw than ayw. A similar MIA-bias has been described by Vanderwald, Mahmood, Goffin, Porter Megson and Cossart (1974).
It appears that HAI is capable of detecting C.I.E.O.P.-negative HBAg carriers which can produce hepatitis if transfused into a susceptible recipient. Had HAI been used at the time of donation, it is probable that 40 percent of the posttransfusion hepatitis cases investigated here would have been avoided. Whether the four remaining cases in which all donations were negative by HAI, RPHA and RIA and the recipient was HBAg negative were in fact due to HB Ag is unclear, particularly in the face of circumstantial evidence of a hepatitis G virus(es) (Prince et al., 1974). Unfortunately, convalescent sera were not available to test for the appearance of HBAb.
CHAPTER 4
EFFECTIVENESS OF HEPATITIS B IMMUNOGLOBULIN IN THE PREVENTION
OF HEPATITIS RESULTING FROM ACCIDENTAL EXPOSURE TO HBsAg

I. Introduction

Normal immunoglobulin (N-IgG) prepared from the pooled plasma of unselected blood donors may prevent or modify infectious (type A) hepatitis. There are, however, conflicting reports regarding its efficacy in relation to serum (type B) hepatitis (Crossman et al., 1945; McCallum, 1965; Drake et al., 1955; Holland et al., 1966). During the recent virulent outbreak of dialysis-associated type B hepatitis in Edinburgh (Marmion and Tonkin, 1972) N-IgG exhibited no apparent protective effect. On the other hand, the use of hyperimmune hepatitis B globulin (HB-IgG) in France and the U.S.A. was associated with a definite protective effect which was particularly evident if administered soon after exposure to infective material (Krugman et al., 1971; Soulier et al., 1972). Soulier et al. (1972) followed 18 recipients of HB-IgG who had been exposed to HBsAg by transfusion of antigen positive blood or by accidental parenteral exposure. A rise in transaminase levels occurred in two patients, but none developed HBs antigenemia, neither did passive immunisation appear to favour the development of an HBsAg carrier state as had been feared by some workers (Prince et al., 1971).

Large clinical trials are currently being conducted in the U.K. and U.S.A. to provide further data regarding the safety and efficacy of HB-IgG, meanwhile this chapter is designed to provide an insight into the use of HB-IgG by considering the initial findings of an 'on-going' study at the South-East of Scotland Regional Blood
Transfusion Centre, Edinburgh. The HB-IgG used in this study was prepared at the Libertorl Protein Fractionation Centre nearby.

Its serologic (HBAb) status was compared with similar preparations from other centres.

II. Materials and Methods

(a) HB-IgG preparations

Since 1971 three batches of HB-IgG have been prepared from pooled donations known to possess specific HBAb by C.I.E.O.P. In each preparation the starting pool and the final product were exhaustively investigated for the presence of HBAg or immune complexes. HB-IgG was supplied as a 10 percent solution of 1 gm. or 0.5 gm. doses for intramuscular use. Similar preparations were obtained from Paris, Amsterdam and the Australian Commonwealth Serum Laboratory (C.S.L.) for comparison of serologic activity with our local product.

(b) Laboratory tests

Tests for HBAg were by HAI and RIA (Austria-2). The amount of HBAb activity was estimated by direct HA titration, while E.M. was used to look for HBAg-HBAb immune complexes. Follow-up liver functions tests consisted of measuring serum bilirubin and transaminase (SGOT and SCPT) levels.

(c) Experimental protocol

0.5 - 1.0 gm. HB-IgG was given within 3 to 5 days following accidental exposure (needle-stick or non-parenteral exposure) to HBAg. Prior to treatment the accident victim was tested for HBAb and HBAg to (i) avoid administering HB-IgG to an individual already carrying high concentrations of HBAb or HBAg and
thereby avoid wasting HB-IgG by unnecessary inoculation, and
(2) determine base level of HB\textsubscript{3}Ab (if any) in the victim prior to
treatment. Serum samples were obtained 3 to 4 days after HB-IgG
administration and, where possible, at fortnightly intervals for
the first three months, then monthly intervals for the next three
months. Each accident was categorised as 'high' or 'low' risk
depending upon the relevant circumstances. Twenty-two high risk
cases of direct parenteral inoculation with HB\textsubscript{3}Ag positive blood
resulted from either 'needle sticks' or cuts with broken glass. A
further twenty-three low risk cases occurred as a result of accident
situations in which infected blood was splashed onto large areas
of unprotected skin, swallowed as a result of mouth to mouth
resuscitation, or parenteral inoculation with blood from a patient
who had previously been HB\textsubscript{3}Ag positive but who was HB\textsubscript{3}Ag negative
at the time of the accident. During the follow-up, a record was
kept of any other treatment the HB-IgG recipient received.

III. Results

(a) Assessment of HB-IgG preparations

HB-IgG prepared in Amsterdam, Edinburgh, Paris and the
Commonwealth Serum Laboratory (Australia) were compared for HB\textsubscript{3}Ab
activity by HA. All preparations exhibited a high concentration of
HB\textsubscript{3}Ab with slight variations within and between centres (table 4.4,III.a).
Table 4.4.111.a. Titration of different preparations of HB-IgG

<table>
<thead>
<tr>
<th>Origin</th>
<th>Batch No.</th>
<th>Reciprocal of HA titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edinburgh</td>
<td>GHB/1</td>
<td>6,000</td>
</tr>
<tr>
<td></td>
<td>GHB/2</td>
<td>512,000</td>
</tr>
<tr>
<td></td>
<td>GHB/3</td>
<td>128,000</td>
</tr>
<tr>
<td>Paris</td>
<td>91C</td>
<td>6,000</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>128,000</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>128,000</td>
</tr>
<tr>
<td>Amsterdam</td>
<td>1</td>
<td>12,000</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1,000</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4,000</td>
</tr>
<tr>
<td>Australia</td>
<td>2</td>
<td>64,000</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8,000</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>32,000</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>16,000</td>
</tr>
</tbody>
</table>

(b) Assessment of response to HB-IgG

Of the 45 recipients of HB-IgG only 22 were available for comprehensive follow-up (Table 4.4.111.b). None developed HB₂-antigenaemia and serum bilirubin levels remained normal. Four HB-IgG recipients (BL, MD, EG and JA) exhibited abnormal transaminase levels. BL had chronic hepatitis with persistently abnormal liver function prior to inclusion in the HB-IgG study, suggesting that the elevated transaminase values were unrelated to recent HB₂Ag exposure. Patients MD and JA were receiving anti-tuberculosis therapy and suffering from glandular fever respectively. However, the liver function data
<table>
<thead>
<tr>
<th>Patient</th>
<th>Occupation</th>
<th>Accident category</th>
<th>HB-IgG Dose/batch</th>
<th>Follow-up period (weeks)</th>
<th>Peak SGPT/SGOT/ at time (weeks)</th>
<th>Reciprocal of peak HB Ab titre at time (weeks)</th>
<th>Time (weeks) of last HB Ab pos. sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK</td>
<td>Nurse</td>
<td>LR</td>
<td>1 gm/ GHB2</td>
<td>28</td>
<td>- / 5/-</td>
<td>256/0-4</td>
<td>17</td>
</tr>
<tr>
<td>HL</td>
<td>&quot;</td>
<td>HR</td>
<td>&quot;</td>
<td>32</td>
<td>26/27/-</td>
<td>128/2</td>
<td>12</td>
</tr>
<tr>
<td>WD</td>
<td>Dr.</td>
<td>&quot;</td>
<td>&quot;</td>
<td>28</td>
<td>18/32/-</td>
<td>256/0-3</td>
<td>8</td>
</tr>
<tr>
<td>BL</td>
<td>Nurse</td>
<td>LR</td>
<td>&quot;</td>
<td>16</td>
<td>59/82/*</td>
<td>16/1</td>
<td>3</td>
</tr>
<tr>
<td>ML</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>32</td>
<td>13/20/-</td>
<td>256/2</td>
<td>9</td>
</tr>
<tr>
<td>B</td>
<td>Dr.</td>
<td>&quot;</td>
<td>&quot;</td>
<td>48</td>
<td>28/21/-</td>
<td>128/1</td>
<td>2</td>
</tr>
<tr>
<td>LR</td>
<td>Nurse</td>
<td>&quot;</td>
<td>&quot;</td>
<td>32</td>
<td>35/30/-</td>
<td>32/4</td>
<td>12</td>
</tr>
<tr>
<td>McD</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>32</td>
<td>46/15/8**</td>
<td>128/2</td>
<td>18</td>
</tr>
<tr>
<td>FW</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>32</td>
<td>21/16/-</td>
<td>64/4</td>
<td>11</td>
</tr>
<tr>
<td>HT</td>
<td>Dr.</td>
<td>&quot;</td>
<td>&quot;</td>
<td>28</td>
<td>17/17/-</td>
<td>128/1</td>
<td>4</td>
</tr>
<tr>
<td>GB</td>
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<td>&quot;</td>
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<td>72</td>
<td>66/-/17</td>
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<td>CY</td>
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<td>35/33/-</td>
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<td>HR</td>
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<td>38/35/-</td>
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<td>28</td>
<td>110/20/4</td>
<td>40/0-1****</td>
<td>-</td>
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LR = low risk; HR = high risk; * chronic hepatitis; ** receiving anti-T.B. drugs;
- = no data available; *** = HB-IgG prepared at Lister Institute (Elstree);
**** pre-existing HB Ab.
relating to patient EG (figure 4.4.III.c) may be related to his accidental exposure to HB₃Ag. EG is a medical laboratory technician who cut his hand on a broken centrifuge tube containing HB₃Ag (ayw) positive blood from a renal unit patient implicated in two fatalities during the 1969-1970 dialysis-associated epidemic in Edinburgh. There was little doubt, therefore, regarding the infectivity and virulence of the inoculum. EG received 20 ml. N-IgG immediately after the accident, the following day and five weeks later. He also received 1 gm. of HB-IgG (supplied by Lister Institute, Elstree) three days after the accident. An influenza-like

Figure 4.4.III.c. Post-HB-IgG follow-up of patient EG.
illness followed by abdominal pain developed nine weeks later with epigastric discomfort 20 weeks post inoculation, the latter being preceded by a moderate but persistent SGPT elevation lasting eleven weeks. The circulating HBAb titre rose prior to the prodromal symptoms but no HBAg was detected throughout the follow-up period. EG has remained well since then.

In all but one of the patients followed the HB-IgG administered contained sufficient activity for HBAb to be detectable (suggesting that antibody was in excess) for an average of 11 weeks. The highest passive HBAb titre usually occurred between 1 to 4 weeks post HB-IgG administration, a feature which may reflect the cumulative effect of the slow release of antibody from the site of intramuscular inoculation into the circulation. Those receiving 1 gm. of GHB 2 usually exhibited higher circulating HBAb levels than those receiving 0.5 gm. of GHB 3. Although one would expect this to be the case, the data cannot be compared directly since initial quality control studies on the HB-IgG preparations suggest that the activity of GHB 2 was greater than that of GHB 3.

As anticipated, the recipients of HB-IgG were all medical, nursing or technical staff, and as such it was interesting to note that among this high exposure risk group, 9 percent showed evidence of previous exposure to HBAg, representing ten times the incidence of HBAb than among healthy blood donors in the same region.

IV. Discussion

The distinction between 'high risk' and 'low risk' exposures to HBAg is difficult if not impossible to make (not all the six staff members who died in the 1969-1970 dialysis-associated outbreak in Edinburgh were obviously parenterally exposed). The type of
accidental involvement and the source of the inoculum should be carefully analysed in each case. Some of the subjects in the present study did not report actual parenteral inoculation, but were given HB-IgG because of the circumstances surrounding their exposure (e.g. mouth to mouth resuscitation of a neonate immediately after delivery of an antigen positive mother). In such cases assessment of the prophylactic value of HB-IgG may prove difficult.

None of the HB-IgG recipients showed evidence of immediate or delayed reaction to the injection. Most had detectable circulating HBsAb for an average of eleven weeks after inoculation, confirming the findings of Samaness, Prince, Goodman, Enrich, Pick and Ansari (1974). The reason for the rapid disappearance of antibody in BL, B, TH and FQ is not clear. None had pre-existing, anti-globulin (GM, Inv) to the administered HB-IgG. BL had chronic hepatitis possibly of autoimmune origin which may have accelerated HBsAb removal by virtue of abnormal protein metabolism, but the other three were clinically and biochemically normal. Since immunoglobulins are suicidal proteins, being catabolised in the performance of their function, it could be argued that accelerated clearance of HB-IgG may reflect the presence of HBsAg in or on the tissues. Millman et al. (1969) reported the occurrence of HBsAg in the hepatocytes of serum negative patients and demonstrated cellular hypersensitivity to HBsAg in nearly 60 percent of a group of HBsAg negative chronic hepatitis patients. Further investigations regarding the occasional rapid clearance of HB-IgG are clearly merited.

Several batches of N-IgG, Rhesus anti-D and vaccinia immunoglobulin were tested for HBsAb. No activity was found in the few anti-D or anti-Vaccinia preparations tested, and only one of the
27 N-IgG preparations exhibited an HB$_A$Ab titre of greater than 1/500 by HA, most showing a titre of less than 1/10. It would seem, therefore, that the fractionation of HB$_A$Ab positive plasma produces a product which, by virtue of its high HB$_A$Ab content, provides a significant degree of protection against type B hepatitis as a result of accidental exposure involving a relatively small challenge dose. The presence of detectable circulating HB$_A$Ab for some weeks after HB-IgG administration suggests antibody excess and infers that any circulating HB$_A$Ag has been complexed and, hopefully, neutralised. It seems logical that HB-IgG should be administered as soon as possible after exposure (except where HB$_A$Ab is already present in high concentration or where the victim is already found to be an HB$_A$Ag carrier) for maximum efficacy before the virus gains the protection of the (hepatic) cells in which it replicates.

The HB-IgG preparations used in this study possessed specific antibody to both the d and y determinants of HB$_A$Ag. It seems logical that the protective value of the product will be dependent in part upon the presence of antibody to all the HB$_A$Ag determinants associated with the infecting virus. The HB$_A$Ab activity of GHB 2 and GHB 3 was similar to that of HB-IgG prepared at other centres. Variation between batches is a feature requiring further assessment. HB-IgG is currently prescribed by volume or weight, whereas it may prove more beneficial to the recipient if doses were to be standardised in terms of biological activity. The feasibility of expressing the antibody activity directly is supported by Ginsberg et al. (1972) who observed a correlation between antibody titre and apparent protection. It is felt that the present study demonstrates the prophylactic value of HB-IgG which in turn emphasises the need to standardise such preparations on a world-wide basis.
PART FIVE

FINAL DISCUSSION AND CONSIDERATION OF
FUTURE LINES OF RESEARCH
I. Identification of HBsAg

Recognition of the significance of the presence of HBsAg in blood has led to a proliferation of laboratory techniques over the past five years, many of which have been reviewed in Part One (Section Two) of this thesis. It is now established beyond doubt that 'third generation' tests (RIA, RPHA, HAI) are superior to C.I.E.O.P. for the detection of HBsAg among blood donors and patients, increasing the number of detectable carriers (blood donor) by up to 50 percent (Korlets, Klahn, Ritman, Damas and Gitnick, 1973; Vandervelde et al., 1974; Hopkins, Robertson, Ross, Turnbull and Das, 1975; Wallace, Barr and Milne, 1975; Polasky and Tawall, 1975; Dodd, Ni, Malin and Greenwalt, 1975). Data presented in Part Four (chapters 1 and 3) suggests that the higher sensitivity of HAI, RPHA and RIA could result in a worthwhile reduction of the number of cases of posttransfusion type B hepatitis.

As one with some experience in the use of most of the newer HBsAg assays, I find the most endearing quality of RIA to be its objectivity. Unfortunately, this is offset by the high cost of reagents and counting equipment, quite apart from the hazards of handling radioactive isotopes. Many laboratories, particularly those responsible for blood donor testing, are reluctant to use RIA because of cost and the time required for completion of the test. Among those who have evaluated some form of RIA a popular choice has been the sandwich solid phase kit (SP-RIA—AUSRIA) available from Abbott Laboratories (see Part One, Section 2, chapter 3). The initial version of the test (AUSRIA-I) utilised HBsAb-coated tubes and required a total incubation time of 21 hours. This was replaced by a modified system (AUSRIA-2) in which the reactive surface area
was increased and the amount of non-specific 'background' isotope binding decreased by using an HB$_3$Ab-coated bead, leading to a slight improvement in both sensitivity and specificity. Kinetic studies based on the AUSRIA test (Prince and Jass, 1974) revealed a marked effect of incubation temperature and time upon sensitivity, being enhanced at incubation temperatures up to 50°C, but declined if kept beyond eight hours at this temperature. Such findings may be explained by (1) the increasing speed of diffusion of HB$_3$Ag towards bound HB$_3$Ab attached to the solid phase, (2) reduction in the background count, and (3) partial denaturation of HB$_3$Ag at higher temperatures leading to the exposure of additional antigenic sites.

To achieve an acceptable compromise providing good sensitivity in a reasonable time, instructions with the AUSRIA-2 kit suggest that serum samples be incubated by floating on a 45°C waterbath initially for 2-4 hours, followed by a 1 hour incubation with labelled antibody.

For those laboratories with facilities for liquid scintillation but not direct gamma counting, Jordan, Spiehler, Haendiges and Helman (1974) have described a method of solubilising the tube-bound radiolabelled (AUSRIA) complex with glacial acetic acid suitable for subsequent liquid scintillation counting. It is claimed the method avoids high background, chemiluminescence and quench.

Duimel and Brummelkamp (1973) have used voluminous m-diazo- benzyleoxymethylene cellulose as a carrier of HB$_3$Ab in a competitive SP-RIA also requiring highly purified $^{125}$I-labelled HB$_3$Ag. The presence of either HB$_3$Ag or HB$_3$Ab in the test sample results in a decrease in the amount of bound tracer (separated by centrifugation) compared with a negative control serum. Differentiation between
HB<sub>s</sub>Ag and HB<sub>s</sub>Ab necessitated a sandwich SP-RIA, although they point out that this may be accomplished by a single competitive SP-RIA test if a mixture of immunoadsorbents (insolubilized HB<sub>s</sub>Ag and HB<sub>s</sub>Ab) are used.

Hollinger, Werch and Melnick (1974) compared the effectiveness of SP-RIA (AU0RL-1) with that of double antibody radioimmunoassay (DA-RIA) as screening tests for reducing the incidence of post-transfusion hepatitis B. Seven out of eight susceptible recipients who received DA-RIA positive blood subsequently developed clinical hepatitis B or showed serologic evidence of exposure to HB<sub>s</sub>Ag. In contrast, SP-RIA failed to implicate a positive donor in five of these, including two in whom clinical hepatitis B developed. Since both tests were of comparable sensitivity for HB<sub>s</sub>Ag detection, it is interesting to postulate that DA-RIA was recognising additional antigenic determinants (HB<sub>c</sub>Ag?) which were not identified by SP-RIA. This situation could result from the use of low pH (pH 2.4) to dissociate antigen-antibody complexes in the preparation of purified HB<sub>c</sub>Ag for raising HB<sub>s</sub>Ab for use in the DA-RIA. It is known that such treatment may disrupt the outer coat of the Dane particle, exposing the inner core (Hollinger et al., 1974). Antisera produced in this way could contain HB<sub>c</sub>Ab in addition to HB<sub>s</sub>Ab and provide the capability for detecting an additional set of antigen-antibody reactions. At the time of writing DA-RIA kits are not commercially available.

