IMMUNOADSORBENTS FOR PROTEIN
PURIFICATION AND RADIOIMMUNOASSAY

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by

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Summary

A variety of methods for preparing human immunoglobulin G (IgG) specific immunoadsorbents have been compared. All preparations have been based on cellulose and its derivatives, and the suitability of this polysaccharide as a carrier has been investigated.

The relative efficiencies of different protein immobilisation procedures have been assessed with particular reference to the success and practicalities of the carrier activation procedure and the capacity to couple antibody protein. Once immobilised the activities of the antibodies were measured in terms of the capacity for antigen using radioimmunoassay procedures and comparisons were made with free antibody. A number of different eluting agents were used to recover antigen, and the relative effectiveness of these agents was determined. The most efficient immobilisation procedures were those involving coupling of the antibody to diazotised N-(3-aminobenzylxoxymethyl) cellulose, glutaraldehyde activated aminoalkyl cellulose or bromoacetamidoalkyl cellulose. These methods were used to make immunoadsorbents for use on a preparative scale and in solid-phase radioimmunoassay.

Immunoadsorbents were used as the basis of sensitive and specific radioimmunoassays for IgG, IgD, insulin and human placental lactogen.

Immunoadsorbents specific for human IgG were used in various purification protocols involving different combinations of batch and column immunoadsorption, washing and elution. The findings were then applied to the purification of IgD from both myeloma and normal sera. The IgD was characterised both physically and immunochemically.
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CHAPTER ONE

Introduction
Introduction.

Some of the most exciting immunological techniques to evolve in recent years are those involving immunoadsorbents. These are insolubilised preparations of antibodies or antigens used for the specific adsorption of antigens or antibodies respectively. The concept was first put into practice by Campbell, Luescher and Lerman (1) who prepared an immunoadsorbent by linking the antigen bovine serum albumin (BSA) to a carrier, p-aminobenzylcellulose. This particular immunoadsorbent was employed to remove the specific antibody to BSA from rabbit antiserum. Subsequently, the potential of immunoadsorption for both antibody and antigen purification has come to be realised.

Specific antibodies can be removed from whole antisera with the appropriate insolubilised antigen, and conversely antigens can be purified from mixtures using insolubilised forms of specific antisera. Other applications include the simple agglutination reactions used for the detection of human chorionic gonadotrophin in urine as being indicative of pregnancy; removal of cross-reacting antibodies from antisera to render them mono-specific; and the estimation of antibody and antigen levels either by adsorption and subsequent elution or by solid-phase radioimmunoassay.

The preparation of immunoadsorbents by immobilising antibodies or antigens allows for the conservation of valuable materials which can be used repeatedly, as well as imparting increased stability to the molecular structure.

The number of published methods of immunoadsorbent preparation continues to grow and would seem to indicate that no one technique has
asserted itself as a good all round method.

There are four basic approaches to the problem of making a soluble antigen or antibody insoluble:- covalent coupling to a support; physical adsorption on to a support; trapping within a lattice; or cross-linking the material with itself or a similar "sacrificial" substance. The introduction of the glutaraldehyde polymerisation method by Avrameas and Ternynck (2) was considered by many to be potentially the most satisfactory method of immunoadsorbent preparation. Similarly, some methods using covalent coupling to polysaccharides have found wide acceptance, particularly the cyanogen bromide method of Axen, Porath and Ernback (3). However, the relative merits of various immunoadsorbent preparations are rarely considered for particular applications, and it is this that has been attempted in the work for this dissertation.

Antibodies to human immunoglobulin G (IgG) have been used as a model in the preparation of immunoadsorbents to study the relative efficiencies of these adsorbents for immunoglobulin purification. Conclusions drawn from this study have been applied to the purification of immunoglobulin D (IgD) from myeloma and 'normal' human sera. Furthermore, solid-phase indirect radioimmunoassays have been developed for immunoglobulins, insulin and human placental lactogen.

a) Immunoadsorbents

The word 'immunoadsorbent' was originally coined to define an insolubilised antigen preparation, but in recent years it has come to describe both antigen and antibody preparations. Campbell, Luescher and Lerman (1) pioneered the technique with insoluble protein antigens which would combine specifically with the antibody, and referred to them as immunologic adsorbents.
Immunoadsorption requires either an antiserum showing specificity for the particular antigen to be purified or, if antibody purification is to be attempted, pure antigen. The protein is first immobilised by linking it to a suitable matrix or trapping it in a lattice, then any protein which has not reacted is washed off. Unreacted sites on the matrix are blocked by reaction with suitable compounds. For the purpose of protein purification the immunoadsorbent is either suspended in the biological fluid containing the protein of interest, or else the fluid is passed through a column packed with the adsorbent. Specific antibody-antigen binding then occurs while all non-specific proteins remain free to be washed from the adsorbent by a suitable buffer. The immunochemically linked protein is then eluted using a solution of suitable pH and/or ionic strength (Fig. 1.2)

Three different approaches may be made to radioimmunoassay using immunoadsorbents. The more generally used method employs insolubilised antibodies, and the assay depends upon the ability of the antibody to bind radio-actively labelled antigen, and the competitive inhibition of this reaction by unlabelled antigen. The antibody-bound antigen is then separated from the unbound by centrifugation. The more specialised immunoradiometric assay developed by Miles and Hales (4) involves the use of radioactively labelled antibodies. Antibodies are isolated on an immunoadsorbent composed of antigen bound to cellulose, labelled with radioactive iodine and then eluted from the adsorbent. An excess of this antibody is incubated with antigen standards and unknowns, and the amount of antibody-antigen complex formed is proportional to the antigen concentration. An antigen immunoadsorbent is then added to remove excess antibody. After centrifugation the supernatant is sampled for measurement of radioactivity.
Fig. 1.1.

Structure of the IgG molecule. a = N-terminal ends, b = C-terminal ends, I = antigen-binding sites.

Fig. 1.2.

Preparation of immunoadsorbents. The activation step may involve more than one reaction.

Immunoadsorption, and elution of the specifically adsorbed protein.
Sandwich techniques employing antibody and antigen immunoadsorbents are used to assay antibody and antigen respectively. The antigen to be assayed is incubated with the appropriate antibody immunoadsorbent, and this is then washed. Radioactively labelled antibodies are then added and these react with the antigen bound to the antibody on the solid matrix. The antigen must be able to bind to two antibodies simultaneously. The uptake of labelled antibody is directly proportional to the amount of antigen present. A similar rationale applies to the assay of antibodies. A more detailed discussion of these assays is included in Chapter 3.

b) Antisera

Although apparently a powerful technique, immunoadsorption presents many problems. The first of these is in obtaining a suitable specific antiserum, or a very pure sample of the antigen. The antiserum of choice must be mono-specific and cause the formation of antibody-antigen complexes that are readily dissociable. Proteins of molecular weight greater than 5000 are usually good immunogens, but smaller molecules may have to be linked to a carrier protein such as bovine serum albumin. A single injection of a particular immunogen will initiate a primary response made manifest by the appearance of antibodies after a lag period. The antibody concentration rises to a maximum and then slowly declines. A booster injection of immunogen at this time will be followed by a transient negative phase due to mopping up of circulating antibodies and then an anamnestic response leading to a higher peak of antibody concentration. Repeated injections at suitable time intervals will lead to the production of a maximum level of antibody which will vary from one individual to another. The primary response to an immunogen initially results in the synthesis of 19 S immunoglobulin M (IgM).
Since IgM is less stable than IgG it is not as suitable for immuno-adsorbent preparation. IgG is synthesised in preference to IgM as the primary response progresses (5). Najjar (6) has suggested that this change over from IgM to IgG synthesis may be because the body responds differently to antigen-antibody complex than to antigen alone. Although the insolubilisation will probably confer greater stability upon the IgM antibodies, antibodies from the IgG class are generally used due to their inherent stability and the fact that they are more easily obtained.