The alternative to RIA is some form of haemagglutination. The best RPHA reagents achieve a sensitivity similar to RIA, are simple to use since they require no special equipment, and are considerably cheaper to purchase (Part One, section 2, chapter 2).
Hepatest (Wellcome Reagents) appears to be the RPHA of choice in the U.K., while 'Hepanosticon' (Organon) is attracting considerable interest on the continent (Reesink et al., 1973; Schuurs and Kacaki, 1974; Hadziyannis, 1974) and Abbott Laboratories are supplying 'Auscell' mainly in the U.S.A. (Germain, Sturdivant and Rightsel, 1973). Hepatest has been evaluated by a number of blood transfusion centres in Britain (Cayzer et al., 1974; Barbara, Denning, Clegham, Dane and Briggs, 1975) and is currently being used by many centres in England and Wales.

It is to be expected that extremely sensitive tests will be prone to occasional false positive reactions. However, recent data suggests that RPHA tests, particularly using animal erythrocytes as antibody 'carriers', may be subject to a higher than expected incidence of false reactions in certain racial groups (Chrystie, Islam and Banatvala, 1974; Wilcox, 1975). Shattock and Smith (1975) compared Hepatest and Hepanosticon with I.D. using heterogenous serum samples derived mainly from patients. While concluding that both RPHA tests satisfied most of the requirements for mass screening they did point out that batch variation in sensitivity does occur.

Another RPHA test (Raphadex E) has recently been described by Prince, Ikram, Chicot, Wright, Vnek, Neurath, Lippin and Swiss (1975). This uses immunochemically purified chimpanzee HB Ab bound to stabilised human erythrocytes. The test has equivalent sensitivity to RIA (AUSRIA-1) and detected a similar number of HB Ag-containing specimens during screening of volunteer blood donors. Unfortunately, there appear to be differences in sensitivity between different reagent batches (Prince, 1975, personal communication).
In parts Two and Four of this thesis, evidence has been presented that the HAI test described possesses both the sensitivity and specificity required of a third generation HB_s Ag technique. Reagent stability combined with the relative ease of large scale preparation results in a highly standardised test system which may be readily introduced into most blood transfusion centres at a cost considerably below that of any comparable commercial reagent.

An interesting new development has recently come to light regarding a haemagglutination procedure with very high sensitivity for the detection of HB_s Ag (Pert and Verch, 1975). Fresh human erythrocytes were coated with HB_s Ab and used to test for the presence of HB_s Ag. Treatment of the reagent with cobra venom factor resulted in inhibition, and there was a marked loss of sensitivity with cells pretreated with formalin or chromic chloride, suggesting that C3 and some type of reactive sites on the cells are required. Sensitivity is claimed to be several orders of magnitude higher than other related tests, and the authors go as far as to suggest that this may be sufficient to detect HB_s Ag if present in virtually any blood sample. Reproducible discrimination between positive and negative settling patterns is often difficult for the untrained observer, but it is hoped to overcome this problem by partially automating the technique (Pert, 1975, personal communication).

Detection of circulating antibody to the core of the Dane particle (HB_c Ab) may provide a further means of identifying blood donors capable of transmitting hepatitis type B, since most individuals who are chronic carriers of HB_s Ag have circulating HB_c Ab (Purcell, Gezin, Almeida and Holland, 1973/4). Development of serological techniques for the detection of HB_c Ab have been
hindered by the limited availability of the antigen. Free HBc.Ag has not yet been found in the serum of either acutely or chronically infected individuals (probably because it becomes heavily coated with antibody). Chronic HBs.Ag carriers could theoretically provide a source of HBc.Ag, particularly if only those whose sera was rich in Dane particles were chosen. Unfortunately, this would involve processing large quantities of potentially infectious material (Fauval, Babiuk, Sheaff and Spence, 1975), a prospect which would not appeal to many laboratories even if they possessed the necessary equipment. Alternatively, HBc.Ag may often be extracted from the nuclei of infected liver tissue (Barker et al., 1974; Maupas, Werner, Larouse, Millman, London, O'Connell and Blumberg, 1975) care being taken to remove free antibody before HBc.Ag is exposed.

Despite the application of RIA to detection of HBc.Ag and HBc.Ab (Purcell et al., 1973/4; Greenman, Robinson and Vyas, 1975) complement fixation (C.F.) has been used extensively to characterise the HBc.Ab response in patients with hepatitis B (Hoofnagle, Gerety and Barker, 1974). HBc.Ab is frequently detectable while the surface antigen is still present during acute infection. The titre of HBc.Ab generally falls to low levels after recovery, but high HBc.Ab titres are routinely found in persistent HBs.Ag carriers. Preliminary investigation of the possibility that HBc.Ab might serve as a marker of chronic infection with hepatitis B virus in some individuals without detectable HBs.Ag has revealed a high prevalence of HBc.Ab in donors (without HBs.Ag) who were implicated in cases of hepatitis following blood transfusion.

The DNA polymerase reaction can be used to radiolabel Dane particle cores for a specific and sensitive DA-RIA for HBc.Ab (Robinson, 1975). DNA polymerase activity appears early in the
period of HB$_s$Ag reactivity (corresponding to peak viral replication) and disappears at about the same time as HB$_s$Ag (Krugman, Hoofnagle, Gerety, Kaplan and Gerin, 1974). Quantitation of DNA polymerase may provide a useful approach in distinguishing the degree of infectivity of HB$_s$Ag positive sera.

II. Subtypes of HB$_s$Ag

The ability to subtype a weakly reacting test sample provides important confirmation of the specificity of the original HB$_s$Ag positive reaction. As detailed in Part Three (chapter 1), determinants a, d, y, w and r are readily definable by HAI. As 'new' HB$_s$Ag (virus-coded) reactivities are discovered the value of a sensitive but simple subtyping test will become even more apparent. Since the information in Part One (Section 1, chapter 1, II) was compiled, some new HB$_s$Ag determinants have been reported or rumoured, for example 'g', 'n', 'q' and 't' (Le Bouvier and Williams, 1975). It must be shown that each new postulant antigen is distinct, associated with the surface of HB$_s$Ag particles, and coded for by the HB-Ag viral genome. Among the newly postulated determinants most data is available regarding 't', a peculiar feature of which is its variable physical behaviour, being overt in association with the adw phenotype, cryptic in association with ayw, and apparently absent from ayr.

Though apparently not attached to the HB$_s$Ag particle surface, the e antigen, first described by Magnus and Espmark (1972), has generated considerable interest in recent months as a possible indicator of liver damage in HB$_s$Ag carriers. e-antigen or specific e-antibody can be detected in the three-to-five-times concentrated serum of most HB$_s$Ag carriers by I.D. Most investigators agree that
e-antigen is associated with active and usually continuing liver disease and may prove a particularly useful prognostic aid, while e-antibody appears to be significantly associated with normal or only mildly abnormal liver function (El Sheikh, Woolf, Galbraith, Edleston, Dymock and Williams, 1975; Kleftheriou, Thomas, Heathcote and Sherlock, 1975; Feinman et al., 1975). My own investigations (as yet unpublished) agree with these findings, since the majority of apparently healthy HB$_3$Ag (blood donor) carriers in the South-East of Scotland are typable for the e-system by I.D. provided test serum is concentrated three to five times with lyphogel. To date the only carriers to exhibit e-antigen are those with persistently elevated transaminase values, representing about 10 percent of carriers tested for the e-system. Since both e-antigen and DNA polymerase appear to be associated with viral replication, infectivity, abnormal liver function and the presence of Dane particles, it is tempting to hypothesise that they may represent the same product.

III. Immunisation against Hepatitis B

Although HB$_3$Ag is distributed throughout the world, there are certain 'high risk' groups who would benefit from some form of active or passive protection. These include patients receiving blood transfusions, drug abusers and health-care personnel such as surgeons, physicians, dentists, nurses, laboratory technicians and blood bank workers. Haemodialysis patients and staff, children of HB$_3$Ag positive mothers, institutionalised patients and persons visiting or living in certain tropical areas where sanitation is poor are also considered to be at risk.
Progress towards the development of a vaccine against hepatitis B has been hampered by the very limited success obtained in growing the virus in culture and the difficulty of obtaining a suitable animal model. Diluted and heat-treated whole serum (Krugman et al., 1971) may be regarded as an inactivated 'vaccine', but it is a very crude way of inducing immunity and it is improbable that such material will be licensed for general use. Isolated coat protein challenges the body's immune mechanism in the same way as the whole infectious agent and the possibility of using purified HB₅Ag free of nucleic acid, and therefore not infective, appears attractive. However, such an approach may be precluded by contaminating host protein which may include various pre-existing structures of the liver cell and may thus induce undesirable immunological reactions (Popper and Mackay, 1972). Subunits of HB₅Ag, in the form of small polypeptides, are considered by some (Zuckerman and Howard, 1975) to possess a greater potential as possible immunogens. In a recent report by Dreesman, Chaires, Suarez, Hollinger, Courtney and Melnick (1975), five purified polypeptides were isolated from HB₅Ag types adw and ayw and injected into guinea pigs. Antibody to each HB₅Ag type was measured by RIA. Although not all the five elicited detectable antibody response, it was concluded that subunits free of antigenically cross-reacting host components and which do not contain infectious viral nucleic acid may yield an effective hepatitis B vaccine. Another approach could be the development of a synthetic peptide, which, when coupled to a macromolecular carrier, could serve as a suitable immunogen. Successful transmission of hepatitis B virus to chimpanzees (Barker et al., 1975) may provide a means of monitoring vaccines when they are developed.
Results of studies on passive immunisation using pooled human immunoglobulin have been inconsistent, probably because most preparations contained little or no HB$_3$Ab. However, the evidence in Part Four (chapter 4) suggests that immunoglobulin with a high titre of HB$_3$Ab (HB-IgG) prepared from plasma of selected donors could offer protection in certain cases. Since the early work of Prince et al. (1971), Gocke (1971), Conrad et al. (1971), Krugman et al. (1971) and Soulier et al. (1972), the protective effect of HB-IgG has been studied on a variety of 'high risk' groups.

Samaness et al. (1974) compared the protective effects of HB-IgG and normal immune globulin (passive haemagglutination titre = 1/16) on children admitted to three institutions and concluded that both preparations were effective in preventing or modifying non-parenterally transmitted hepatitis in an endemic setting during a 1.5 to two year follow-up period. Kohler, Dubois, Merrill and Bowes (1974) found HB-IgG to be effective, compared to no treatment, in preventing neonatal hepatitis B infection in babies born to HB$_3$Ag positive mothers, and Redeker, Mosley, Gocke, McKee and Pollack (1975) obtained evidence that HB-IgG appeared effective in suppressing not only disease, but infection itself in spouses of patients with acute type B hepatitis, compared with normal immune globulin.

The majority of reports concerning the effectiveness of HB-IgG have been with regard to prevention of type B hepatitis in dialysis units and in individuals accidentally exposed to HB$_3$Ag as a result of needle-stick-type accidents. Half as many cases of hepatitis B developed among patients treated with HB-IgG as compared with the group receiving normal globulin during a clinical trial.
in two haemodialysis centres in New Jersey (Szmuness, Prince, Hoofnagle, Ribot and Jacobs, 1975), while in a double blind study reported by Desyter, Bradbourne, Vermylen, Deneels and Boelnert (1975) HB-IgG significantly protected haemodialysis patients against development of HBs antigenemia, compared to control patients receiving normal gamma globulin. Results of a large multicentre study (Prince, Szmuness, Mann, Vyas, Grady, Shapiro, Suki, Freidman and Stenzel, 1973) in which haemodialysis patients and staff were given either high, intermediate or low titre HB-IgG suggest that high titre material may be most protective particularly among patients.

Preliminary evaluation of patients who received prophylactic HB-IgG following accidental needle-stick exposure to HBsAg indicated that HB-IgG significantly reduced the frequency of both clinical and subclinical hepatitis during the first 3 to 4 months after injection (Seeff, Zimmerman, Wright, Felscher, Finkelstein, Garcia-Pont, Greenlee, Diets, Hamilton, Koff, Leevy, Kiernan, Tamburro, Schiff, Vlahcevic, Zemel, Zimman and Nath, 1973). However, less than 10 percent of the recipients had detectable HBsAb at the sixth month after injection, suggesting that HB-IgG might need to be given every 3 to 4 months to continually exposed individuals. This is in agreement with my own findings reported in Part Four (chapter 4).

HB-IgG is administered to 'high risk' groups in the hope that circulating HBsAb will prevent the hepatitis B virus (should it enter the blood) from reaching its target organ and replicating. The logic of this seems to be borne out by the findings of De la Concha, Ortiz, Hernandez-Guio and Hernando (1975) who found immunity to the disease to be directly dependent on the titre of circulating HBsAb.
Given that high titre HB-IgG appears to be initially more protective than normal gamma globulin in certain circumstances, the question of when and where it should be used is not as clear-cut as might be supposed. As follow-up is extended there are indications that normal gamma globulin is more effective than HB-IgG in inducing longer-lasting passive-active immunity to HB$_A$G. Grady and Lee (1975) found that 12 months after injection more recipients of normal gamma globulin possessed HB$_A$Ab than in a group receiving high titre HB-IgG, suggesting that the latter treatment may inhibit active stimulation. A similar finding was noted by Seeff et al. (1975) with over 30 percent of normal gamma globulin recipients possessing HB$_A$Ab after six months, compared with only 8 percent of the HB-IgG group. This is an important point because the need to stimulate active immunity must be one of the considerations in the decision to use HB-IgG or normal gamma globulin.

On the basis of available data, it is difficult to decide upon specific guidelines for the use of either product, or indeed the frequency of administration. Theoretically immunoprophylaxis need not be given to persons who already have circulating HB$_A$Ab. The main requirement for HB-IgG would seem to be in cases of accidental HB$_A$Ag inoculation where one should aim for maximum immediate protection and be less concerned with long term immunity. HB-IgG does not seem to be indicated in the prophylaxis of transfusion-associated hepatitis since approximately 90 percent of such complications are not now caused by hepatitis B virus (Prince et al., 1974). Posttransfusion (type B) hepatitis should continue to be prevented mainly by using the least amount of blood possible, using all-volunteer blood and screening donors by the most sensitive
It is difficult to know whether immunoprophylaxis should be given to persons who are repeatedly exposed to HB Ag, and if so, whether it should be in the form of HB-IgG or normal gamma globulin. Experimental hepatitis B vaccines are being prepared but several years will be required before they are accepted as safe and effective. In the interim, the use of HB-IgG or normal gamma globulin will depend upon the availability of the former and upon their relative ability to induce passive-active immunity.

IV. The Way Ahead (future research arising from work reported in this thesis).

At the time of writing the haemagglutination-inhibition test for detection of HB_Ag, described in this thesis, is already proving its worth in three Scottish Regional Blood Transfusion Centres and the Iranian National Blood Transfusion Service, while laboratories as far afield as South Africa and the U.S.A. are evaluating reagents prepared in Edinburgh. However successful a technique may appear, there is invariably room for improvement. Automation of HB_Ag testing may be desirable in centres required to test very large numbers of donations daily, and to this end I am pleased to report that Mr. John Lockyer at the Blood Transfusion Centre in Bristol is employing HB_Ag-coated, gluteraldehyde-fixed spherocytes prepared in Edinburgh to develop a fully automated test procedure using the Technicon autoanlyser. Although many laboratories may not consider automation of HB_Ag testing worthwhile, the fact remains that the main weakness of the present (manual) HAI test is its lack of
objectivity. Automation at the point of reading, possibly in the form of an optical scanning device in conjunction with a numerical scale, would be desirable provided sensitivity did not suffer.

The discovery that HB_{s} Ag-coated gluteraldehyde-fixed spherocytes could be 'overcoated' with specific antibody and subsequently used for a direct RPHA-type test for HB_{s} Ag has meant that extremely stable RPHA reagents could be available to any laboratory capable of preparing HB_{s} Ag-coated, gluteraldehyde-fixed spherocytes. Not only do overcoated cells constitute a more stable reagent than any of the commercial RPHA kits (which are extremely unstable when reconstituted), but the fact that stabilised HB_{s} Ag-coated spherocytes 'select' specific HB_{s} Ab from the environment infers that specificity should also be improved. These are features of the overcoated spherocytes which are in the process of investigation.

The work of Purell et al. (1973/4) concerning the association of HB_{c} Ab with HB_{s} Ag carriage and possible infectivity suggests that a simple direct haemagglutination test for HB_{c} Ab could prove useful. To this end, one aim of current research is the extraction of HB_{c} Ag from serum and/or infected liver for subsequent spherocyte coating. Preliminary experiments using a crude HB_{c} Ag-HB_{s} Ag preparation suggest that it may be possible to coat HB_{c} Ag in the same way as HB_{s} Ag, although this may require de-purifying the 'purified' HB_{c} Ag with normal serum prior to coating.

Perhaps the most exciting prospect looming ahead of virologists working in the hepatitis field is that of soon identifying other posttransfusion hepatitis viruses. Their existence seems certain since with the
introduction of 'third generation' \(^*\) HB\(_b\)Ag testing most posttransfusion (viral) hepatitis cases are negative for HB\(_b\)Ag and subsequently show no evidence of antibody response to either surface or core components (Prince, et al., 1974; Feinstone, Kapikian, Purcell, Alter and Holland, 1975; Alter, Purcell, Holland, Feinstone, Morrow and Moritsugu, 1975; Galbraith, Portmann, Edlestein and Williams, 1975; Villarejos, Visora, Eduarte, Provost and Hilleman, 1975).

In order to eliminate hepatitis A virus (HA-Ag) as the causative agent of non-B hepatitis, I have obtained antiserum to the MS-1 strain of HA-Ag from the National Institutes of Health (Bethesda, U.S.A.) which I am currently using to attempt to identify the causative agent of 'epidemiological' hepatitis A outbreaks in Edinburgh. When sufficient of the causative (virus) agent has been isolated from pre-and acute phase stool extracts, it is hoped to attempt spherocyte coating. Meanwhile electron microscopy and immune adherence haemagglutination appear to be identifying a virus-like particle serologically related to MS-1.
APPENDIX A

Phosphate Buffered Saline (P.B.S.)

Stock Solutions:

1. Disodium hydrogen orthophosphate (Na$_2$HPO$_4$, B.D.H., analar grade, molecular weight = 177.99) - a 0.15 molar solution was prepared by dissolving 53.597 gms. in 2000 ml. of sterile distilled water. Two grams of sodium azide was also added to prevent microbial growth.