A protein as large as human IgG, which has a molecular weight of 160,000, will possess several antigenic determinants of different immunogenicity. Consequently an antiserum raised to such a protein will contain several antibodies showing specificity for different antigenic determinants. Further, the free binding energies of the different antibody molecules for any one determinant are distributed according to the Gauss error function (7). The most weakly reacting antibodies probably do not reflect the true structure of the antigenic determinants and are often referred to as being the least avid, whilst the most strongly reacting antibodies have the greatest avidity for the antigen. When using immunoadsorbents for preparative purposes the avidity of the antibodies should ideally fall between the two extremes. Avidity can be measured by means of a Scatchard plot (8) derived from a radioimmunoassay standard curve. A plot of the ratio of antibody-bound to free antigen against bound antigen (moles litre\(^{-1}\)) gives a linear response, the slope of which represents the avidity of the antibody. Immunoadsorbents prepared with weakly reacting antibodies are liable to suffer from non-specific adsorption problems, whilst with highly avid antibodies it will be very difficult to effect dissociation of the antigen once it has reacted with the specific antibody. Another point
to be borne in mind is that of early and late antibodies (9). Late antibodies from hyperimmune sera may show reactivity with antigen determinants not exhibited by sera taken from the same animal on a previous occasion. The antisera often become less specific as an immunisation course progresses (10). The antiserum obtained from an animal very often shows cross-reactions to other proteins, therefore before using the antiserum to prepare an immunoadsorbent the cross-reactions must be removed. This is achieved by either liquid adsorption* or by use of an immunoadsorbent prepared from the cross-reacting proteins (11).

c) Types of Immunoadsorbents and Support Materials

The methods available for either coupling proteins to insoluble supports, or trapping or polymerising them are numerous and diverse, reflecting the fact that no one technique is generally applicable to all systems. One can only ascertain which system is best suited to a particular application by testing each one in turn and assessing it according to a set of established criteria.

There are basically four approaches to the problem of protein insolubilisation: physical adsorption on to a carrier, covalent bonding to a carrier, trapping within a lattice structure and polymerisation.

Immunoadsorbents prepared by physical adsorption of antibody to a carrier are unsuitable for preparative work because conditions of pH and ionic strength which will dissociate the protein from the carrier are almost certain to be encountered. However, such preparations do find applications in the field of immunological tests and assays. Polystyrene latex particles have been used in a passive agglutination test for the rheumatoid factor (12), and more recently in a number of

* The cross-reacting protein is added to the antiserum, and the immunoprecipitate formed on incubation is removed.
pregnancy diagnostics. In the latter case, latex particles are sensitised with human chorionic gonadotrophin (HCG). Addition of antiserum to HCG will cause agglutination. If urine containing a significant level of HCG is added to the antiserum it will neutralise the antibodies and so prevent agglutination. Hagiwara (13) used latex particles coated with antigen to bind specific antibody, and after centrifuging and washing the particles he dissociated the latex-antigen-antibody complex. Other carriers used for physical adsorption include tanned (14) or formalised (15) red blood cells, glass beads (16), and bentonite, which has been used for solid-phase radioimmunoassay (17).

Preparations involving trapping of the antibody molecules within a lattice structure, such as that formed by polyacrylamide, also have their shortcomings. Provided that the antibody molecules cannot escape through the pores of the lattice-work and that the antigen is small enough to enter, the immunoadsorbents should perform satisfactorily. These problems were overcome by Carrel and Barandun (18) who used a macroporous gel to prepare a number of immunoadsorbents of high capacity, and by Carrel et al (19) in isolating normal immunoglobulin E. It is possible that the protein is covalently coupled to the polyacrylamide gel!

Protein polymers are easily made by reaction of protein with cross-linking reagents such as ethylchloroformate (20), glutaraldehyde (2), and bis-diazotised benzidine (21). Disulphide-linked antibodies have been prepared successfully by thiolating the protein with N-acetyl homocysteine thiolactone and then cross-linking with a potassium ferricyanide reagent (22, 23). Bifunctional organomercurial compounds may also be used to cross-link through thiol groups (24).
Methods of coupling proteins to insoluble carriers through covalent bonds account for the majority of immunoadsorbent preparations. Several of these methods were originally developed for the preparation of insolubilised enzymes, but have proved to be equally useful for immunoadsorbent preparation. Linking of antigens or antibodies to a suitable carrier by covalent binding eliminates the danger of desorption during elution. The most commonly used insoluble carriers are cellulose and its derivatives, beaded agarose, Sephadex, beaded polyacrylamide, ethylenelmaleic anhydride copolymer, polystyrene, nylon and glass. The applications of these carriers will be discussed in subsequent sections.