2. Potassium dihydrogen orthophosphate (K$_2$HPO$_4$, B.D.H., analar grade, molecular weight = 136.09) - a 0.15 molar solution was prepared by dissolving 40.827 gm. in 2000 ml. sterile distilled water. Two grams sodium azide was also added (see above).

3. Sodium chloride (NaCl, B.D.H., analar grade, molecular weight = 58.44) - a 0.15 molar solution was prepared by dissolving 17.532 gm. in 2000 ml. sterile distilled water. Sodium azide was added as above.

Stock solutions were stored at 4°C or room temperature.

P.B.S. pH 6.4 was prepared by mixing the following volumes of stock solutions:

- Na$_2$HPO$_4$ 280 ml.
- K$_2$HPO$_4$ 520 ml.
- NaCl 800 ml.

Total volume = 1600 ml.

P.B.S. pH 7.2 was prepared by mixing the following volumes of stock solutions:

- Na$_2$HPO$_4$ 715 ml.
- K$_2$HPO$_4$ 285 ml.
- NaCl 1000 ml.

Total volume = 2000 ml.

Working solutions were kept at 4°C.
APPENDIX B

Preparation of HB_s Ag from serum by extraction with polyethylene glycol (PEG)

Twenty-five ml. HB_s Ag positive serum was centrifuged at 120,000 g. and the pellet resuspended to the original volume in P.B.S. pH 6.4. The pH was adjusted to 4.6, and 2 gm. PEG 6000 were added. After stirring for 10 minutes, the mixture was centrifuged at 1500 g. for 10 minutes, the supernatant discarded and the precipitate resuspended in 25 ml. of sterile distilled water. The PEG was subsequently removed by filtration through Sephadex G200. Fractions possessing HB_s Ag activity were pooled, concentrated ten times using an Amicon cell, dialysed against P.B.S. pH 6.4 and adjusted to contain 128 latex units of activity.
APPENDIX C

Preparation of HB$_3$Ag from serum by density gradient centrifugation

HB$_3$Ag positive serum was centrifuged at 120,000 g, and the pellet resuspended to 1/20 of its original volume in P.B.S. pH 6.4. One ml. of this concentrate was layered onto a 4 ml. caesium chloride (Cscl, B.D.H. analar grade, molecular weight = 168.36) density gradient (2 gm. Cscl dissolved in 4 ml. sterile distilled water). The gradient was then centrifuged at 130,000 g, for 24 hours at 4°C, after which 0.2 ml. fractions were collected from the bottom of the tube. The refractive index of each fraction was measured and the density determined from tables. Fractions having a density between 1.1943 - 1.2502 g./cm$^3$ were pooled, dialysed against P.B.S. pH 6.4 and re-gradiented. Those fractions possessing most HB$_3$Ag (as determined by immune electron microscopy) were pooled, dialysed against P.B.S. pH 6.4 and adjusted to contain 128 latex units of HB$_3$Ag activity.
APPENDIX D

Treatment of HbAg positive serum with betapropiolactone (B.P.L.) and ultra-violet light (U.V.L.)

B.P.L. was diluted 1 in 10 in sterile distilled water. This 10 percent solution was further diluted 1 in 10 (to give final B.P.L. concentration of 1% percent) in saline bicarbonate solution. (Above procedures should be carried out at 0°C to 4°C).

One volume of 1% B.P.L. solution was mixed with two volumes of HB.Ag-positive serum for 2 hours at 37°C and left overnight at 4°C.

The serum B.P.L. mixture was then decanted into plastic petri dishes (just sufficient to cover bottom of dish) and exposed to U.V.L. in a Luckham chamber for 2 hours.

*B.P.L. (Betapone: Fellows and Testagar) should be stored at minus 15°C to minus 20°C when not in use, and care should be taken to avoid inhalation of fumes.

**Saline bicarbonate solution - The following reagents were dissolved in 100 ml. sterile distilled water containing 1 mgm./ml. sodium azide;

1.68 gms. NaHCO₃ (B.D.H., analar grade, mol. wt. 84.01)
0.30 gms. NaCl ( " " " " mol. wt. 58.44)
1 mgm. phenol (M & B, detached crystals, mol. wt. 94.11)
APPENDIX E

Phosphate Buffer pH 7.2

Stock solutions consisted of:

A. 0.2M Na$_2$HPO$_4$ (B.D.H., analar grade) prepared by dissolving
B. 0.02M K H$_2$PO$_4$ (B.D.H., analar grade)

in sterile distilled water containing 0.1 percent (w/v) sodium
azide.

Working solutions were prepared by dissolving stock solutions
in sterile distilled water containing 0.1 percent (w/v) sodium
azide.
### APPENDIX F

Summary of HB Ag testing data from five English Blood Transfusion Centres.

#### SUMMARY OF RESULTS OF TESTS FOR AUSTRALIA ANTIGEN AND ANTIBODY IN DONORS RECEIVED FROM RTCs UP TO SEPTEMBER 1972

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>SHEFFIELD</td>
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<td></td>
</tr>
<tr>
<td>All donations</td>
<td>56,057</td>
<td>41 (1: 1367)</td>
<td>19 (1: 2950)</td>
<td>15 (1: 3459)</td>
<td>2 (1: 25,797)</td>
<td>1 (1: 213)</td>
<td>1 (1: 278)</td>
</tr>
<tr>
<td>General public</td>
<td>54,557</td>
<td>38 (1: 1462)</td>
<td>17 (1: 3208)</td>
<td>13 (1: 3846)</td>
<td>2 (1: 25,002)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>University etc.</td>
<td>1,222</td>
<td>1 (1: 1222)</td>
<td>1 (1: 1222)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Prisons etc.</td>
<td>278</td>
<td>2 (1: 139)</td>
<td>1 (1: 278)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>51,594</td>
<td>49 (1: 1465)</td>
<td>19 (1: 377)</td>
<td>15 (1: 374)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>General Public</td>
<td>50,005</td>
<td>38 (1: 1737)</td>
<td>16 (1: 292)</td>
<td>13 (1: 3846)</td>
<td>2 (1: 25,002)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>University etc.</td>
<td>1,163</td>
<td>1 (1: 1751)</td>
<td>1 (1: 292)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Prisons etc.</td>
<td>426</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>New donors</td>
<td>2,000</td>
<td>8 (1: 265)</td>
<td>8 (1: 71)</td>
<td>2 (1: 213)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>51,594</td>
<td>50 (1: 1138)</td>
<td>1117 (1: 486)</td>
<td>1117 (1: 486)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BRISTOL</td>
<td></td>
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<tr>
<td>All donations</td>
<td>49,207</td>
<td>30 (1: 1640)</td>
<td>84 (1: 586)</td>
<td>84 (1: 586)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>General public</td>
<td>47,299</td>
<td>30 (1: 1640)</td>
<td>84 (1: 586)</td>
<td>84 (1: 586)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>University etc.</td>
<td>1,904</td>
<td>6 (1: 67)</td>
<td>10 (1: 100)</td>
<td>10 (1: 100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>51,203</td>
<td>36 (1: 1820)</td>
<td>94 (1: 696)</td>
<td>94 (1: 696)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CAMBRIDGE</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>All donations</td>
<td>66,799</td>
<td>42 (1: 1550)</td>
<td>40 (1: 1670)</td>
<td>40 (1: 1670)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>General public</td>
<td>61,044</td>
<td>34 (1: 1801)</td>
<td>32 (1: 1908)</td>
<td>32 (1: 1908)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>University etc.</td>
<td>2,618</td>
<td>2 (1: 1309)</td>
<td>2 (1: 1309)</td>
<td>2 (1: 1309)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>2,164</td>
<td>4 (1: 541)</td>
<td>5 (1: 433)</td>
<td>5 (1: 433)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>ARMS</td>
<td>975</td>
<td>2 (1: 486)</td>
<td>1 (1: 973)</td>
<td>1 (1: 973)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>66,799</td>
<td>46 (1: 1550)</td>
<td>45 (1: 1670)</td>
<td>45 (1: 1670)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>WESSEX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All donations</td>
<td>46,752</td>
<td>18 (1: 2597)</td>
<td>12 (1: 1941)</td>
<td>12 (1: 1941)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>General public</td>
<td>42,675</td>
<td>9 (1: 4742)</td>
<td>10 (1: 1944)</td>
<td>10 (1: 1944)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Armed Forces</td>
<td>2,401</td>
<td>3 (1: 600)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Prisons etc.</td>
<td>1,676</td>
<td>6 (1: 279)</td>
<td>2 (1: 488)</td>
<td>2 (1: 488)</td>
<td>0</td>
<td>0</td>
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</table>


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The incidence and significance of hepatitis B (surface) antibody in a burns unit

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Robert Hopkins
South-east Regional Transfusion Centre, Royal Infirmary, Edinburgh

Summary
The incidence of hepatitis B (surface) antigen and its specific antibody among patients and staff of a burns unit were investigated using a sensitive haemagglutination test. Comparison with appropriate age- and sex-matched controls showed a higher incidence of specific antibody among both patients and staff within the unit. No antigen was detected.

INTRODUCTION
Severe burns (in excess of 40 per cent total body surface) are associated with profound alterations in plasma volume during the first few hours (Kukral and Shoemaker, 1970). Specific therapy frequently employs the transfusion of plasma during the initial resuscitation period (Muir and Barley, 1973), followed by whole blood for those with more than 20 per cent full-thickness burns (Sanders, 1974). Further transfusion may be required during subsequent skin grafting.

Hepatitis B (serum hepatitis) has emerged as a significant complication following the transfusion of blood and blood products, and it has been estimated that 25-60 per cent of recipients of hepatitis B (surface) antigen (HBs-Ag)-positive blood develop antigenemia, and in many it is subsequently associated with hepatitis (Goeke and Kavey, 1969). Each unit of dried plasma is prepared from a pool of 10 donations, and since a severely burned adult may receive as many as 12 units of plasma during the initial period of resuscitation, the maximum total exposure might be as high as 120 blood donations for plasma alone. The outcome of a challenge with HBs-Ag in the burned patient may be influenced by the transient immune deficiency state which follows major thermal injury (Rapaport et al., 1968; Munster et al., 1970; Daniels et al., 1971; Mahler and Batchelor, 1971) since a high incidence of HBs-Ag has been found in association with conditions characterized by underlying immune deficiency (Blumberg et al., 1967).

The following article describes the results of a study designed to detect HBs-Ag and HBs-Ab in the sera of 118 burned patients who received plasma and whole blood prior to the introduction of routine HBs-Ag blood-donation testing in 1971.

PATIENTS AND METHODS
Sera from 118 patients previously treated in the burns unit and 26 staff (medical and nursing) were collected and stored at -20°C until tested for HBs-Ag and HBs-Ab. The controls consisted of 118 age- and sex-matched, healthy blood donors being tested for the first time and with no history of blood transfusion, and 25 members of the medical and nursing staff from a general surgical ward in the same hospital. A haemagglutination technique (Hopkins and Das, 1973) was used to test the sera for the presence of HBs-Ag and HBs-Ab. All specimens were coded and their origin not revealed to the person responsible for testing. Sera giving positive reactions were confirmed and subtyped by a modification of this technique (Hopkins and Das,
RESULTS
The overall results are summarized in Table I and show that HBs-Ag was not detected in any of the patients, appropriate matched controls, or members of the burn unit staff. HBs-Ag, however, was present in 6.9 per cent of the patients but in none of the control blood-donor population.

Table I. Incidence of HBs-Ag and HBs-Ab amongst patients and staff of a burns unit and their respective controls

<table>
<thead>
<tr>
<th></th>
<th>No. tested</th>
<th>No. HBs-Ag</th>
<th>No. HBs-Ab-Pos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>118</td>
<td>0</td>
<td>8 (6.9)†</td>
</tr>
<tr>
<td>Controls</td>
<td>118</td>
<td>0</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Staff</td>
<td>26</td>
<td>0</td>
<td>2* (7.6)</td>
</tr>
<tr>
<td>Controls</td>
<td>23</td>
<td>0</td>
<td>1** (4.3)</td>
</tr>
</tbody>
</table>

† Figures in parentheses represent percentages
* Titres were 1/80 and 1/40; only anti-a was detectable when subtyped
** Titre was 1/640 and subtyped as anti-ad

This difference was statistically significant at the 1 per cent level (McNamar test). In the staff of the burns unit the incidence of HBs-Ab was 7.6 per cent compared to 4.3 per cent in a similar group working in a general surgical unit. The difference between these two was not significant.

Further details of the HBs-Ab-positive patients are summarized in Table II. With the exception of 1 patient, the HBs-Ag titre was low. In 5 patients it was so low that it was not possible to subtype. One of these patients had a slightly abnormal elevation of SGPT.

DISCUSSION
Since February, 1974 all blood donations in the South-east region of Scotland have been tested for HBs-Ab and HBs-Ag by haemagglutination and haemagglutination-inhibition respectively (Hopkins and Das, 1973). Current evidence, using this technology on 44,053 donations, shows an HBs-Ab incidence of 0.7 per cent which compares with 0.04 per cent as determined by counter-electrophoresis (CIEOP) in the same laboratory during the previous 12 months (Hopkins et al., 1975). If this frequency (0.7 per cent) is taken as a baseline for the regional population, then the incidence of HBs-Ab in burned patients, following treatment, is 9.8 times greater. This difference was highly significant. In a recent publication Payne et al. (1974) found that the combined incidence of HBs-Ab in patients admitted to medical and surgical wards of a general hospital was almost 3 times greater than that of the local blood donor population. It seems likely, therefore, that burned patients are exposed to HBs-Ag to a considerably greater extent than most other groups of hospitalized patients. This is hardly surprising in view of the fact that resuscitation and subsequent plastic surgery may expose a burned individual to more than 100 different blood donors and that the immune status of these patients at the time of exposure could be abnormal.

Within the group of HBs-Ab-positive patients no correlation was found between the degree of exposure (number transfusions given), the time elapsed since exposure, and the HBs-Ab titre. In all but one sample (patient no. 6) the amount of antibody was too low to be detected by CIEOP, a feature which may account for the fact that in 5 of these patients only the anti-a specificity
could be identified. SGPT values were only slightly elevated in one of the antibody-positive patients.

The incidence of HBs-Ab among the surgical and nursing staff of the burns unit was found to be 10-8 times greater than in the regional blood-donor population and almost twice that of comparable staff in a general surgical ward of the same hospital. Because the numbers in this part of the study are small, it is not possible to come to firm conclusions, but it does suggest that the high risk seen in the burned patients is likely to be transferred to the staff. Such a conclusion is supported by the observation that in haemodialysis units 1 or 2 members of staff suffer some exposure for every 2 patients (Editorial, British Medical Journal, 1972).

CONCLUSION
The patients studied in this series were transfused with blood and plasma prior to routine HBs-Ag testing of blood donations, and thus the clinical significance of the results so obtained should not be interpreted in light of existing practices. Further studies are in hand to ascertain the incidence of HBs-Ab in burned patients managed with blood and plasma which has been HBs-Ag tested. The data do suggest, however, that burned patients may represent a population at especially high risk and that this risk is transferred to their attending staff. Burned patients may constitute a particularly sensitive hospital population on which the quality of HBs-Ag testing by the Blood Transfusion Service can be readily assessed.

REFERENCES


Present and cough

A 57-year-old Hospital, Hong 39

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Received for

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Doorschodt separate

particles, and the few reported

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monospecific antisera. As

population.

Increasingly, hepatitis reference labora¬

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four

(Bancroft and the immune

status

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1972; Levine and Soulier

mutually exclusive.

haemodialysis

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is

being adw (D), adr, ayw (Y), and ayr. The last is exceedingly rare in a European population. Increasingly, hepatitis reference labora¬
tories are able to test for d and y specificities using monospecific antisera. As a rule, the two common

phenotypes, D and Y, are mutually exclusive. The finding of d and y specificities in the same serum is rare. It is even less common to find both d and y on separate particles, and the few reported cases were all on long-term haemodialysis (van Kooten-Kok Doorschodt et al, 1972; Soulier and Courroucé-Pauty, 1973). In our present report immunoadsorption

studies indicate that the two specificities reside on separate particles, suggesting dual infection.

To the best of our knowledge there are no previous reports of this finding in hypogammaglobulinaemia. Such patients, if treated with fresh frozen plasma infusions, may be at particular risk of becoming hepatitis B virus (HBV) carriers.

Case Report

A 57-year-old Caucasian man had had recurrent respiratory infections since adolescence. In 1957, aged 39 years, he was investigated for recurrent cough and purulent sputum. The bronchogram was normal and a diagnosis of chronic suppurative bronchitis was made. In 1962 he was admitted to hospital with bilateral bronchopneumonia and found to have a serum y-globulin level of 0-2 g/l. Regular therapy with pooled normal human y-globulin was begun and he was much improved subsequently. After four years he began to have anaphylactic reactions to the y-globulin and treatment was discontinued. Despite continuous prophylactic antibiotic therapy he deteriorated and in 1969, after an attempt at desensitization, y-globulin therapy was resumed. Further anaphylactic reactions ensued and in September 1971 he was started on infusions of fresh frozen plasma, initially one per week and later on every second week, along with continuous oral ampicillin. In March 1972 he suffered an episode of general malaise, anorexia, and upper abdominal pain. He was not jaundiced and his serum bilirubin, SGOT, SGPT, and LDH levels were normal, but the serum alkaline phosphatase was raised at 263 IU/l. A liver biopsy was not done and serum was not tested for HBsAg. Subsequently the alkaline phosphatase returned to normal. In September 1973, plasma infusions were reduced to three-weekly intervals and on this regimen he has since remained reasonably well.

In June 1974 his immune status was fully investigated, with the following results:

Peripheral blood:

Absolute lymphocyte and granulocyte counts were normal.

Serum immunoglobulins:

IgG 30 IU/ml, IgA 23 IU/ml, IgM 28 IU/ml (2½ weeks after plasma infusion).

Serum complement:

C3 and C4 levels normal.

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ad and ay subtypes of hepatitis B antigen in a case of hypogammaglobulinaemia

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From the South-East Regional Blood Transfusion Service, Royal Infirmary, Edinburgh, and Respiratory Unit, Northern General Hospital, Edinburgh

SYNOPSIS We describe the finding of d and y specificities of hepatitis B surface antigen (HBsAg) in a case of hypogammaglobulinaemia of the 'common variable' type treated with fresh frozen plasma infusions. Absorption studies show that the two specificities are on separate particles, suggesting dual infection. It raises important questions regarding the relationship between HBsAg persistence and the immune status of the carrier.
Red cell serology:
Direct and indirect antiglobulin tests and screen for anti-erythrocyte antibodies-negative
Iso-agglutinin titre:
Iso-agglutinins not detectable (red cells, group O)
Phagocytic function:
Phagocytosis and intracellular killing of Staphylococcus aureus by blood leucocytes, stimulated NBT test, and leucocyte chemotaxis (migration through 3µ Millipore filter), all normal
Lymphocyte function:
Stimulation of blood lymphocytes (incorporation of 3H-thymidine into DNA) by mitogens (phytohaemagglutinin, concanavalin A, and pokeweed mitogen) and by pooled allogeneic cells, all normal
Blood lymphocyte subpopulations:
The T cell count (E rosettes) was normal; B cells (EAC rosettes) 6% (normal 10-20%)
Skin testing:
Positive delayed hypersensitivity response to mumps antigen; no response to PPD and Candida
Antibody response to tetanus toxoid:
No antibody detected (passive haemagglutination technique) before and 14 days after immunization
These results confirmed hypogammaglobulinaemia and demonstrated intact cell-mediated immunity, both phagocytic and lymphoid.
In September 1974 routine testing in a diagnostic laboratory (Regional Virus Laboratory, City Hospital, Edinburgh) showed that the patient's serum was positive for HBsAg (by counter-immunoelectrophoresis, CIEP). At this time the liver was not enlarged and liver function tests (serum bilirubin, GOT, GPT, and alkaline phosphatase) were normal.

HBsAg testing and subtyping
Methods used in our laboratory to confirm HBsAg positivity were haemagglutination inhibition (HAI), as previously described by Hopkins and Das (1973), haemagglutination (turkey cell HA kit, Welcomes Reagents Ltd), and radioimmunoassay (Austria 1 RIA kit, Abbot Labs, Ltd). To identify and titre d and y specificities, monospecific antisera were used in the HAI assay as previously described (Hopkins and Das, 1974).

Immunoadsorption
In order to determine whether d and y were associated with the same or separate particles, 0.5 ml of a 1 in 5 serum dilution was incubated with an equal volume of monospecific anti-y serum^1 at 37°C for 1 hour, and at 4°C for 16 hours. The mixture was then centrifuged at 30,000 × g for 60 minutes to pellet immune complexes. The supernatant and pellet were tested for s, d, and y antigenic specificities by HAI and the supernatant for the presence of anti-y antibody by direct haemagglutination.

Electron Microscopy
Serum was centrifuged at 120,000 × g for 4 hours and the pellet washed once in saline and reconstituted to 1/20th of the original volume. One drop of this suspension was mixed with an equal volume of 2% phosphotungstic acid (pH 6.8) and absorbed onto a collodial coated copper grid (300 mesh) for examination in the electron microscope (Hitachi 12A).

Results

<table>
<thead>
<tr>
<th>Antigen/Subtype</th>
<th>Titres</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum^1</td>
</tr>
<tr>
<td></td>
<td>Dilution</td>
</tr>
<tr>
<td></td>
<td>1/10</td>
</tr>
<tr>
<td>d</td>
<td>1/20</td>
</tr>
<tr>
<td>y</td>
<td>1/10</td>
</tr>
</tbody>
</table>

Table: Immunoadsorption with monospecific anti-y^1

^1Monospecific anti-y antisera prepared by affinity chromatography
^2Titres performed on whole serum; figures corrected for 1:10 dilution incurred during absorption

Reciprocal absorption of a separate serum sample using monospecific anti-d antisera removed d antigen leaving y antigen undiminished (see text).
ad and ay subtypes of hepatitis B antigen in a case of hypogammaglobulinaemia

Figure  Electron micrograph (× 210 000) showing 20nm spherical and rod-like structures of HBsAg and 42nm Dane particles
presence of a in both the pellet and supernatant provided good supporting evidence that we were dealing with two distinct populations of HBsAg particles.

**Electron Microscopy**

EM studies (figure) showed the characteristic spherical and rod-like structures, 30 nm in diameter, as well as numerous 42 nm Dane particles, believed to represent the complete infectious virion.

**Review of Patient's Sera and Plasma Donors**

Retrospective investigation of a serum sample of 7 February 1973 showed the presence of both HBsAg specificities (at the same titre as in 1974), but a sample of 2 December 1970 was negative. We were unable to locate any serum samples taken between these dates. From the beginning of plasma infusions on 7 September 1971 until 7 February 1973 he had received 72 donations of plasma. Screening of all blood donations for HBsAg by the CIEP method was begun in September 1971. Initially the patient received four donations of plasma that had not been tested. The remaining 68 donations were HBsAg negative by the relatively insensitive CIEP test. None of the 72 donors involved has subsequently been found to be positive by CIEP or by the more sensitive HAI technique which has been in routine use since the beginning of 1974.

**Review of Other Hypogammaglobulinaemic Patients**

Recognizing the possibility that other similar patients may have become HBsAg carriers, we tested all the hypogammaglobulinemic patients known to us in the Edinburgh area. There were nine such patients in all; seven had received γ-globulin therapy at some stage, and one had been receiving fresh frozen plasma infusions for two and a half years. All were negative for HBsAg by the HAI method.

**Discussion**

Generally speaking the d and y specificities of HBsAg are mutually exclusive, and it is exceptional to find both specificities in the same serum. There are three reports where d and y specificities may have been present on separate particles in patients on haemodialysis. Ling et al. (1973) reported two such cases indicated by the results of radioimmunoassay, but cross absorption studies were not done. Van Kooten Kok-Doorschot et al. (1972) described a case and Sollier and Couroucé-Pauty (1973) reported two further cases where d and y sub-

specificities could be separated by absorption. The absorption studies which we described clearly indicate that the ad and ay subtypes were present as distinct populations. The simplest and most likely explanation is that the patient underwent dual infection from two positive donors. Unfortunately, serum samples were not available to show whether the two subtypes appeared sequentially. Therefore there is the small possibility that he was infected by one donor who carried both subtypes.

It is now widely believed that the immune response to HBV determines the nature and degree of tissue damage and whether the subject develops the carrier state. The HBV per se is probably not cytotoxic to hepatocytes (Hand and Finlayson, 1973) but cell damage results primarily from a cell-mediated response of the delayed hypersensitivity type (directed against the virus or altered cell components). This cellular immune response is heightened by giving transfer factor in chronic active hepatitis (HBsAg positive), then there is a transient rise in serum transaminase levels (Shulman et al., 1974). It has been repeatedly shown, using leucocyte-migration inhibition and lymphocyte transformation tests, that clinical disease is associated with a positive in vitro response to HBsAg, while in the chronic carrier there is a negative response (Dudley et al., 1972; Laiwah et al., 1973).

Specific antibody alone is protective if present before infection (Sutnick et al., 1972) or when given prophylactically after accidental inoculation (Das and Hopkins, 1974). Antibody may also mediate cellular cytotoxicity to liver cells in chronic active hepatitis (Eddleston and Williams, 1974). In established HBV infection the cell mediated immune response would seem to act in conjunction with circulating antibody to eliminate the virus; that is, cytotoxic T or K cells cause lysis of infected hepatocytes, and the liberated viral particles are then neutralized by circulating antibody. Thus it can be postulated that both the cellular and humoral arms of the immune response must be impaired (or inadequate) to allow the development of the HBsAg carrier state with little or no liver damage. In the case we describe here, no deficiency in T cell function could be detected; however, it is possible that a specific defect existed which was not detectable by the screening tests used, none of which was specific for HBsAg.

There is ample epidemiological evidence indicating that impaired immune function predisposes to the carrier status. There is a relatively high incidence of HBsAg carriers among institutionalized cases of Down's syndrome (Sutnick et al., 1968), chronic renal disease on haemodialysis (Turner and White, 1969), and haemophiliacs (Dus and Paterson, 1973). McLean et al. (1972) found an impaired immune response to HBV in patients with haemophilia. A high proportion of HBV antigen carriers (HBsAg) were in patients who had undergone non-accidental haemodialysis (Weinberg, 1974).

It is interesting that the HBsAg carrier status in every one of our cases was accompanied by persistently positive tests for anti-HBs when both antigen and antibody have been repeatedly negative in patients and donors who have not received HBV antigens. For example, in a recent review Paterson et al. (1974) reported that of 69 patients only 12 (18%) had anti-HBs positive and of these, 11 were HBsAg negative. Anti-HBs is of course a marker for exposure to HBV. The positivity in our cases may be due to other factors.

We thank Dr. J. A. Paterson of the Western Infirmary Hospital, Glasgow, and Dr. P. J. Meehan of the Western Infirmary in Edinburgh for permission to use cases that were under their care. We are grateful to Dr. E. P. O. White, Director of Health Services, Eastern Region, Edinburgh, for permission to publish this paper.

**References**


Australia Antigen (HB-Ag) Subtyping by a Sensitive Tanned Cell Haemagglutination-Inhibition Technique

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(Received 11 December 1973; accepted for publication 2 January 1974)

Summary. The tanned cell haemagglutination-inhibition technique was used to subtype Hepatitis B antigen (HB-Ag) sera according to their D (ad+, y−) and Y (ay+, d−) specificity. The results were in agreement with those obtained by workers in the United States and Sweden using immunodiffusion. The majority (35 out of 39 tested) of HB-Ag blood donors detected during the last 2 yr in South-East Scotland were found to possess the D subtype. The Hepatitis B antibody subtype content of two preparations of immunoglobulin prepared for clinical use has also been studied.

Analysis of the surface antigens of Hepatitis B antigen (HB-Ag) have revealed one common specificity 'a' and three additional determinants, 'd', 'y' and 'x' (Le Bouvier, 1971). Specificities a, d and y appear to reflect the genotype of the antigen, while x may be a component of the host which has become integrated into the particle or firmly attached to its surface (Le Bouvier, 1972; Le Bouvier et al., 1972). It is now possible to divide HB-Ag positive material into at least two subtypes, i.e. 'Set D' (ad+, y−) or 'Set Y' (ay+, d−). A third subtype may be included within a miscellaneous collection of HB-Ag samples which do not fall into these categories and designated 'Set A' (a+, dy−). Most, and perhaps all, of these are more likely to reflect the inability of immunodiffusion to detect very low levels of d and y. Two additional antigenic determinants designated 'w' and 'r' have recently been described (Bancroft et al., 1972) in a study in which w predominated in the Western hemisphere, and r characterized HB-Ag of Eastern origin. HB-Ag appears to breed true in the sense that a given specificity gives rise to HB-Ag of the same specificity in the recipient (Krugman & Giles, 1970). This makes it possible to trace the spread of both subtypes independently through the community. Iwarson et al. (1973) have reported evidence for a change in the dominant subtype associated with HB-Ag positive clinical hepatitis in Sweden, from D to Y over a period of 20 yr. A number of other recent publications suggest that subtype D is often found in the chronic asymptomatic carrier (Holland et al., 1972; Wenzel et al., 1972). The Y subtype has also been associated with outbreaks of serum hepatitis in renal dialysis units, including the exceptionally severe outbreak in Edinburgh (Marmion & Tonkin, 1972). Recently Nielsen & Le Bouvier (1973) have studied the relationship between HB-Ag subtypes and the degree of liver damage in acute viral hepatitis. They too identified subtype Y in the majority of cases, but surprisingly found that when acute disease did occur in association with

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subtype D, it was usually more severe than that associated with Y. Immunodiffusion (ID) was the first technique to be used for the detection of HB-Ag (Blumberg, 1964) and remains a useful means of subtyping (Gust, 1971). Counter-electrophoresis (CIEOP) employing antisera rendered monospecific by absorption has also been applied to subtyping (Holland et al., 1972), and solid phase radioimmunoassay (RIA) promises to provide a sensitive means of subtyping HB-Ag not detectable by ID or CIEOP (Ginsberg et al., 1972).

Prince et al. (1972) were the first to apply haemagglutination inhibition (HAI) to the subtyping of HB-Ag, but the chronic chloride technique used (Vyas & Shulman, 1970) has proved unsuitable for mass donor screening. We report the application of a simple, rapid and sensitive tanned cell HAI (Hopkins & Das, 1973) to the subtyping of HB-Ag derived from local blood donors, dialysis patients and sera from standard panels supplied by the National Institute of Health and the American Red Cross.

MATERIALS AND METHODS

A number of sera containing Hepatitis B antibody (HB-Ab) were tested by ID and CIEOP against previously subtyped HB-Ag sera. It was apparent that one of the antisera reacted strongly with antigen of subtype D, but weakly, if at all, with antigen of subtype Y, suggesting a predominance of anti-a and anti-d. Anti-a was removed by absorption with HB-Ag of subtype Y by concentrating the antiserum five times with lyphogel and incubating with different concentrations of Y at room temperature for 1 hr, followed by overnight incubation at 4°C. Immune complexes were removed by centrifugation at 20,000 rpm (Sorval RC2B) for 1 hr and the supernatant tested for the presence of anti-d by direct haemagglutination (HA) with D-sensitized cells (Fig 1). Removal of anti-a activity was indicated by agglutination of D-sensitized cells, but not of Y-sensitized cells (Table I). Anti-y was prepared in the same way by absorbing the appropriate HB-Ab with HB-Ag of subtype D.

<table>
<thead>
<tr>
<th>Reciprocal of HB-Ag dilutions used for absorption</th>
<th>Reciprocal of haemagglutination titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neat 5</td>
<td>2 4 8 16 32 64 128 256 512 1024 2048 4096</td>
</tr>
<tr>
<td>10</td>
<td>Agglutination due to anti-d</td>
</tr>
<tr>
<td>100</td>
<td>Agglutination due to anti-d plus some unabsorbed anti-a</td>
</tr>
<tr>
<td>1000</td>
<td></td>
</tr>
</tbody>
</table>

Fig 1. Titration of HB-Ab (anti-ad) after absorption with different concentrations of HB-Ag of specificity Y.

HB-Ag was subtyped by HAI (Hopkins & Das, 1973) using anti-d and D-sensitized cells. Absence of agglutination was indicative of HB-Ag specificity d while the presence of agglutination showed that anti-d had not been neutralized by the antigen, which may possess specificity y. This was confirmed by retesting with anti-y antibody when a true Y antigen would not allow agglutination of Y-sensitized cells.
Australia Antigen Subtyping

Table I. Confirmation of monospecific HB-Ab following absorption

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Cells sensitized with HB-Ag of subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monospecific anti-d (prepared by absorption)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
</tr>
<tr>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

The reliability of the technique was tested by subtyping HB-Ag positive sera from three different sources: five sera from a standard panel supplied by the National Institute of Health, Bethesda, U.S.A., and five sera from a panel supplied by the American Red Cross. These sera had previously been subtyped at source; three sera from healthy carriers detected during routine donor screening by CIEOP at the Blood Transfusion Centre, Royal Infirmary, Edinburgh, and two sera from dialysis patients involved in the Edinburgh outbreak of 1969-70, which had already been subtyped (Dr G. Le Bouvier, Yale University Medical School, Connecticut, U.S.A.) using ID. The sera from all three sources were double coded and an equal number of HB-Ag negative sera included, before being subtyped by HAI.

For the past 2 yr all blood donated in the South-East Region of Scotland has been tested for HB-Ag by CIEOP. During this period 39 HB-Ag positive sera have been identified. In order to determine the prevalence of each HB-Ag subtype in the local donor population these sera were investigated by HAI using monospecific HB-Ab as described above. Most of these sera had already been subtyped by Dr Le Bouvier (Yale University Medical School, Connecticut, U.S.A.) or by Dr L. Magnus (National Bacteriological Laboratory, Stockholm) using ID.

The technique has also been used to titrate the anti-ad and anti-ay specificity in the hyperimmune (HB-Ab) gammaglobulin (HB-Ab, IgG) prepared at the Protein Fractionation Centre, Royal Infirmary, Edinburgh, and used prophylactically in cases of accidental exposure to HB-Ag.

RESULTS

The results of subtyping HB-Ag positive sera by HAI and ID are given in Table II. Complete agreement was found between the two techniques. The prevalence of D and Y amongst HB-Ag positive blood donors in South-East Scotland is shown in Table III. The majority appear to possess HB-Ag of subtype D. Table IV shows the differences in reactivity to two batches of HB-Ab, IgG prepared at the Protein Fractionation Centre, Royal Infirmary, Edinburgh.

DISCUSSION

At the present time most HB-Ag is subtyped by ID or CIEOP. Kim & Tillies (1971) were first to note the greater negative charge associated with the Y subtype. This was subsequently confirmed by P. E. Gibson & Y. E. Cossart (personal communication, 1973) who showed that
Table II. Comparison of subtyping by HAI and ID of HB-Ag positive sera from three different sources

<table>
<thead>
<tr>
<th>Sample</th>
<th>Origin</th>
<th>Immunodiffusion</th>
<th>Haemagglutination inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>208</td>
<td>National Institutes of Health, Bethesda, U.S.A.</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>209</td>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>213</td>
<td></td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>217</td>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>228</td>
<td></td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>1001</td>
<td>American Red Cross</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>1018</td>
<td></td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>1006</td>
<td></td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>2002</td>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>2005</td>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>BD-2-Edin</td>
<td>Local HB-Ag positive blood donors</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>BD-7-Edin</td>
<td></td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>BD-9-Edin</td>
<td></td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>H226</td>
<td>HB-Ag positive dialysis patients</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>GC</td>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

Table III. Prevalence of D and Y subtypes of HB-Ag as determined by haemagglutination-inhibition amongst antigen positive blood donors in South-East Scotland

<table>
<thead>
<tr>
<th>No. of carriers</th>
<th>No. of donors with D subtype (%)</th>
<th>No. of donors with Y subtype (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35 (89.7)</td>
<td>4 (10.3)</td>
</tr>
</tbody>
</table>

Table IV. Haemagglutination titre of HB-Ab IgG prepared at the Protein Fractionation Centre, Edinburgh

<table>
<thead>
<tr>
<th>Batches of gamma-globulin</th>
<th>Reciprocal titre of cells sensitized with HB-Ag of subtype D and subtype Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma-G(2)/H₂</td>
<td>D: 32000, Y: 512000</td>
</tr>
<tr>
<td>Gamma-G(3)/H₃</td>
<td>D: 32000, Y: 64000</td>
</tr>
</tbody>
</table>
HB-Ag of subtype Y migrated further than D in immunoelectrophoresis. Because of their limited sensitivity, ID and CIEOP are wasteful of precious monospecific typing antisera and are unable to subtype HB-Ag detected by more sensitive means (Shaffer et al., 1972; Ling & Overby, 1972). Solid phase RIA offers a means by which such sera may be subtyped. Unfortunately, this technique seems prone to give false positive results with certain sera (Sgouris, 1972; Prince et al., 1973) requires expensive counting equipment and specially trained personnel to handle radioactive material. At the time of writing, solid phase RIA subtyping kits are not commercially available. HAI (Hopkins & Das, 1973), on the other hand, is a simple technique using human 'O' Rh negative erythrocytes sensitized with beta-propiolactone/UVL-treated HB-Ag. It has overcome many of the problems associated with the chromic chloride technique. The application described here permits a rapid (within 2 hr) differentiation of D and Y subtypes of HB-Ag with a sensitivity similar to that of RIA. The discovery of at least two subtypes of HB-Ag clearly implies that techniques and reagents used for routine testing of blood donors and hepatitis patients should be capable of detecting both subtypes. The apparent predominance of D amongst healthy HB-Ag positive blood donors in South-East Scotland is in agreement with other Western European donor populations (Ivarson et al., 1973; Holland et al., 1972).