d) The choice of support

Immunoadsorbents may be used either in columns or in a 'batchwise' procedure (2) which involves stirring the adsorbent in suspension and recovery by centrifugation. Ideally, adsorbents used in columns should have a loose, porous network which permits uniform and unimpaired entry and exit of macromolecules. The adsorbent particles should be of uniform size, spherical and rigid (25), so imparting good flow properties to the column. Cellulose is not ideal in these respects, but it does form columns with acceptable flow rates. Weliky and Weetail (26) prepared immunoadsorbent columns based on carboxymethylcellulose for the study of antigen-antibody interactions. Boegman and Crumpton (27) concluded that myoglobin immunoadsorbents prepared from aminoethylcellulose and carboxymethylcellulose were suitable for column applications. The very small particle size of many cellulose derivatives causes clogging of columns, but this problem has been overcome by mixing the immunoadsorbent with swollen Sephadex G-25 (28,29). The Sephadex serves not only as a solid diluent but also as a molecular sieve, a property which has contributed to its success as an immunoadsorbent support in its own right (30). Beaded
agarose (Sepharose) comes even nearer to the ideal because of its loose network (30), & beaded polyacrylamide (Biogel) also shows promise (31). Glass beads were used by Baum, Ward and Weetall to couple acetylcholinesterase (32), and are now used as the basis of antibody-immunoadsorbents in certain radioimmunoassay kits. Polyaminostyrene was used by Williams and Kunkel (33) for the insolubilisation of rabbit and human IgG, and has also been used to prepare insolubilised enzymes, but references to its use are few and far between.

As pointed out by Porath (30), and Hornby, Lilly and Crook (34), it is very important to make allowance for fixed charge groups on the support material. Hydrophobic carriers such as polystyrene may tend to denature the covalently linked protein, while hydrophilic carriers may stabilise its configuration. The hydrophilic nature of hydroxyl-containing carriers such as agarose may be enhanced by reaction with polyhydric phenols or alcohols and epichlorohydrin (35). Strongly anionic or cationic carriers will produce electrostatic interactions with the insolubilised protein and so influence its stability and activity. Further, such carrier properties can be expected to affect the optimum pH of the antibody-antigen reaction and its sensitivity to temperature and ionic strength. Hornby, Lilly and Crook (36) studied insolubilised ficin preparations with oppositely charged supports, and concluded that charge-charge interactions between substrate and support strongly influenced the enzyme's activity. Since a charged support has the ability to adsorb proteins with an opposite charge, such physical adsorption may occur during either the coupling reaction or immunoadsorption (37). The chemical nature of the support may also affect its affinity for a given protein and hence its ability to covalently bind that protein. Weliky and Weetall (26) studied the influence of carboxymethylcellulose as a
support material on some antigen-antibody reactions. The adsorbent contributed strongly to the interaction of ionisable haptens with antibody, with a pH dependency typical of acid-catalysed reactions. Dissociation of antibody from haptenic and protein immunoadsorbents showed no significant discontinuities in the acid range. Antibodies to haptens which did not ionise in acid did not dissociate from the immunoadsorbent at low pH, but did so at high pH. Unmodified and hapten-substituted carboxymethylcellulose exhibited non-specific adsorption of gammaglobulin and albumin between pH 3.0 and 4.0. Hydrogen bonding to hydroxy groups in the cellulose was considered to be an important factor, and this could only be prevented by using highly ethylated or acetylated carboxymethylcellulose. After such modification, non-specific gammaglobulin was not retained by the adsorbent column between pH 2.0 and 7.0.

The surface area of the support plays a major role in determining the amount of protein which may be covalently bound, and its subsequent stability. By dissolving m-aminobenzyloxymethylcellulose in a cuprammonium solution and then reprecipitating it by the addition of water followed by rapid cooling and addition of 10% sulphuric acid to discharge the blue colour, Gurvich et al (38) obtained a finely divided cellulose derivative. After diazotisation the reprecipitated cellulose bound in excess of 500mg of protein per gram as compared with 50mg per gram for the untreated cellulose. Jagendorf et al (39) chose cellulose as a solid phase because of its enormous surface area and its hydrophilic nature which serves to minimise the degree of non-specific adsorption one would expect from a hydrophobic support.

Silman and Katchalski (37) predicted that functional groups closely attached to the backbone of a polymeric carrier should display lower
reactivity than similar groups situated on the ends of long, flexible, hydrophilic side chains. Therefore by employing spacer side-chains it should be easier to couple protein to the carrier, and in greater amounts. Cuatrecasas (25) pointed out that attaching proteins to a carrier in this manner should help in overcoming steric difficulties when interactions with macromolecules are studied. This is more likely to be the case when low molecular weight antigens are insolubilised for immunoadsorption of their respective antibodies than when the roles are reversed.

Following the covalent coupling of an antigen or antibody to an insoluble support it is often necessary to block any unreacted groups on the carrier. Unreacted diazo-groups are commonly blocked by \(\beta\)-naphthol. Weetall and Weliky (40) followed such a procedure after coupling BSA to diazotised \(p\)-aminobenzylcellulose and arylaminocellulose derivatives with different levels of free amino groups. The retention and subsequent release of non-specific protein by these immunoadsorbents appeared to be dependent upon the number of \(\beta\)-naphthol groups present. One gram of the \(p\)-aminobenzylcellulose derivative retained 0.10 mg of non-specific protein per ml of serum passed over the adsorbent, whereas the arylaminocellulose derivative which has twice the number of \(\beta\)-naphthol groups retained twice the quantity of non-specific protein. The possible influence of unmodified amino groups was not considered. An immunoadsorbent prepared by direct coupling of BSA through an amido link to a carboxymethylcellulose in the presence of dicyclohexylcarbodiimide required no blocking agent, and retained only 0.011 mg of non-specific protein per ml of serum passed over one gram of the adsorbent. Following diazotisation of polyaminostyrene and subsequent reaction of the diazo groups with \(\beta\)-naphthol, Yagi et al (41) found that antigen was bound to the carrier
by physical adsorption. By decreasing the number of amino groups in the polyaminostyrene-based immunoadsorbent the affinity of the antigen for its antibody was increased. This suggested that any charge on the carrier attributable to the amino groups influenced antibody-antigen binding. Appreciable amounts of non-specific protein were released with the antibody on elution (42).

Ethanolamine and propanolamine have been employed to block any excess of reactive groups occurring in cyanogen bromide activated polysaccharides and bromoacetyl derivatives. In this way hydroxyl groups are restored to the carrier.

Amino acids are employed as blocking agents for groups exhibiting specificity for amino or carboxyl functions. However, it must be borne in mind that their use will introduce ionisable groups to the carrier which may have the disadvantage of causing non-specific protein adsorption. In a few cases serum albumin has been used to quench unreacted groups on the carrier, as for example, after coupling immunoglobulins to cellulose carbonate (43).

e) Methods for Coupling Proteins to Insoluble Carriers by Covalent Bonds

The chemistry and use of cellulose derivatives is largely covered in a comprehensive paper by Weliky and Weetall (44). Reviews of methods available for covalent bonding of proteins to insoluble supports include those by Silman and Katchalski (37), Sehon (42), Campbell and Weliky (45), Goldman et al (46), Cuatrecasas (25), Cuatrecasas and Anfinsen (47) and Cuatrecasas (48).

The functional groups available in proteins for covalent bond formation are the N-terminal and ε-amino groups, C-terminal, β- and γ-carboxyl groups, the hydroxy groups of serine and threonine, the phenolic
ring of tyrosine, the thiol group of cysteine, the imidazole group of histidine, the indole group of tryptophan and the guanidino group of arginine.