Subtyping of HB-Ag is an important epidemiological tool in view of the possible relationship between subtype and disease. The prophylactic use of HB-Ab, IgG in 'accident' circumstances may be effective, but as a therapeutic measure in the treatment of disease requires further investigation. Its efficacy may be enhanced by using HB-Ab, IgG of the appropriate subtype specificity.

Addendum

Since acceptance of this paper for publication the authors have received further confirmation of the subtypes relating to the antigen-positive blood donors detected locally. Results obtained from Dr Overby (Abbott Laboratories) using radioimmunoassay show complete agreement with those obtained by HAI. Dr Overby has also reported that each of the donor-carriers tested possessed the antigenic determinant w by immunodiffusion.

ACKNOWLEDGMENTS

We are greatly indebted to Dr G. Le Bouvier (Yale University Medical School, Connecticut, U.S.A.) for initially subtyping many of our HB-Ag positive samples, and for reading the typescript and providing helpful suggestions. We also wish to thank Dr L. Magnuis (National Bacteriological Laboratory, Stockholm) for subtyping a number of the HB-Ag positive donors, Professor B. P. Marmion (Edinburgh University Medical School) and Dr R. A. Cumming and Dr J. D. Cash (South-East Scotland Blood Transfusion Service) for constant encouragement and helpful criticism during the course of this project. Our thanks also to Mr J. Watt and Dr J. Smith (Protein Fractionation Centre, Royal Infirmary, Edinburgh) for kindly supplying hyperimmune (HB-Ab) gammaglobulin. We also wish to thank the N.I.H., Bethesda, U.S.A., and the American Red Cross Blood Transfusion Centre, Washington, for providing HB-Ag panels.
REFERENCES


A Tanned Cell Haemagglutination Test for the Detection of Hepatitis-Associated-Antigen (Au-Ag) and Antibody (Anti-Au)

R. Hopkins and P. C. Das

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(Received 22 March 1973; accepted for publication 2 May 1973)

Summary. A tanned cell haemagglutination technique using inactivated Au-Ag to sensitize human 'O' Rh negative erythrocytes is described. The method may be used to detect Au-Ag by haemagglutination inhibition (HI) and anti-Au by direct haemagglutination (HA). Sensitivity and specificity are demonstrated and its application to mass donor screening is considered.

Although not all post-transfusion jaundice is caused by hepatitis B virus, transmission of hepatitis B is one of the most serious complications of the use of blood and blood products. This situation is likely to persist until all donated blood is screened for Australia antigen (synonyms: Au-Ag, HAA, HB-Ag) by a rapid, specific and sensitive test system. The techniques currently used in most Transfusion Services are counter electrophoresis (CIEOP) (Prince & Burke, 1970), which lacks sensitivity, and latex agglutination (Leach & Ruck, 1971), which is both rapid and sensitive, but lacks specificity (Hopkins & Das, 1973; Ziegenfuss, 1972; Burrell et al., 1972). The radio-immuno techniques (Lander et al., 1971; Ling & Overby, 1972) are extremely sensitive research tools, but are costly and not yet applicable to the rapid mass screening demanded by the Blood Transfusion Service.

Most laboratories have experience of some form of haemagglutination, and many have found it amenable to automation. Juji & Yokochi (1969) described a haemagglutination test using formalized erythrocytes coated with anti-Au (synonyms: anti-HAA, Hb-Ab). Unfortunately they observed non-specific agglutination, and a tendency for sensitized cells to lyse within a few days. Vyas & Shulman (1970) published details of a haemagglutination technique using chromic chloride as a coupling agent. This very sensitive technique has gained acceptance in many research laboratories since it compares favourably with CIEOP (Shaffer et al., 1972). When considering the logistics of its application to routine donor screening, limitations have arisen due to the highly purified antigen required (Vyas et al., 1972), and great variability observed between different batches of cells (Reesink & Duimel, 1972). Furthermore, it is desirable to use inactivated antigen for coating erythrocytes which will subsequently be used as a routine test reagent. In our experience the outcome of coupling inactivated Au-Ag to human erythrocytes by chromic chloride has proved very unpredictable.

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This communication describes a tanned cell haemagglutination technique, incorporating inactivated antigen, which we believe goes a long way towards filling the Au-Ag screening requirements of the Blood Transfusion Service.

**MATERIALS AND METHODS**

**Standard Sera**

Known Au-Ag positive sera, used as controls, were derived from panels obtained from the NIH (Bethesda), American National Red Cross, Blood Research Laboratory, and apparently healthy carriers found locally. Sera from six healthy males (39–64 yr), repeatedly tested serologically and electron microscopically, were used as negative controls. Known specific anti-Au capable of reacting with both ad+ and ay+ antigens (Le Bouvier, 1972), were used in the inhibition reaction to detect Au-Ag.

**Test Material**

Samples were obtained from selected donors, known contacts of Au-Ag, renal dialysis patients and a subject involved in an 'accident' with Au-Ag positive blood, who subsequently received hyper-immune anti-Au IgG prepared by the Scottish National Plasma Fractionation Centre, Edinburgh. Au-Ag positive and Au-Ag negative normal sera were coded and included among the test samples.

**Phosphate Buffered Saline (PBS)**

This was prepared from 0.15 M Na₂HPO₄, 0.15 M KH₂PO₄ and 0.15 M NaCl. Two batches were made, one adjusted to pH 6.4 and the other to pH 7.2. Cell diluent consisting of PBS, pH 7.2, containing 1% normal rabbit serum (previously inactivated at 56°C for 25 min and absorbed with washed packed red cells from the batch being sensitized) was used to suspend the sensitized cells. Diluting Fluid (D.F.) consisting of 50% normal human serum in PBS 7.2 was used to make serial dilutions for end-point titrations. Diluting media were stored at 4°C.

**Preparation of Antigen**

Au-Ag positive serum collected from apparently healthy carriers was inactivated using β-propiolactone and ultraviolet light according to Lo Grippo (Lo Grippo & Hartman, 1958; Lo Grippo et al, 1971). The inactivated material was pelleted by centrifugation at 120 000 g for 4 hr in a M.S.E. Superspeed 65 centrifuge, using an 8 x 25 ml angle head, washed twice in sterile physiological saline and finally resuspended in a small volume of PBS 6.4 and adjusted to give an end point titre of 1/256 by latex agglutination (Pfizer Ltd, Kent, Batch No. Li 13/4; Leach & Ruck, 1971), or a titre of 1/4 by CIEOP (Das et al, 1971), against a commercial anti-Au serum (Batch T2160C) supplied by Hoechst Pharmaceuticals (Hounslow). Au-Ag, thus prepared and still containing some serum proteins was stored at –20°C until required, when it was thawed and heated at 60°C for 30 min prior to sensitization.

**Tanning and Sensitization Procedure**

Human 'O' Rh negative blood was collected in heparin, washed three times in physiological saline and packed by centrifuging at 800 g for 5 min. The packed cells were then resuspended
in PBS, pH 7.2, to give a suspension of 6.6% (v/v) to which was added an equal volume of 1:10 000 tannic acid (w/v) freshly prepared in the same buffer. The mixture was incubated in a 37°C water bath for 15 min, shaking gently every 3 min. The tanned cells were washed once in an equal volume of PBS, pH 6.4, and resuspended in the same buffer to a 4% cell suspension, which was divided into two aliquots. Au-Ag was added to one aliquot in the ratio of 1 volume Au-Ag to 4 volumes of cells, mixed and incubated for 1 hr at room temperature (20°C) on a rotostat (Luckham Ltd, Sussex) to ensure thorough mixing. The remaining aliquot of cells was sensitized in the same way, with 1/125 dilution (in PBS, pH 6.4) of pooled normal human plasma (Au-Ag and anti-Au negative) for use as a plasma sensitized cell control. The sensitized cells were washed three times in equal volumes of cell diluent then made up to a 1.5% suspension in the same diluent and left at 4°C overnight. Next morning the supernatant was replaced with fresh cell diluent and the cells stored at 4°C in small aliquots.

**Test Procedure**

Two systems were employed. One used disposable U-bottomed microtitre plates (Flow Laboratories, Irvine) into which reagents were dispensed as drops from a pasteur pipette—referred to as the microtitre system (M.T.S.). In the other system Terasaki microtest tissue culture trays with lids (Bio Cult Laboratories, Paisley) were used. Reagents were dispensed in 5 μl volumes using an Eppendorf pipette with disposable tips (Alderman and Co. Ltd, London). This was designated the mini-microtitre system (M.M.T.S.). In M.T.S. 0.75% sensitized cells were used, while a similar degree of sensitivity was obtained in M.M.T.S. with 0.3% sensitized cells.

(i) **Microtitre system.** For detection of anti-Au by direct haemagglutination (HA), the test serum was diluted 1-4 in PBS 7.2 and mixed with an equal volume of sensitized cells. The plate was covered to diminish evaporation, and incubated at 37°C for 1 hr. Antibody-containing wells showed positive agglutination characterized by a smooth 'mat' of cells, while absence of antibody was characterized by a 'button' of cells at the bottom of the well (Fig 1). Once confirmed, the test serum was further diluted and the end point titre, the lowest concentration of antiserum producing complete agglutination, was obtained and defined as 1 HA unit. Controls included (a) virus control = sensitized cells + cell diluent, (b) positive control = sensitized cells + known anti-Au and (c) negative control = sensitized cells + normal serum.

Au-Ag was detected by a two-stage haemagglutination inhibition reaction (HI). The test serum was diluted 1-4 in PBS 7.2 and mixed with an equal volume of standard anti-Au containing 4 HA units of antibody activity and the plate incubated at 37°C for 30 min. Sensitized cells were then added, the plate mixed and reincubated at 37°C for a further hour. Agglutination indicated that no antigen was present. Controls included (a) positive control = anti-Au + known Au-Ag + sensitized cells, and (b) negative control = anti-Au + normal serum + sensitized cells.

(ii) **Mini-microtitre system.** For detection of anti-Au, test serum was diluted 1/2 in PBS 7.2 and added to an equal volume of sensitized cells, mixed on a rotostat at room temperature (20°C) for 15 min and then transferred to a 37°C incubator for a further 15 min. The tray was then spun for 10-15 s at No. 1 speed (0-25 g) in a bench
centrifuge equipped with a serological head (Griffin and George, Middlesex). Finally the tray was inclined at an angle of 45-50 degrees, at room temperature and read against a light background at 5 min intervals until control reactions were complete. If no agglutination occurs the cells slide down to the lower part of the base of the wells (Fig 2).

For detection of Au-Ag, the test serum was diluted 1/2 in PBS pH 7.2 mixed with an equal volume of anti-Au containing 4 HA units and incubated for 1 hr at 37°C. Sensitized cells (5 µl) were then added to each well, and the two-phase incubation procedure repeated. The tray was centrifuged and read as described. Controls for HA and HI were the same as for M.T.S.

Confirmation
Positive reactions for anti-Au were confirmed by (a) retesting the sample with plasma sensitized cells and (b) using the test sera as anti-Au in an inhibition reaction with known Au-Ag positive controls and normal sera.

Positive Au-Ag reactions were confirmed by the ability of the sample to inhibit the titre of known anti-Au sera, when compared with normal serum.

Determination of Sensitivity
The ability of HI to detect Au-Ag was compared with immuno diffusion (ID), electrophoresis (Das et al, 1971; Hopkins & Das, 1972), latex agglutination (Leach & Ruck, 1971) using two commercial reagents (Pfizer Ltd and Hoechst Pharmaceuticals), immune electron microscopy (Kelen et al, 1971), radio-immunoassay (RIA) using reagents from Abbot Laboratories (Kent), and radio-immuno precipitation (RIP) performed at the Bacteriology Department, Edinburgh University Medical School.

The sensitivity of HA was measured by comparing Anti-Au titres with CIEOP and also by its ability to detect circulating anti-Au after administration of hyperimmune gammaglobulin to 'accident cases'.

Specificity
This was investigated by coding known Au-Ag positive and Au-Ag negative sera and distributing them randomly among the test samples. All positive results were repeated for confirmation.

RESULTS
Fig 3 shows partially purified Au-Ag prior to sensitization, while Fig 4 shows a sensitized cell with Au-Ag bound to its surface. No free Au-Ag can be seen in the supernatant surrounding the cell.

Pooled Au-Ag containing both ad + and ay + subtypes was titred and compared with a variety of other techniques (Fig 5). It is clear that the haemagglutination-inhibition method has a similar degree of sensitivity to the radio-immuno methods, and is considerably more sensitive than most of the techniques commonly used in routine laboratories for blood donor or patient screening.

The direct haemagglutination test is capable of detecting very low levels of anti-Au as compared with CIEOP (Fig 6). This was further evidenced by its successful application in
Detection of Hepatitis-Associated-Antigen and Antibody

Fig 1. Appearance of positive and negative haemagglutination patterns in microtitre system.
Fig. 2. Appearance of positive and negative haemagglutination patterns in mini-microtitre system, including artist's impression.
Detection of Hepatitis-Associated-Antigen and Antibody

Fig 3. Partially purified Au-Ag prior to sensitization (E.M. mag. = 50,000).

Fig 4. Sensitized cell with Au-Ag bound to surface. No free Au-Ag visible in supernatant (E.M. mag. = 28,000).
monitors the levels of passively administered hyperimmune anti-Au (details of dose and incubation schedule to be published elsewhere) in a number of cases where CIEOP has consistently failed to detect any antibody. Fig 7 shows the levels of anti-Au detected in one such case. No antibody could be detected prior to administration of the gamma-globulin, while circulating anti-Au could be detected within 48 hr after injection and persisted up to 15 weeks later.

Fig 8 shows the results obtained when Au-Ag positive and negative sera were coded and tested by a variety of techniques. The haemagglutination-inhibition test detected all positive samples as did CIEOP and RIA, and gave no false positive result, while one batch of latex, ID and IEM failed to detect several of the positive (Au-Ag) sera, and latex agglutination from both commercial sources gave false positive reactions.

| Table 1. Results obtained when sera from selected donors and dialysis patients were screened for Au-Ag and anti-Au by haemagglutination-inhibition and CIEOP |
|---------------------------------|--------|--------|--------|--------|--------|----------------|
|                                 | CIEOP  | Tanned-cell haemagglutination |
|                                 | No. tested | Anti-HAA positive | HAA positive | Anti-HAA positive | HAA positive | 'Plasma-cell' agglutination |
| Donors                          | 76     | 4       | 7       | 4       | 7       | 0             |
| Dialysis patients               | 14     | 2       | 0       | 2       | 0       | 2*            |

* Different samples from those possessing anti-HAA.

The results shown in Table 1 confirm the specificity of the haemagglutination method, showing complete agreement with CIEOP when sera from selected donors and dialysis patients were tested for Au-Ag and anti-Au. In two of these patients low levels of antibody capable of agglutinating antigen sensitized cells were, in fact, due to reactions against serum protein(s) as demonstrated by agglutination of plasma sensitized cells. This antibody did not inhibit the reactivity of purified ad* or ay* antigens whereas a known anti-Au (diluted to a similarly low level) did have an inhibitory effect under the same circumstances.

DISCUSSION

The technique described in this report provides a simple, rapid, economical and highly sensitive test within the scope of most routine laboratories. Preparation of reagents is straightforward, and the use of inactivated antigen reduces the risk from accidental exposure to personnel involved in routine screening. The use of a 0.75% cell suspension (microtitre system) allows a reasonable incubation time, removes the need to centrifuge plates and facilitates interpretation of results by personnel receiving only a short period of instruction, while retaining a sensitivity of the same order as that of the radio-immuno techniques. The mini-microtitre system, on the other hand, reduces the volume of reagents considerably although it then becomes advisable to centrifuge the trays to obtain the best definition between positive and negative results. The apparent absence of variation amongst different batches of cells eliminates any dependence upon a select group of red cell donors and reflects
Fig 9. Situation which may arise in a multitransfused patient possessing antibodies to both Au-Ag and certain plasma proteins.
the stability of the technique. The extreme sensitivity suggests that antisera may be used at least 100-1000 times more dilute than for immunodiffusion or counter-electrophoresis, thereby alleviating problems arising from expense or shortage of reagents. A shelf life of 3 weeks is acceptable if routine sensitization is performed fortnightly. Storage of fresh cells in liquid nitrogen and the use of glutaraldehyde fixed cells are at present under investigation in this laboratory with a view to increasing the shelf life of the reagents. It remains to be seen whether or not tanned cells sensitized with Au-Ag will prove suitable for application to an autoanalyser.

Experience in a variety of other biological systems, including hormone estimation, where the tanned cell haemagglutination system has been used extensively, has shown that non-specific inhibition can occur in the assay system despite the use of specific antibody (Stavisky & Ingraham, 1964). However, the specificity of the present technique for detection of Au-Ag seems good as no false positive inhibition reactions have occurred amongst the number of samples so far tested. A prozone effect was noted in the direct haemagglutination test when testing hyperimmune sera containing high titre antibody; this was eliminated by using a starting dilution of test sera of 1/2.

It is probable that a proportion of the normal, apparently healthy, population may show positive reactions due to low levels of antibody against plasma proteins when screened by this sensitive method. Brumelhuis et al (1971) found 13 such reactions in 30,000 samples tested by the Netherlands Red Cross Transfusion Service using the less sensitive technique of immunodiffusion. Patients, particularly multitransfused patients, are likely to produce such reactions due to the development of iso-precipitins (Blumberg, 1964; Langenhuijzen, 1971). One possible way of circumventing this problem is to use highly purified Au-Ag for coating the cells. However, when 'purified' Au-Ag was used in the haemagglutination method of Vyas & Shulman (1970), as modified by Prince et al (1971), the sensitized cells were agglutinated by commercial anti-whole human sera and an anti-human IgG (Prince, 1972). This finding may indicate the presence of anti-Au in the commercial antiserum, but is more likely to represent contaminating serum protein(s) in the 'purified' antigen. Whether the contaminant(s) is a part of the Au-Ag moiety (Millman et al, 1971), or plasma protein, is not known. However, the practical conclusions from these observations are that any serum giving a positive reaction, particularly for antibody, must be retested using plasma sensitized cells, and further confirmed by the means outlined in this paper. Fig 9 represents the situation which may arise in a multitransfused patient possessing antibodies to both HAA and certain plasma proteins (Vierucci et al, 1970). Further investigation of this phenomenon is required, but such results are of interest in the light of the high incidence of apparently low levels of anti-Au in multi-transfused patients reported by Lander et al (1971) and Levy & Hawrisiak (1972).

ACKNOWLEDGMENTS

We wish to thank Dr R. A. Cumming and Dr J. D. Cash for encouragement and constructive criticism, Professor B. P. Marmion for providing electron microscope facilities, Dr C. J. Burrell (Edinburgh University Medical School) for providing results from radio-immunoprecipitation, Dr A. M. Prince (New York Blood Centre) for supplying data regarding the chromic chloride haemagglutination technique, and last but by no means least, Dr A. E.
Robertson, Dr S. Parker and Dr J. Pope (Edinburgh) without whose co-operation much of this work would not have been possible.