The first reference to the preparation of an immunoadsorbent by covalent binding of an antigen to a support was that employing bis-diazobenzidine to couple ovalbumin to sheep erythrocytes (83). The coupling of protein to cellulose was also effected through a diazo bond by Campbell et al (1) and subsequently by Gurvich et al (38). They prepared p-aminobenzylcellulose and m-aminobenzyloxymethylcellulose respectively by etherification reactions of cellulose and diazotised these derivatives by reaction with hydrochloric acid and sodium nitrite at 0°C. Mildly alkaline pH favours reaction with the phenolic ring of tyrosine and amino groups, while at pH 6.0 tyrosine reacts more slowly and the imidazole groups of histidine may also react. An advantage gained with diazonium salts is that they are able to react with proteins under a range of conditions. Comparable coupling levels are obtained between pH 6.0 and 9.0, and between 0°C and 37°C (49). An aminoarylcellulose, derived by coupling benzidine to carboxymethylcellulose in the presence of N,N'-dicyclohexylcarbodiimide (40), was diazotised and coupled with rabbit anti-human IgG (50). Diazo coupling was employed in the linking of α-amylase to 3-(p-aminophenoxy)-2-hydroxypropyl ethers of cellulose with different degrees of substitution (51). The lower the substitution level the higher was the retention of α-amylase activity. This effective loss of activity at higher substitution levels was more marked when coupling at pH 6.3-6.4 than at 7.6-7.7. It was considered probable that at pH > 7.0 coupling took place via tyrosine residues, whereas at pH < 7.0 coupling via histidine and tryptophan residues was of importance.

Sehon (52) bound albumin and human IgG to polyaminostyrene by a diazo bond, and the method has been used in numerous other applications.
Marked variations in the physical and chemical properties of polyaminostyrene result in immunoadsorbents with considerably different properties. Bovine serum albumin was covalently coupled to a diazonium salt derivative of polyacrylamide to the extent of 300 mg per gram of acrylamide (31). Diazonium-Sepharose derivatives (25) were prepared by treating amino-Sepharose with p-nitrobenzyol azide in 50% dimethylformamide, followed by reduction with dithionite and diazotisation with nitrous acid. Coupling of the diazo-group to the protein occurred through imidazole and phenolic groups of the protein. Blocking of unreacted diazo-groups, following the coupling of the protein, was achieved by reaction with β-naphthol forming a red azo dye.

Enzymes have been coupled to carboxymethylcellulose by modifications of the method of Michael and Ewers (53), which involves the synthesis of carboxymethylcellulose hydrazide and its conversions to the azide for reaction with the protein. The technique has been applied to the insolubilisation of chymotrypsin (54), alkaline phosphatase (55) and ficin (34). Bovine serum albumin was coupled to an acyl azide derivative of beaded polyacrylamide, and the product contained 78 mg protein per gram (31). Unreacted azide groups were reconverted to the amide by the addition of either ammonium hydroxide or hydrazine and ammonium chloride, to the reaction mixture. The acyl azide group can react with amino, hydroxyl and thiol groups of the protein by nucleophilic substitution producing amide, ester and thioester bonds respectively.

Esterification of cellulose by bromoacetic acid and bromoacetyl bromide yields bromoacetylcellulose (39, 56). Proteins are coupled by an alkylation reaction via their amino groups at pH 8.5-8.9. Coupling also occurs via the phenolic and imidazole substituents. The weakness

* See Fig. 1.3, p.21.
of the method lies in the labile ester linkage produced in the first reaction. Jagendorf et al (39) employed an initial physical adsorption step at pH 4.6 to immobilised rabbit IgG. Covalent bonding was effected by adjusting the pH to 8.5. Approximately 98% of the protein was linked to the cellulose support, equivalent to 400 mg of protein per gram of bromoacetylcellulose. Blocking of the free bromoacetyl groups was achieved by reaction with ethanolamine.

Kay and Lilly (57) favoured the use of coupling agents which react with hydroxyl groups on the support. It was then possible to choose the net electrostatic charge on the insoluble support by using such materials as DEAE- or CM-cellulose. Cyanuric chloride (2,4,6-trichloro-s-triazine) and various of its derivatives were employed in the preparation of insolubilised enzymes. It was concluded that 2-amino-4,6-dichloro-s-triazine was the most convenient and produced the best results. One of the chlorines reacts with an hydroxyl on the cellulose giving a monochloro-s-triazinyl cellulose. The second chlorine reacts with stronger nucleophiles such as amino groups of proteins. Chymotrypsin was coupled to various support materials at pH 8.75.

Activation of hydroxylated polymers can readily be achieved by reaction with divinylsulphone which simultaneously introduces a side chain on to the support. The active vinyl groups are some considerable distance from the base polymer and will react with protein amino groups in the pH range 8-10 (35). Pretreatment of the polymer with polyhydric phenols or alcohols and epichlorohydrin was advocated in order to increase the number of available hydroxyl groups, some of which are in sterically more favourable positions and possess higher reactivities.