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Stavisky, A.B. & Ingram, J.S. (1964) Haemagglutination and haemagglutination-inhibition reactions with tannic acid- and bis-diazotised benzidine-protein-conjugated erythrocytes; with an Appendix: Photometric haemagglutination titra-


direct haemagglutination. Sera positive for HBsAg were confirmed by subtyping (adag) using a modification of the test assay.19

Other Assays.—C.E.P. was performed as previously described,6 and the plates were washed with 1% tannic acid before reading. Radioimmunoassay (Austria, Abbott Laboratories) and haemagglutination (Austria, Abbott Laboratories; Hepronostic, Organon, and turkey cells; Wellcome Reagents) were performed as recommended by the manufacturers.

Blood Donor Testing.—Three Scottish regional transfusion centres have been evaluating E.H.A.I. for large-scale donor testing: the North of Scotland Blood Transfusion Centre have tested 16,771 donations by E.H.A.I. and C.E.P. since August 1973; the Edinburgh and South-East of Scotland Blood Transfusion Centre have tested 44,053 donations since February 1974; 4086 of which were also tested with the turkey-cell reagent; the East of Scotland Blood Transfusion Centre have tested 9600 donations by E.H.A.I. Antigen suitable for cell sensitization was prepared centrally at Edinburgh, but cell sensitization and subsequent standardization were performed at each centre independently.

Panel Studies.—In addition to sera from the N.I.H. panel (no. 2), which we have studied before,19 the following antigen panels were investigated: (a) Public Health Laboratory panel (A) supplied by the Standards Laboratory for Serological Reagents and consisting of 25 antigen-positive sera of varying concentrations; (b) Canadian Red Cross panel containing weakly antigen-positive sera (and one antibody specimen)—this has recently been used in a multicentre C.E.P. study;19 (c) American Red Cross panels (1) containing antigen-positive material of varying concentration, negative controls, and which gave false-positive reactions by radioimmunoassay with antigen originally tested in the U.S.A. (2) containing 25 antigen-positive coded sera of different subtypes.

Comparative Titration Sensitivity.—Seven techniques were used to assess relative sensitivity against a standard ad antigen and a standard ay antigen.

Post-transfusion Hepatitis.—Since mid-1973 the South-East region has stored 0.5-ml aliquots of serum at —20°C from all donations, so we were able to test the original donations given to eight patients referred with suspected post-transfusion hepatitis by E.H.A.I., E.H.A.I., and C.E.P. E.H.A.I. was also used to assay two similar specimens supplied by Manchester Regional Blood Transfusion Centre. These sera were obtained at a return visit of the donors and were therefore not truly representative of the original donation. They had all been tested by C.E.P. and electron microscopy and found to be antigen negative before their arrival in our laboratory.

Results and Comments

E.H.A.I. detected seven HBsAg-positive carriers (two previously implicated in post-transfusion hepatitis and five donating blood for the first time) which were missed by counterelectrophoresis (table I), each centre finding at least one additional positive. E.H.A.I. compares well in sensitivity to commercial haemagglutination and radioimmunoassay kits (table II), a finding borne out by the results obtained from the antigen panels studied (table III). All antigen-positive sera were correctly identified by E.H.A.I. with no non-specific reactions. Hepanostic, however, missed two positive sera and the turkey cells failed to detect the weakest antigen-positive sample in the American Red Cross panel (1). During the screening of 4086 blood donations with the turkey cells, C.E.P., and E.H.A.I. each method detected the three positive sera, but there were 20 false-positive reactions with turkey cells.

The retesting of more than 100 donations implicated in eight cases of post-transfusion hepatitis in Edinburgh (cases 1-8, table IV; five

### Table I: Incidence of HBsAg Among Blood Donors Tested at Three Centres

<table>
<thead>
<tr>
<th>No. of Donations</th>
<th>No. Positive on C.E.P.</th>
<th>No. Positive on E.H.A.I.</th>
</tr>
</thead>
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<tr>
<td>Edinburgh</td>
<td>41,665</td>
<td>18</td>
</tr>
<tr>
<td>Inverness</td>
<td>38,711</td>
<td>5</td>
</tr>
<tr>
<td>Dumbarton</td>
<td>9,000</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>70,776</td>
<td>26</td>
</tr>
</tbody>
</table>

### Table II: Comparison of Titration Sensitivity of Haemagglutination Tests, C.E.P., and R.I.A. for HBsAg. Results are Reciprocals of Maximum Titres Recorded

<table>
<thead>
<tr>
<th></th>
<th>C.E.P.</th>
<th>Heparanostic</th>
<th>Turkey Cells</th>
<th>Auscell</th>
<th>E.H.A.I.</th>
<th>E.H.A.I.</th>
<th>Auscell</th>
<th>Auscell</th>
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<tbody>
<tr>
<td>ad</td>
<td>400</td>
<td>1000</td>
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<td>2000</td>
<td>32,000</td>
<td>16,000</td>
<td>8000</td>
<td></td>
</tr>
<tr>
<td>ay</td>
<td>800</td>
<td>4000</td>
<td>32,000</td>
<td>8000</td>
<td>64,000</td>
<td>2000</td>
<td>4000</td>
<td></td>
</tr>
</tbody>
</table>

* With four units of antibody for inhibition.
† With three units of immune absorbed high affinity antibody for inhibition.

### Table III: Results of Antigen Panel Studies

<table>
<thead>
<tr>
<th></th>
<th>Public Health Laboratory Panel</th>
<th>Canadian Red Cross Panel*</th>
<th>American Red Cross Panel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>C.E.P.</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Turkey cells</td>
<td>25</td>
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<td>3</td>
</tr>
<tr>
<td>Austria I</td>
<td>25</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>U.K.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepanostic</td>
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<td>2</td>
<td>10</td>
</tr>
<tr>
<td>E.H.A.I.</td>
<td>25</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

* This panel also contained another specimen with antibody (anti-HBsAg).
† 13 ad, 13 ay.

### Table IV: Results of Reinvestigation of Donors implicated in 10 Cases of Post-transfusion Hepatitis. All Donations were Originally Found to be Negative by C.E.P.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.B.sAg</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Detection of Hepatitis B Surface Antigen among Scottish Blood Donors: Evaluation of Sensitive Tanned-cell Haemagglutination-inhibition Test

R. HOPKINS, M. ROBERTSON, D. ROSS, W. M. TURNBULL, P. C. DAS

Summary
A total of 70,224 blood donations were tested at three Scottish blood transfusion centres for hepatitis B surface antigen (HBsAg) by an economical haemagglutination-inhibition method (E.H.A.I.) and the results compared with those of counter-electrophoresis (C.E.P.). A further 4088 donations were tested using the Wellcome turkey cell haemagglutination test, C.E.P., and E.H.A.I. E.H.A.I. was also compared with commercial haemagglutination and radioimmunoassay reagents for sensitivity and specificity against several established antigen panels and used to reinvestigate counter-electrophoresis-negative blood donations implicated in post-transfusion hepatitis.

E.H.A.I. combines the inherent specificity of an inhibition reaction with a sensitivity equal to that of commercial radioimmunoassay and haemagglutination kits but at a fraction of the cost. The assessment of 70,224 blood donations in three regions showed that E.H.A.I. detected more antigen-positive blood donations than C.E.P. Results of retesting more than 100 blood donors implicated in 10 cases of post-transfusion hepatitis suggested that the use of E.H.A.I. or a test of similar sensitivity in place of C.E.P. may significantly reduce the incidence of this complication.

Introduction
Despite the identification of the causal agent of type B post-transfusion (serum) hepatitis, and the subsequent removal of blood identified as positive for HBsAg by counter-electrophoresis (C.E.P.) hepatitis continues to complicate the therapeutic use of blood and blood products. More sensitive serological tests for surface antigen might lead to a reduction in the number of cases of post-transfusion hepatitis but are unlikely to eliminate it completely because of the potential infectivity of blood containing antibody to the Dane particle core and the possible existence of a reputed hepatitis C virus—quite apart from other causes such as hepatitis virus A (infectious hepatitis) and other microbial agents.

The relatively insensitive technique of C.E.P. is most commonly used in blood banks. Much more sensitive assays, using principles of haemagglutination or radioimmunoassay, are now commercially available.12,13 Some reports suggest that these kits can detect carriers of HBsAg too weak to be positive on C.E.P.14 Unfortunately, their effective use in the blood transfusion service will prove expensive and only time will tell whether such expense is justified.

We report here the experience of three Scottish blood transfusion centres in evaluating a sensitive and economical tanned-cell haemagglutination-inhibition test (E.H.A.I.).11 In addition to a field assessment involving 70,224 blood donations the test was compared with commercial haemagglutination and radioimmunoassay kits for specificity and sensitivity. We also present data on the retesting of 138 blood donors' sera which were HBsAg negative on initial screening by C.E.P. but were later implicated in post-transfusion hepatitis in 10 patients.

Methods
Haemagglutination Inhibition.—The test used was the “mini-micro” version of that described by Hopkins and Das11 in which washed, β-propiolactone/ultra-violet light-treated antigen was used to sensitize human O Rh-negative erythrocytes. Test serum was diluted 1/10 or 1/5 in phosphate-buffered saline and the test conducted in Terasaki trays using only 5 μl of reagent. Antibody was detected by
direct haemagglutination. Sera positive for HBsAg were confirmed by subtyping (adjacent) using a modification of the test assay.

Other Assays.—C.E.P. was performed as previously described, and the plates were washed with 1% tannic acid before reading. Radioimmunoassay (Ausria, Abbott Laboratories) and haemagglutination (Ausria, Abbott Laboratories; Hepanosticon, Orgaran; and turkey cells; Wellcome Reagents) were performed as recommended by the manufacturers.

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The retesting of more than 100 donations implicated in eight cases of post-transfusion hepatitis in Edinburgh (cases 1-8, table IV; five

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<th>No. Positive on C.E.P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edinburgh</td>
<td>43,053</td>
<td>38</td>
</tr>
<tr>
<td>Inverness</td>
<td>49,571</td>
<td>1</td>
</tr>
<tr>
<td>Dundee</td>
<td>9690</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>70,224</td>
<td>26</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th></th>
<th>C.E.P.</th>
<th>Hepanosticon</th>
<th>Turkey Cells</th>
<th>Ausria</th>
<th>E.H.A.I.</th>
<th>E.H.A.I.</th>
<th>Austria 1</th>
<th>Austria 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ad</td>
<td>32</td>
<td>400</td>
<td>1000</td>
<td>64,000</td>
<td>2000</td>
<td>64,000</td>
<td>16,000</td>
<td>4000</td>
</tr>
<tr>
<td>4p</td>
<td>16</td>
<td>600</td>
<td>6000</td>
<td>32,000</td>
<td>8000</td>
<td>64,000</td>
<td>2000</td>
<td>4000</td>
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</tbody>
</table>

* With four units of antibody for inhibition.

** With three units of immune absorbed high affinity antibody for inhibition.

Table III—Results of Antigen Panel Studies

<table>
<thead>
<tr>
<th>Public Health Laboratory Panel</th>
<th>Canadian Red Cross Panel</th>
<th>American Red Cross Panel</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Positive</td>
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<tr>
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<td>0</td>
</tr>
<tr>
<td>Turkey cells</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>Ausria</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>U.S.A.</td>
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<td>0</td>
</tr>
<tr>
<td>Hepanosticon</td>
<td>23</td>
<td>2</td>
</tr>
<tr>
<td>Alabard</td>
<td>22</td>
<td>0</td>
</tr>
</tbody>
</table>

* This panel also contained another specimen with antibody (anti-HBsAg).
† 13 ad, 15 ay.

Table IV—Results of Reinvestigation of Donors implicated in 10 Cases of Post-transfusion Hepatitis. All Donations were Originally Found to be Negative by C.E.P.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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</thead>
<tbody>
<tr>
<td>No. of donations</td>
<td>4</td>
<td>8</td>
<td>14</td>
<td>18</td>
<td>18</td>
<td>32</td>
<td>18</td>
<td>14</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

patients were antigen positive) showed that in two cases an antigen-positive unit of blood had been transfused which was undetectable by the electrophoresis method during the initial screening as well as on re-investigation. In both cases antigen was detected only by the more sensitive tests (table IV). On re-investigation of the two cases from Manchester (cases 9 and 10) a positive unit was detected in both cases, though one was missed by turkey cells and the other by Austria I. The antigen-positive unit transfused in case 9 was subtyped as ad, while that transfused in case 10 was found to be Be, confirming the finding of the titration study (table II) in which Austria I showed a bias towards detection of the ad subtype, despite that all other tests except Auscel found ad to be weaker. A similar bias in favour of the ad subtype was recorded by Vanderveld et al., but this fault seems to have been corrected to some extent in the newer Austria II test (table II).

Discussion

Recent reports have highlighted the inadequacies of C.E.P. in detecting HBsAg in blood donations. The American Red Cross seem to have opted for R.I.A., while regional transfusion centres in the UK seem to favour haemagglutination. Three commercial haemagglutination assays, all using agglutination of antibody-coated erythrocytes, are currently available. The cheapest of these (about 10 p per test if ordered in bulk) is marketed by Wellcome Reagents, and consists of fixed turkey erythrocytes coated with affinity column purified antibody raised in horses. In terms of speed and simplicity it is well suited to the large-scale testing of blood donations, and its sensitivity is much greater than that of C.E.P. but less than that of R.I.A. The other two haemagglutination tests—Hepanostic, which consists of fixed sheep erythrocytes coated with antibody raised in sheep, and Auscel, which consists of fixed human erythrocytes coated with antibody raised in guinea pigs—have been found to be more sensitive than C.E.P. All three tests are susceptible to a few false-positive reactions, the problem being solved by absorption-titration experiments.

Our E.H.A.I. test is about as sensitive as R.I.A. and, being an inhibition reaction, has an inherent specificity not found in the haemagglutination tests. The use of monospecific antibody (prepared by absorption or affinity chromatography) allows E.H.A.I. to be used to subtype antigen-positive sera, which provides an essential confirmatory step.

The cost of E.H.A.I. is negligible owing to the availability of raw material, the considerable expertise in haemagglutination technology within the Blood Transfusion Service, and the simplicity of reagent preparation combined with the small quantities (5 µl) needed for testing. In one week 500 ml of high-titre HBsAg-positive serum was processed to provide enough antigen for the sensitization of sufficient cells to test 720 000 donations for both antigen and antibody. We estimate that about 40 tests can be done for £1.

The prime criterion in evaluating a new screening test must be the number of additional antigen carriers detected in comparison with current techniques. Our results indicate that E.H.A.I. detects several donations possessing HBsAg in concentrations below the sensitivity of C.E.P. The importance of these findings is emphasized by the results obtained from the retesting of blood donations implicated in post-transfusion hepatitis, which serves as a measure of the efficiency of HBsAg testing within the Blood Transfusion Service. On the three occasions in which a C.E.P. false-negative donation was detected by R.I.A. it was also detected by E.H.A.I.; furthermore, E.H.A.I. detected one out of 17 units transfused which remained R.I.A. negative (table IV). This tends to confirm the findings of Karetz et al., who reported that a positive haemagglutination reaction was more closely related with hepatitis or seroconversion than was a positive R.I.A. reaction.

E.H.A.I. may prove acceptable in large-scale blood donor testing. The incidence of antibody, detected by direct haemagglutination, is about 10-20 times greater than that detected by C.E.P. (252/31 999 vs. 13/31 999), thus not only providing valuable materials for laboratory reagents but also increasing the quantity of antibody for subsequent fractionation of hepatitis B immunoglobulin for therapeutic use. It seems that with appropriate training the technique may be readily introduced into any regional transfusion centre, thereby keeping reagent costs to a minimum. The basic technology has been adapted to a rapid microcapillary test for use in emergencies and is being automated in Inverness and modified for the detection of antibody to the Dane particle core in Edinburgh.

We thank Dr. J. D. Cash, Dr. I. A. Cook, and Dr. C. Cameron, regional directors of the South-East, North, and East regions respectively, for continued support and encouragement during this project. We also thank Mr. R. Y. Dodd and Dr. T. J. Greenland (American National Red Cross), Dr. B. P. L. Moore (Canadian Red Cross Society), and Dr. P. Bradstreet (Public Health Laboratory Service) for kindly providing the antigen panels. We are grateful to Dr. L. D. Wadsworth (Manchester Blood Transfusion Service) for allowing us to report on two cases of post-transfusion hepatitis. Finally, we thank Abbott Laboratories for the timely arrival of an Austria II evaluation kit.

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Bulk preparation of reagents for hepatitis B testing

A. D. WATT, M. ROBERTSON AND R. HOPKINS
Technical method

Bulk preparation of reagents for hepatitis B testing

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Hepatitis B surface antigen (HBsAg) is a marker of one of the causative agents of post-transfusion (serum) hepatitis (Giles et al, 1969) and as such must be identified in donor blood before transfusion. Over the past few years, a number of techniques have been applied to the detection of HBsAg, ranging in sensitivity and sophistication from agar-gel diffusion (AGD, first generation test) to radioimmunoassay (RIA, third generation test). The experience of many laboratories clearly indicates that third generation methodology must be adopted if maximum efficiency of blood donor testing is to be achieved (Ling and Overby, 1972; Cayzer et al, 1974; Hopkins et al, 1975). Unfortunately, third generation reagents are available to the majority of blood transfusion centres only on a commercial basis, the cost ranging from 10 pence per test (Hepatetst—Reverse Passive Haemaggglutination (RPHA) Wellcome Reagents) to 50 pence per test or more, apparently depending on geographical location (AUSRIA-II, Radioimmunoassay (RIA), Abbott Laboratories).

In 1973 details were published of a tanned-cell haemaggglutination inhibition (HAI) technique utilizing Terasaki tissue culture trays, which achieved a sensitivity similar to RIA but at a fraction of the cost (Hopkins and Das, 1973). This technique has recently been evaluated by three Scottish regional transfusion centres testing over 70 000 blood donations together with various well documented HBsAg panels (Hopkins et al, 1975). The results indicated that, with appropriate training, the technique could be introduced into most regional transfusion centres, thereby keeping reagent costs to a minimum. A valid comparison between centres was possible only because reagents were prepared in bulk at one centre and were thus completely standardized.

This communication describes the bulk preparation of HBsAg coated glutaraldehyde fixed spherocytes in a quantity sufficient for approximately one million tests.

Preparation of Fixed Spherocytes

**PREPARATION OF SPHEROCYTES**

Reagent standardization should be enhanced if a uniform cell population is used for HBsAg coating. Uniformity is, in this case, achieved by selection of the most robust cells using a controlled hypotonic medium.

One unit (approximately 450 ml) of human group O rhesus negative blood collected in anticoagulant (citrate phosphate dextrose), and not more than three weeks old, was obtained from the blood bank. It was first necessary to determine the molarity of phosphate buffer pH 7.2 required for the selection process. Isotonic (0-2 M) phosphate buffer containing 0-1 % sodium azide was serially diluted in deionized water, and 0-1 ml of fresh, well mixed blood was added to 2 ml of each dilution of phosphate buffer in a test tube, mixed thoroughly, and allowed to stand for 60 minutes. That solution showing at least 50% haemolysis was arbitrarily taken as optimum. The molarity at this point was usually 0.045 M to 0.050 M. The remainder of the unit of blood was then mixed with phosphate buffer (adjusted to optimum molarity) in the ratio 1 volume of cells to 4 volumes of buffer, and the bulk volume was split into 200 ml aliquots for ease of subsequent washing. After 60 minutes at room temperature the aliquots were spun at 1000 rev./min for 15 minutes at 20°C or room temperature using an MSE 6L centrifuge, the supernatant was removed, and fresh hypotonic buffer was added. This procedure was repeated until only a minimum of haemolysis was observed in the supernatant, and the remaining cells accounted for approximately 50% of those originally present. Wet film microscopy showed that the cells had lost their conventional biconcave morphology and become rounded. They were subsequently referred to as 'spherocytes'.

**FIXATION OF SPHEROCYTES**

In a pilot experiment some spherocytes were divided into aliquots to which glutaraldehyde (Koch Lights, 25%) was added to give the following concentrations: 1 in 100, 1 in 200, 1 in 400, 1 in 800, 1 in 1600, 1 in 3200, and 1 in 6400. After thorough mixing, the cell aliquots containing fixative were left at room temperature overnight. The following morning cells from each aliquot were examined microscopically and subjected to a 'water-resistance' test, which
involved adding 25 μl of ‘settled’ spherocytes to 2 ml of deionized water, when properly fixed cells resisted haemolysis. Dilutions of glutaraldehyde up to 1 in 1600 (0.06%) produced acceptable fixation, although dilutions up to 1 in 400 (0.25%) tended to distort the shape of the spherocytes, causing them to revert to biconcave discs. The remaining spherocytes (from the unit pack) were fixed in the appropriate concentration of glutaraldehyde (approximately 0.06%) as above and stored at 4°C in phosphate buffer containing 0.1% sodium azide. All solutions contained 0.1% sodium azide.

TANNING, SENSITIZATION, AND STABILIZATION OF FIXED SPHEROCYTES (for one million HAI tests)

Sixteen ml of packed glutaraldehyde-fixed spherocytes were washed twice with 500 ml volumes of physiological saline (MRC bottle spun at 1000 rev/min for 5 minutes in an MSE 6L centrifuge at room temperature). The deposited cells were resuspended in 500 ml of tannic acid (M & B at a 1 in 60 000 dilution in phosphate buffer 0.15 M pH 7.2 containing 0.1% sodium azide and previously heated to 37°C) and left in a 37°C waterbath for 15 minutes with occasional mixing. The tanned cells were spun down as before, washed twice with 500 ml of phosphate buffered saline (PBS) pH 6.4 containing 0.1% sodium azide, and finally resuspended in 400 ml PBS pH 6.4.

Twenty millilitres HBsAg, prepared as previously described (Hopkins and Das, 1973) and containing both ‘d’ and ‘y’ antigenic determinants, was added, and the spherocyte-antigen suspension was mixed continuously at room temperature (see addendum). After 20 hours at 1 ml aliquot was removed, stabilized as previously described (Hopkins and Das, 1973), and used to titrate a known standard HBsAb containing serum. Sampling was repeated at approximately 20-hour intervals until a satisfactory sensitivity was achieved. The bulk reagent was removed from the mixer and the cells were recovered by centrifugation as before. The sensitized cells were then stabilized by resuspension and mixing for 4 hours at room temperature in 500 ml PBS pH 7.2 containing 2% normal human serum to give a cell suspension of approximately 3-2% (or 10 times working concentrations).

Storage of these cells overnight at 4°C was followed by a repetition of the stabilizing procedure. Thereafter, the 3-2% suspension of cells was dispersed into 50 ml aliquots and stored at 4°C ready for pasteurization.

PASTEURIZATION AND STORAGE OF HBsAg COATED SPHEROCYTES

Immediately before pasteurization the coated spherocytes were pelleted and resuspended to the same volume with PBS pH 7.2 containing 0.05% phenol. They were then left for 12-16 hours in a 60°C waterbath, allowed to cool, divided into smaller aliquots (for convenience), and stored at 4°C until required when each aliquot would be diluted 10-fold with PBS pH 7.2 to achieve working strength.

The coated cells could also be stored at –20°C or in the lyophilized state without appreciable loss of sensitivity.

Comment

Recent reports have highlighted the inadequacies of first and second generation methods of HBsAg testing (Ling and Overby, 1972; Cayzer et al., 1974; Hopkins et al., 1975). The American Red Cross appear to have opted for RIA as a means of blood donor screening, while transfusion centres in the United Kingdom seem to favour haemagglutination.

It is the purpose of this communication to emphasize that third generation testing efficiency is available at a fraction of the cost of purchasing commercial reagents. Preparation of sufficient HAI reagents for approximately one million tests is described in detail using a process which is readily reproducible (we have recently prepared a second batch of reagents sufficient for 3.5 million HAI tests) and requires relatively unsophisticated laboratory equipment. The cost of one million RPHA tests is £80 000, while a similar number of RIA tests bought commercially could cost £500 000.

Experience has shown that HAI reagents are extremely stable in that they will survive 4°C for over one year, repeated freezing and thawing, lyophilization and exposure to 2.5 mega rads of gamma irradiation from a cobalt source (Courtesy of Ethicon Ltd, Edinburgh), neither does transport over thousands of miles via air freight appear to have an adverse effect, even when cells are maintained in the liquid state.

The safety of laboratory staff handling hepatitis B testing reagents is of paramount importance. Until it is possible to evaluate the biological activity of HBsAg it is essential that every reasonable precaution should be taken to ensure the safety of such reagents. For this reason we have attempted to incorporate into reagent preparation accepted methods of virus inactivation, combining the principles of chemical and physical inactivation, namely, (a) betapropiolactone treatment and ultraviolet irradiation of raw serum (LoGrippo et al., 1971), (b) pasteurization of HBsAg coated glutaraldehyde fixed spherocytes, and, if required, (c) gamma irradiation of the lyophilized reagents.

We wish to express our sincere thanks to Drs J. D. Cash and P. C. Das, of the South East of Scotland
Regional Blood Transfusion Centre, for their support and encouragement in the course of this project.

References


Addendum

We observed that cell sensitization was successful when prepared by mixing in an MRC 500 ml bottle by mechanical turntable but unsuccessful when performed in the same type of bottle using a magnetic mixer with plastic-coated follower. Adsorption of HBsAg onto the plastic follower has been eliminated but the electrochemical or electrophysical possibilities for the failure have yet to be investigated.
Rapid Identification of Hepatitis Associated Antigen and Antibody by Counter-Immunoelectroosmophoresis

P. C. Das, R. Hopkins, J. D. Cash and R. A. Cumming

Blood Transfusion Centre, and Blood Products Unit, The Royal Infirmary, Edinburgh EH3 9BB

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SUMMARY. A modification of the counter-immunoelectroosmophoresis method is described which will allow rapid identification of hepatitis associated antigen and antibody within 2 hr by producing a reaction of identity with known reference materials. The specificity of the reaction is established by the demonstration of non-identity and partial identity by this technique.

The exact relationship between Australia/SH antigen, presently known as hepatitis associated antigen (HAA), and A or B hepatitis virus remains to be clearly defined. However, there is little doubt that products containing HAA are capable of transmitting the infection to susceptible recipients. Amongst the various immunological techniques used for the detection of HAA, the counter-immunoelectroosmophoresis (CIEOP) (Passendorfer et al., 1970) has recently gained popularity for its sensitivity and the rapid formation of precipitin lines due to an enhanced reaction between the antigen and antibody. Combining the principle of electrophoresis and immunoprecipitation simultaneously, the basic technique involves two wells per sample between which a precipitin line is observed when the antibody immunoglobulin present in the anode well reacts with the material in the cathode well containing HAA. When a precipitin line does occur, it is necessary to confirm its specificity by demonstrating its identity with known reference material. Occasionally, erroneous results may be obtained because the antisera of human origin which are presently used for detection of HAA, are mostly derived from multitransfused patients and may contain, besides anti-HAA, other antibodies to plasma proteins (Blumberg, 1964). Zuckerman & Taylor (1970) and Passendorfer et al. (1970) suggested that CIEOP is unable to produce reactions of identity readily and recommended the Ouchterlony double diffusion method for this purpose. This has been a major drawback to the adoption of CIEOP for identification of HAA and its antibody.

It was suggested (F. Reicht, personal communication 1970) that CIEOP could be used for identification purposes if the precipitin bands are allowed to form sufficiently close between the wells containing reference material such as known HAA and the test substance reacting simultaneously against the complementary antibody present in a third well. This paper describes such a modification and allows the identification of antigen and/or antibody within an hour by showing reaction of identity, partial and non-identity between the reactants.

MATERIAL AND METHOD

Materials

HAA. Plasma and serum containing HAA were obtained from a hepatitis patient; the reference antigen was received from Dr Okochi, Tokyo.
Fibrinogen. Normal plasma obtained from a healthy blood donor was used as a source of human fibrinogen and hooded rat plasma for rat fibrinogen.

Anti-human fibrinogen (Behringwerke AG, Germany) produced in the rabbit was used. This antiserum reacts only with fibrinogen but not with other plasma proteins.

Anti-HAA was obtained from a multiply transfused haemophiliac and its reactivity had been tested and compared with other reference antisera (Das et al, 1971).

Preparation of Plates

A 0.9% agarose solution in 0.025 M veronal buffer (pH 8.6) was used. Aliquots of 25 ml molten agarose were poured onto a 10 cm-square plastic petri dish (Sterilin) on a levelled table and allowed to solidify. Circular wells 2 mm in diameter were cut in the agarose 3 mm apart along the electrophoresis axis (Fig 1). If HAA was suspected, the pair of wells 1 mm apart were placed towards the cathode and vice versa.

\[
\text{Anode} \quad + \quad \text{Cathode} \quad -
\]

\[
\begin{align*}
&3\text{mm} \\
&2\text{mm} \{ \text{A} \} \\
&1\text{mm} \{ \text{B} \}
\end{align*}
\]

Fig 1. Position of wells in the agarose gel for the CIEOP technique. A: HAA control; B: suspected HAA; C: anti-HAA serum.

Method

The anode well(s) were filled with antiserum by means of a capillary tube. The petri dish was then placed in the electrophoresis chamber (Shandon, Model U.177) and maintained at 30°C by means of a water jacket (Shandon) through which water was circulated at the rate of 1 litre/2.5 min from a water bath using a peristaltic pump. Glass fibre paper (Whatman) was used as connecting wicks with veronal buffer (pH 8.6, 0.05 M) in the electrophoresis chamber and a current of 40 mA (about 200 volts) was passed for 5 min. The current was then switched off and the antiserum well(s) 'topped' up. Antigen was added to the cathode well(s) and and the current re-started. Under these conditions, the precipitin lines appear in 1 hr. If the reactants in the two vertical wells both contain HAA (or antibody) a line of identity will be obtained. The specificity of the technique is further demonstrated by the production of reactions of non and partial identity.

RESULTS

Fig 2(a) shows a complete reaction of identity by CIEOP using serum and plasma from the same patient. Complete identity was also obtained when the test serum was compared with the reference antigen (Fig 2b). This basic observation was not exclusive to the HAA and anti-HAA system and could be extended to plasma-fibrinogen as shown in Fig 2(c). However,
Fig 2. Different types of immunological reaction obtained by the CIEOP. (a), (b) and (c) Reaction of identity; (d) non-identity reaction; (e) reaction of partial identity.

(Facing p 674)
when the patient's plasma was allowed to react simultaneously against the anti-HAA and anti-fibrinogen serum, a reaction of non-identity showing the characteristic intersected precipitin lines was obtained (Fig 2d). When human and rat plasma were allowed to react against an anti-human fibrinogen by this method, the resultant precipitin lines showed partial-identity due to the known serological differences in rat and human fibrinogen. The size and direction of the 'spur' indicate the degree of dissimilarities between the antigens (Fig 2e).

DISCUSSION

In examining 158 transfused thalassaemic patients, Vierucci et al (1970) reported that 20% of those showing anti-Ag (low density lipoprotein) also contained anti-HAA and suggested that use of such antisera in HAA detection might give rise to false positives. Establishment of the nature of reaction by identifying the precipitin bands is therefore of major importance since much of the anti-HAA presently available is derived from multiply transfused patients in whom iso-precipitins, not related to HAA may be present (Blumberg, 1964). During the last year at least one such reaction has been demonstrated in this laboratory in a para-protein-aemic patient's serum which produced a precipitation line against anti-HAA, but failed to demonstrate virus like particles on electronmicroscopy.

In using the CIEOP technique, the size of the wells does not appear to be critical and some workers preferred to alter the well-diameter according to the concentration of the reagents used. The optimal distance between the anode and cathode wells seems to be 3 mm (Alter et al., 1971). For accurate identification one must ensure that the margins of the wells are intact (Ouchterlony, 1964). We have found that layering the gel with 1% freshly made tannic acid solution at room temperature for 10-15 min significantly increases the sensitivity of the system by sharpening the weak precipitation bands (Alpert et al., 1970), and has the added advantages of time and simplicity over the conventional staining methods.

Despite the increasing use of CIEOP for the detection of HAA many laboratories still base their final conclusion on a reaction of identity produced by the technique of Ouchterlony. A major disadvantage of this method is that it is relatively time consuming requiring 1-7 days (Prince & Burke, 1970) before a definite answer is available. It is therefore ill suited for blood transfusion purposes, particularly in making available fresh whole-blood and platelet concentrates which may be required for transfusion within a few hours of withdrawal. The technique we describe seeks to reduce substantially this delay, so that in less than 2 hr initial screening and identification is possible. Besides the HAA system, this method could also be applied in other antigen-antibody systems such as plasma fibrinogen and alpha-feto-protein (Kohn, 1970).

ACKNOWLEDGMENTS

We thank Dr K. Okochi for the reference antigen and Dr F. Reicht for helpful suggestions. This work is supported by a grant from the Scottish National Blood Transfusion Service and the Scottish Hospital Endowment Research Trust.
REFERENCES


Improved sensitivity of the electrophoresis method by tannic acid for detection of Australia antigen

R. HOPKINS AND P. C. DAS
Improved sensitivity of the electrophoresis method by tannic acid for detection of Australia antigen

R. HOPKINS AND P. C. DAS

From the Regional Transfusion Service, Royal Infirmary, Edinburgh

For detection of Australia antigen (Au-Ag) by counter-immuno-electroosmophoresis (CIEOP) staining the agarose gel plates with certain dyes has been claimed to improve the sensitivity (Combridge and Shaw, 1971). In our experience, however, a simpler and less time-consuming procedure is that of layering the gel plates with 1% freshly made tannic acid for 10 minutes (Alpert, Munroe, and Schur, 1970) after the routine CIEOP procedure (Das, Hopkins, Cash, and Cumming, 1971). This has resulted in a significantly increased sensitivity by improving visualization of precipitin lines.

Serial dilutions of Au-Ag containing serum and anti-Au (human origin) were set up in the test system using a 'chessboard' design. After the electrophoresis 'run' the gel plates were observed at an angle under direct light over a dark background. The results were scored as + for sharp precipitin line, ± for weak precipitation, and — for no reaction. Table I shows that the titre of Au-Ag against the neat antiserum was 1/4, and no significant improvement was noticed when the same plate was reviewed after overnight incubation. Tannic acid was now added and the plate read after 10 minutes: the titre was now 1/16. This improvement reflects an increased sensitivity of the system as a whole; thus, before tannic acid treatment, the total number of

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positives in the 'chessboard' was 14, and after tannic acid treatment they were 21. This was further confirmed over a period of two weeks during which 212 selected specimens from patients, including drug addicts, with clotting disorders and hepatitis, as well as blood donors, some of them already known to be carriers of Au-Ag, were subjected to the procedure described above. Results show (Table II) that the number of positive samples were eight before and 12 after tannic acid treatment; the additional

<table>
<thead>
<tr>
<th>No. of Samples Tested</th>
<th>Blood</th>
<th>Patient</th>
<th>Laboratory</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donor</td>
<td>Staff</td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Before tannic acid</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>After tannic acid</td>
<td>4</td>
<td>6</td>
<td>0</td>
<td>12</td>
</tr>
</tbody>
</table>

Table II  Number of positive specimens amongst 212 selected samples before and after tannic acid treatment of CIEOP plates

positive results consisted of three from the patient group and one from the blood donors. In view of these results the above procedure was extended to the 'routine' laboratory where every unit of blood donated was screened for the presence of Au-Ag. During a period of five months a total of 19,423 donors were tested; 10 were found to be positive by routine procedure and no additional positive appeared, however, after tannic acid treatment of the CIEOP plates.

The mechanism by which tannic acid increases the sensitivity of the system is not clear, but from a practical point of view there is no doubt that it is capable of bringing out Au-Ag-antibody precipitin lines, especially amongst the patient's sera. The present procedure in this laboratory is to score the results immediately after the routine CIEOP 'run', the plates are washed, then treated with tannic acid, re-read and photographed immediately. All positive samples are re-investigated for identity reaction, if necessary, after concentration.

We are grateful to Dr R. A. Cumming and Dr J. D. Cash of this Department for constant encouragement, support, and reading the typescript.

References


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Received for publication 11 May 1972.
positives in the 'cheesboard' was 14, and after tannic acid treatment they were 21. This was further confirmed over a period of two weeks during which 212 selected specimens from patients, including drug addicts, with clotting disorders and hepatitis, as well as blood donors, some of them already known to be carriers of Au-Ag, were subjected to the procedure described above. Results show (Table II) that the number of positive samples were eight before and 12 after tannic acid treatment; the additional positive results consisted of three from the patient group and one from the blood donors. In view of these results the above procedure was extended to the 'routine' laboratory where every unit of blood donated was screened for the presence of Au-Ag.

During a period of five months a total of 19,423 donors were tested, 10 were found to be positive by routine procedure and no additional positive appeared, however, after tannic acid treatment of the CIEOP plates.

The mechanism by which tannic acid increases the sensitivity of the system is not clear, but from a practical point of view there is no doubt that it is capable of bringing out Au-Ag-antibody precipitin lines, especially amongst the patient's sera. The present procedure in this laboratory is to score the results immediately after the routine CIEOP 'run', the plates are washed, then treated with tannic acid, re-read and photographed immediately. All positive samples are re-investigated for identity reaction, if necessary, after concentration.

We are grateful to Dr R. A. Cumming and Dr J. D. Cash of this Department for constant encouragement, support, and reading the typescript.

References


Latex agglutination test for detection of Australia antigen (HB-Ag) among blood donors and patients

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SUMMARY The application of the latex test for the detection of Australia antigen (Au-Ag) was investigated. Reagents from two commercial sources were compared with the electrophoresis method with regard to sensitivity and specificity using samples from blood donors, hospital patients, and plasma fractions. Discordant results were further investigated by electron microscopy and radioimmunooassay.