* See Fig. 1.3, p.22.
Studies of the cyanogen halide activation of polysaccharides (58), cellulose, Sephadex and Sepharose in particular, have shown cyanogen bromide to be the most satisfactory activator. Procedures based on this method are now widely used for both immunoadsorption and affinity chromatography. Activation results in the substitution of imidocarbonate esters and carbamate groups into cellulose and Sephadex. However, Sepharose has no vicinal hydroxyl groups and therefore imidocarbonate groups are not formed. The degree of activation can be controlled by varying the reaction pH, activation being maximal at pH 11 or above. The method of protein coupling is gentle and therefore particularly useful for immunoadsorbent preparation. A slightly alkaline pH is preferable.

Cyanogen bromide has been used as the first step in the preparation of various polysaccharide derivatives (25, 59). Aliphatic amines of various chain lengths were substituted directly into the activated polysaccharide giving rise to ω-aminoalkyl derivatives. Proteins were linked to these through their carboxyl groups in the presence of carbodiimide. Trypsin was coupled to aminoethylcellulose with the bifunctional reagent glutaraldehyde at pH 7.0 (60). Carboxylic acid derivatives, prepared by reacting ω-aminoalkyl derivatives with succinic anhydride at pH 6.0, coupled through the amino functions of proteins in the presence of carbodiimide. This procedure has been used extensively for coupling proteins to carboxymethyl cellulose (26, 40, 51, 61, 62), and to an aminocellulose derived by reacting carboxymethylcellulose with a diamine in the presence of a carbodiimide (61). Bromoacetyl polysaccharides were prepared by the action of O-bromoacetyl-N-hydroxy succinimide on the ω-aminoalkyl derivatives (25), and the sulphydryl derivatives by reaction with N-acetylhomocysteinethiolactone. Proteins couple to thiol groups in the presence of carbodiimide, through thiol ester bonds.

* See Fig. 1.3, p.22.
The method of activation of aminoethylcellulose mentioned above, was also employed to activate polyacrylamide beads for the preparation of immunoadsorbents (63). Various proteins, including rabbit, sheep and human IgG and BSA, were immobilised by coupling them to the activated polyacrylamide at pH 7.4. The quantity of protein coupled was of the order of 1 to 2 mg per 1 ml of beads. Unreacted aldehyde groups were subsequently blocked by reaction with lysine.

Cellulose carbonate has several characteristics in common with the cyclic imidocarbonate of cellulose prepared by activation with cyanogen bromide. Immunoadsorbents are relatively simple to prepare, covalent coupling occurring by nucleophilic substitution under relatively mild reaction conditions via terminal- or ε-amino groups in the protein. The preparation of cellulose carbonate was described by Barker et al (64), and covalent coupling of β-D-glucosidase was effected at pH 7.8 (65). Catty et al (44) have since employed the cellulose carbonate to prepare immunoadsorbents by coupling rabbit and human immunoglobulins. The effect of charged groups on the carrier has been demonstrated by Gray and Yeo (66) with DEAE-cellulose carbonate coupled enzymes. At the coupling pH the protein, β-D-glucosidase, was adsorbed on to the DEAE groups and then covalently linked by reaction between amino groups on the protein and the cyclic carbonate groups.

Barker et al (67) reported the formation of titanium-cellulose chelates by reaction with titanous or titeanic chloride. The ions bind six molecules of water which are easily replaced by other ligands such as proteins. The titanium chelates were considered to be particularly suitable for the insolubilisation of enzymes, especially those with poly(n-acryloyl-4 and -5-aminoalicyclic acid) which have the advantage of being non-biodegradable (68).
Another insolubilisation procedure which has application to immuno-
adsorbent preparation, is the oxirane method (69). Sepharose was
activated with 10% epichlorohydrin in 1 M sodium hydroxide at 60°C for
2 hours, and after exhaustive washing the protein was coupled in 0.5 M
bicarbonate buffer. Unreacted groups were subsequently blocked with
ethanolamine.