Differences were noted in the results between these reagents and the significance of the findings together with suggestions for minimizing false positive results are discussed.

The introduction of a latex agglutination test for the detection of Au-Ag (HAA/HB-Ag) by Leach and Ruck (1971), which can be read in minutes and is claimed to be as sensitive as the generally recognized counter immunoelectrophoresis technique, may be regarded as an important advance. This report describes our experience with this technique applied to blood donors and patients and compares it with the counter immunoelectrophoresis method. Electron microscopy and radioimmunooassay were performed on those specimens showing consistent discrepancy between the results by counter immunoelectrophoresis and latex agglutination.

Materials and Methods

STANDARD SERA

Known Au-Ag positive serum and plasma samples from our reference panel were collected either locally or from abroad including the reference panel no. 2 of the Department of Biologics Standard, NIH (Bethesda, Maryland), and the American Red Cross (Washington). Serum and plasma taken into different anticoagulants (citrate, oxalate, heparin, and EDTA) from nine healthy males (39-64 years) were tested both serologically including radioimmunooassay, and by electron microscopy for use as negative controls. Further, a group of sera selected from the panel that had been tested by different laboratories (by multiple methods) and giving concordant results, were used in this study as positive and negative controls (table I).

TEST SAMPLIES

Test samples were obtained from several sources: from plasmapheresis donors, from known contacts to Au-Ag, from miscellaneous patients including some with rheumatoid arthritis, and from coagulation factor concentrates prepared by the Scottish National Plasma Fractionation Centre. Also, 10 known Au-Ag positive sera were derived from the screening, by counter immunoelectrophoresis, of 15,000 donors. Of these 10 Au-Ag positive sera, four were subtyped as ad+ and a further four as ay+ by Le Bouvier (1971) at Yale University; all 10 were coded and randomly dispersed among the other test samples.

LATEX AGGLUTINATION TEST

Two separate batches (PF1, PF2) of latex particles coated with anti-Au-Ag, prepared in guinea-pigs and supplied by Pfizer Ltd, were tested according to the method of Leach and Ruck (1971). A further set of reagents (anti-Au-Ag prepared in rabbits) was also supplied by Hoechst Pharmaceuticals Ltd, and the tests were performed as recommended by the manufacturers. By the same procedure further tests were carried out using latex tagged with normal rabbit IgG and with guinea-pig immunoglobulin (obtained commercially from Wellcome Reagents)

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and Pfizer Ltd. The results were scored as + for strong, ± for weak, and – for no agglutination.

OTHER METHODS
Counterimmunoelectroosmophoresis was performed by the modification of Das, Hopkins, Cash, and Cumming (1971) and read after tannic acid treatment (Hopkins and Das, 1972).

Basic electron microscopy and immune electron microscopy were performed as described by Kelen, Hathaway, and McLeod (1971). Solid phase radioimmunoassay using radiiodinated marker, anti-Australia antigen 125I(19/Austria-125), obtained from the Abbott Laboratories, was used in accordance with the manufacturer's directions.

Results

Preliminary studies on the various latex preparations set up against known standard Au-Ag positive sera demonstrated that although all apparently reacted satisfactorily, differences in sensitivity could be shown between the reagents (table II). Further, the 10 positive sera derived from routine screening of Queenborough, Kent, UK.

<table>
<thead>
<tr>
<th>Samples with (+) or Without (−) Au-Ag</th>
<th>Latex Pfizer (2)</th>
<th>Latex Hoechst</th>
<th>Counter Immuno-electroosmophoresis</th>
<th>Electron Microscopy</th>
<th>Radio-immunoassay</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<td>B</td>
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</table>

Table II Comparative antigen titration with different batches of latex and by counter immuno-electroosmophoresis

1Figures in parenthesis are before addition of normal animal serum.

15,000 blood donors, when tested with one batch of latex (PF1), all reacted strongly; four of these which were subtyped ad+ had a mean reaction time of 1.9 minutes (range 0.8-2.5 minutes) and four which were subtyped ay+ had a mean reaction time of 2.8 minutes (range 1.5-4.5 minutes).

Based on these findings 100 selected test samples were set up against the PF2 and Hoechst latex preparations. The results are summarized in table III; all samples shown to be positive by counter immuno-electroosmophoresis were also strongly positive with the PF2 latex preparation. However, the Hoechst material failed to detect one out of six of the Au-Ag positive samples, and both the latex preparations produced five false positives.

As the one false negative reaction occurred in a serum from a patient, a further 10 patients' sera were tested. These sera were kindly supplied by the Edinburgh University Department of Bacteriology's diagnostic service and were tested against the PF2 and Hoechst reagents. The results are summarized in table IV which shows that the PF2 reagent detected all six Au-Ag positive sera. However, the Hoechst latex preparation reacted to only two of these sera. Both reagents gave false positive reactions all of which were also negative to electron microscopic and immune electron microscopic examination. Prior heat treatment of these sera (56°C for 30 minutes) did not influence the non-specific latex agglutinin titre, except in one sample out of the six so treated, and the addition of normal guinea-pig serum to the test as recommended by one manufacturer produced false negative result in a haemophilic (table IV), probably due to physical dilution. However, in all of these sera the presence of non-
specific latex agglutinins could be readily demonstrated by latex-coated normal rabbit or guinea-pig immunoglobulins.

In order to ascertain whether this latter observation could be used in monitoring false positive results, sera from 19 patients with rheumatoid arthritis (seven of whom were positive for rheumatoid factor) were tested with rabbit anti-Au-Ag coated latex (Hoechst) and with normal rabbit immunoglobulin sensitized latex (Wellcome Reagents). Table V shows that nine samples were negative to both preparations, one serum showed a weak positive reaction to anti-Au-Ag coated latex only, but a further nine samples reacted to both specific and non-specific latex reagents; the agglutination titres against the specific anti-Au-Ag coated latex (mean 24 ± 25) and non-specific gamma globulin-coated latex (13 ± 11) showed highly significant correlation (r = 0.91, p < 0.001) in the later group of nine sera. All 19 samples were negative by counterimmunoelectrophoresis.

Attempts to reduce further the time required for the latex technique by using plasma instead of serum proved disappointing. Fresh normal plasma, irrespective of the anticoagulant used, produced weak but definite latex agglutination and heat treatment (56°C for 30 minutes) did not modify this reaction. Sera from the same donors (tested after two hours at 37°C) were negative.

In a further investigation, radioimmunoassay was used in parallel with both preparations of anti-Au-Ag coated latex (Hoechst and Pfizer). Positive and negative specimens from the Reference Panel were coded and tested by a variety of other methods.
Table I shows that all the eight known positive samples were correctly identified by radioimmunoassay, counter immunoelectroosmophoresis, and Pfizer latex (PF2). However, one remained negative with the Hoechst reagent and three with electron microscopy. No false positive results were obtained by radioimmunoassay, counter immunoelectroosmophoresis, and electron microscopy, but one occurred with both specific latex reagents. This was confirmed by negative results obtained by these three techniques.

Discussion

The surprising feature of this study was the good agreement between both the latex preparations and the counter immunoelectroosmophoresis technique when applied to sera obtained from known Au-Ag positive blood donors. On the other hand undoubted false negatives occurred in sera obtained from Au-Ag positive patients with the Hoechst and PF1 preparations. This may have been due to a limited specificity of the coating antibody, for preliminary studies in this laboratory have suggested that the antigen typed as ay+ may not react as effectively as those designated ad+ with the latex reagents used. It seems more likely, however, that the problem, which is of some clinical importance, may be more closely related to the low sensitivity of these reagents. This conclusion is supported by the absence of false negatives with the PF2 preparation.

False positive reactions to both the latex reagents used in this study have been reported previously (Banatvala, Besl, Almeida, and Dain, 1971; Cossart, Field, March, and Porter, 1972; Burrell, Dickson, Gerber, McCormick, and Marmion, 1972) and our results confirm these observations. In investigating the proficiency of anti-Au-Ag coated latex, Perkins, Perkins, Chen, and Vyas (1972) noted about 2-5 times higher false positivity amongst hospital samples compared with those from normal volunteer donors, and while using basically a similar latex agglutination principle for the detection of rheumatoid factor, Caplan (1963) noted a very high degree of reactivity amongst the non-rheumatoid patients compared with blood donors. The increased reactivity in patients' sera may reflect the presence of substances such as rheumatoid factor, heterophil antibody, or species specific substances, whose diverse properties may affect comparisons between normal individuals and patients (Hoq, Cash, Das, and Cumming, 1971). In addition Langenhuysen (1971) demonstrated the presence of antibodies against gamma globulin causing agglutination in the latex fixation test in patients following transfusions and cytomegalovirus infection. One factor responsible for these non-specific reactions in some sera seems to be associated with the IgM fraction (Zulan, Wilson, and Labzofsky, 1972), for three out of five rheumatoid sera which agglutinated both specific and non-specific latex reagents became negative following mercaptoethanol treatment (table V).

With regard to the false positive results, none of the preparations tested appeared to have any advantage over the others. The fact that different latex preparations react falsely with different Au-Ag negative sera may reflect differences in animal species from which the immunoglobulins were derived, and in the methods of coating the latex, all of which may influence the primary structure of the immunoglobulin molecule (Stanworth and Parloe, 1967).

To eliminate this problem heating at 56°C for 30 minutes has been claimed to be effective (Zielenfuss, 1972), but this was not confirmed by our experience. However, our findings do suggest that latex coated with appropriate normal immunoglobulin introduced as 'control' would help to identify spurious positive results, although simultaneous presence of Au-Ag and non-specific agglutinin(s) could cause agglutination of both 'test' and 'control' reagents. Since the positive Au-Ag sera gave 85% agreement between the two manufacturers' reagents tested, this approach might be useful for emergency screening, provided that the reagents are of high quality and sensitivity; the result, however, is available in about five minutes and repeating the test after an absorption procedure with appropriate normal gamma globulin-coated latex could augment the specificity of this reaction. The simplicity and short length of time involved in the latex test for detecting Au-Ag appear to make it an ideal reagent for rapid monitoring of purification stages of Au-Ag containing materials, and this approach has been successfully employed as one of the methods for standardization of the antigen for sensitizing human red cells employed in a passive haemagglutination test (Hopkins and Das, 1973) for detection of Au-Ag and its antibody.

We are grateful to Drs R. A. Cumming and J. D. Cash for constant help and encouragement, to Dr George L. Le Bouvier of the Department of Epidemiology and Public Health, Yale University School of Medicine, for the subtyping study, to Professor B. P. Marmion of the Department of Bacteriology, Edinburgh University, for electron microscopy, to Mr J. M. Leach of Pfizer Ltd, and to Mr D. Evans of Hoechst Pharmaceuticals, for a generous supply of latex agglutination kits. Tests for rheumatoid factor were carried out by Dr M. S. Hoq.
References


A rapid micro-capillary haemagglutination method for detection of Australia antigen (HB-Ag) and its antibody

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A rapid and a simple micro-capillary haemagglutination test is described for screening and identification of HB-Ag and its antibody in sera. While retaining the specificity and very small volume of reagents required it has a similar degree of sensitivity to the microtitre plate method. The reagents are glutaraldehyde pre-fixed human RBCs coupled to beta propiolactone-inactivated HB-Ag, which retain their reactivity for at least 6 months after storage.

Introduction

With the demonstration of the association between Australia antigen (HB-Ag) and Hepatitis B, blood has been screened for its presence by a variety of immunologic methods, including haemagglutination (HA) which requires 1–2 hr for completion of the test and has a similar degree of sensitivity to radioimmune assay (Hopkins & Das, 1973). This communication describes a modification of the conventional HA plate procedure to a micro-capillary system which, while retaining the sensitivity and specificity of the former, has the primary advantage that the tests can be read within 20 min, and also requires smaller amounts of the reagents. Other advantages are that the antigen-coated human erythrocytes may be stored for at least 6 months, and the presence of both HB-Ag and HB-Ab can be detected and confirmed by the same technique. A similar technique has been described for the detection of serum fibrin degradation products by Israels, Rayner, Israels & Zipursky (1968).

Materials and Methods

Human erythrocytes (group O) were fixed with glutaraldehyde and treated with tannic acid according to the procedure of Hoq & Das (1971). Partially purified HB-Ag, treated with beta-propiolactone was coupled to the tanned cells by following the method of Hopkins & Das (1973). A 4% (v/v) suspension of these cells in phosphate buffered saline (PBS) at pH 7.2, containing 1% normal absorbed rabbit serum and Na-azide (1 mg/cm², PBS) was prepared and stored at 4°C in aliquots sufficient for one day's expected requirements. PBS was used as diluent in all experiments and the supernatant of the stored cells replaced with fresh PBS at monthly intervals. Under these conditions the cells retain their reactivity for at least six months for both the capillary and microtitre plate tests. Fixed cells similarly coated with normal pooled plasma, and uncoated cells, were used as controls. In the preliminary experiments 38 samples containing either HB-Ag or HB-Ab were obtained from previously screened blood donors. Of these, 19 were coded and dispersed at random among 18 normal sera (Table 1).

The tests were performed with appropriate laboratory precautionary measures as described in a report by a scientific group of the WHO (1973). Capillary tubes with blunt
ends, 90 mm in length and 0.5 mm internal diameter were selected. The details of the methodological procedures are shown in Fig. 1. During the entire performance of the test the capillary tube was held (while wearing surgical gloves) only at the upper end (U) which remains uncontaminated throughout the procedure. By capillary action test sera, reagents, and control sera enter the capillary tube through the lower end (L). The index finger of the performer rests gently on the upper end of the tube, releasing the capillary lumen at appropriate times, thereby allowing the reactants to reach the desired levels inside the capillary.

The capillaries were marked at intervals of 55 and 60 mm from the lower end. For HB-Ab detection serial dilutions of the test serum were drawn by capillary action to the 55 mm mark [Fig. 1(a)] followed by sensitized cells to the 60 mm mark [Fig. 1(b)]. For the

### Table 1

<table>
<thead>
<tr>
<th>Nature of sample</th>
<th>Test Number</th>
<th>HB-Ag</th>
<th>Results of HB-Ab</th>
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<tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Normal sera</td>
<td>18</td>
<td>0</td>
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<td>18</td>
</tr>
</tbody>
</table>

**Fig. 1. Stages of microcapillary method.** Capillary was held only at the upper end (U) throughout the test procedure. Reagents and tests enter by capillary action through the lower end (L). (a) Sera containing HB-Ab. (b) HB-Ag coated RBC. (c)-(d) Known antibody plus test expelled out of capillary, mixed and refilled via L. (e)-(f) Inversion and embedding of capillary (note RBC now positioned at the top). (f) RBC gravitating down to form negative or positive reaction pattern.
detection of HB-Ag, the antiserum was selected by determining the titration end point of a given serum and using a ten-fold concentration for the test (e.g. the working dilution of an antibody with a titre of 1 in 10 000 would be 1 in 1000). By capillary action the antiserum was drawn to the 55 mm mark [Fig. 1(a)] followed by test serum to the 60 mm mark, then a rubber teat was fitted at the upper end of the capillary and the contents gently expelled to the bottom of a well of a microtitre plate [Flow Laboratory, Irvine] plastic plate [Fig. 1(c)]. The teat was removed and with the capillary held at the upper end, the index finger resting over the capillary lumen (U), the contents were mixed inside the well with the tip of the lower end of the capillary (L) for 10 sec. The finger was removed and the mixture was re-drawn by capillary action to the 55 mm mark [Fig. 1(d)], followed by coated cells to the 60 mm mark [Fig. 1(b)]. After filling the tubes an air gap (30 mm) remained between the column of reagent (inside the capillary tube) and the upper end of the capillary [Fig. 1(b)]. The capillaries were inverted thus the red cells were positioned at the top of the liquid [Fig. 1(e)], and the ends (U) embedded in plasticine fixed to a sheet of translucent glass, tilted to produce an angle of 55° and mounted in a metal viewing box illuminated by a fluorescent lamp [Fig. 1(f)]. Observations were made at intervals of 2-3 min over a period of 10-20 min, after which the results were recorded. As the red cells gravitate down from the top and through the liquid in the capillary, they form their characteristic pattern in approximately 5 min. In the absence of agglutination this takes the form of a well-defined line tapering to a pencil point [Fig. 1(f)]. In the presence of haemagglutination the characteristic 'fir tree' is first formed followed eventually by frank agglutinates [Fig. 1(f)]. Reactions were recorded as + (agglutination), − (no agglutination) and ± (intermediate). For antibody titrations reactions were designated +++, ++ and +.

The presence of HB-Ag was confirmed by further tests using two specific antibodies. Samples showing direct agglutination (antibody) were retested using red cells coated with normal plasma and the specificity confirmed by utilizing the test serum as anti-HB-Ag in an inhibition reaction with HB-Ag positive controls and normal serum. The following controls were incorporated in each batch of tests:

- **Positive:** Anti-HB-Ag with PBS and normal serum
- **Negative:** Anti-HB-Ag with known HB-Ag positive serum
- **Test control:** PBS with test samples
- **Cell control:** PBS with sensitized cells

**Results and Discussion**

Table 1 shows that all 38 samples in the preliminary experiments were correctly identified. Titration of HB-Ab material showed that, when undiluted, some immune sera of animal origin (chimpanzee and rabbit) produced an unusual reaction pattern suggestive of a prozone effect. The cells remained on the surface of the column even after standing for 3 hr, in contrast to the expected negative (non-agglutination) pattern. Human serum containing HB-Ab has not so far shown this phenomenon (Table 2). Comparative titrations of HB-Ab

<table>
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<tr>
<th>HB-Ab</th>
<th>Neat</th>
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</table>

* Not all animal antisera show this effect.
containing materials so far suggest that the capillary method as described has 'one tube' of advantage over the plate procedure, but by comparison with doubling dilutions of known HB-Ag the capillary method is shown to be slightly less sensitive (1 tube difference). This is consistent with our experience in the determination of serum fibrin degradation products (Hoq & Das, 1971) and is probably related to the larger volumes and to the higher concentrations of antisera used in the capillary method. The sensitivity of the micro-capillary test for the detection of antigen could be increased by pre-incubation of equal volumes of test serum and antibody, drawing the mixture to the 55 mm mark followed by cells to the 60 mm mark, or by reducing the concentration of antibody. For antigen screening however, larger volumes of antisera were used to improve definition. The test does not require costly equipment and even some of the items described above could be simplified. Its simplicity, rapidity, and sensitivity render it superior to the rapid, but problematic latex test (Hopkins & Das, 1974b) for emergency requirements involving a limited number of samples. The red cells are capable of HB-Ag subtyping into ad and ay varieties and can be used for this purpose in the analysis of the specific antibody content of HB-IgG preparations (Hopkins & Das, 1974a).

We are grateful to Dr R.A. Cumming for his encouragement and for reading the typescript, to Dr J. D. Cash for his interest, and to Dr J. E. Maynard, Phoenix Laboratories, Arizona, U.S.A., for the gift of chimpanzee antisera.

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