A simple method for activation of polysaccharides was described by
Sanderson and Wilson (70). Cellulose, Sephadex and Sepharose were
oxidised with sodium periodate, and the aldehyde groups thus generated
reacted with the amino functions of BSA. Cellulose bound 25 mg of
protein per gram following treatment with 0.1 M periodate for 25 hours.
Sephadex G-75 (fine) was degraded by 0.1 M periodate. Treatment for
1 hour with a 0.1 M solution of periodate had no visible effect, and
1 gram of the product coupled approximately 190 mg of protein. Unreacted
groups were reduced with a 1% solution of sodium borohydride.

Reaction of the p-aminophenoxyhydroxypropyl ether of Sephadex with
thiophosgene produces the corresponding isothiocyanate (71). Oxytocin,
insulin, glucagon and IgG were coupled to this derivative, IgG to the
extent of 34 mg per gram.

Organic isocyanides have been used to couple α-chymotrypsin to
CM-Sephadex and periodate-oxidised Sepharose (72). 3-Dimethylamino-
propylisocyanide, acetaldehyde (not in the case of oxidised Sepharose) and
chymotrypsin were mixed with the insoluble carrier and the reaction
maintained at pH 6.5. The CM-Sephadex preparation contained 345 mg of
the enzyme per gram, but only 8% of the original activity was retained.
The Sepharose derivative, whilst containing only 20 mg of protein per gram,
possessed 50-60% of the original activity.
Hydrophilic copolymers based on polyacrylamide are marketed under the trade name Enzacryl. Aromatic amino residues of Enzacryl AA may be activated either by diazotisation or by conversion to isothiocyanate groups by thiophosgene. Coupling to the protein is achieved through tyrosine and imidazole groups in the first instance and through amino groups by formation of a thiourea in the second instance. An Enzacryl acid hydrazide may be converted to the azide, by the method of Michael and Ewers (53), which then reacts with protein amino groups with formation of amide bonds. The syntheses and some of the applications of such polyacrylamide derivatives have been reported by Inman and Dintzis (31).

An alkylaminosilane derivative of porous glass, obtained by reacting the glass with aminoalkyltriethoxysilane in toluene (73), has been used to immobilise protein. This was achieved after activation of the aromatic groups either by diazotisation or by conversion to the isothiocyanate with thiophosgene. The porous glass was found to lend itself well to column chromatography giving very good flow rates.

Centeno and Sehon (74) prepared 'versatile and high capacity' immunoadsorbents by coupling antibodies or antigens to ethylene maleic anhydride copolymers. The anhydride reacts with amino groups forming an amide linkage and a carboxyl group. Goldstein (75, 76) converted the copolymer to a polyamine by reacting it with 1,6-diaminohexane in the presence of N,N'-dicyclohexylcarbodiimide. Proteins were then coupled through either their carboxyl groups in the presence of a water soluble carbodiimide or their amino groups after activation of the carrier with glutaraldehyde.

The use of glutaraldehyde as a protein cross-linking reagent (2,77) has given rise to another widely used type of immunoadsorbent. These can
be made either by direct polymerisation of the protein in solution or by linking the protein to a carrier polymer prepared by cross-linking BSA with glutaraldehyde. Different proteins exhibit different pH optima for polymerisation by glutaraldehyde and ethylchloroformate (20), a property associated with the ionisation state of the amino groups.

An alternative method of protein polymerisation was reported by Stephen, Gallop and Smith (22) involving disulphide linkages. Antibodies were thiolated by reaction with N-acetylhomocysteine thiolactone at pH 10.6. Cross-linking was achieved with a mixture of potassium ferricyanide, ammonia and ammonium chloride. Alternatively organo-mercurial compounds may be used to effect the cross-linking.

This section has out of necessity included only the briefest details of the methods of protein immobilisation involving covalent bond formation. However, it is felt that the list of methods presented is comprehensive, omitting only those which involve minor modifications to those already described.

\[\text{Fig. 1.3}\]

**Diazotisation**

\[\text{cellulose-O-CH}_2\text{-C-OH} \xrightarrow{\text{CH}_2\text{N}_2} \text{-O-CH}_2\text{C-O-CH}_3\] diazomethane

\[\text{NH}_2\text{NH}_2 \xrightarrow{\text{Hydrazine}} \text{-O-CH}_2\text{C-NH-NH}_2\] hydrazine

\[\text{O} \xrightarrow{\text{HNO}_2} \text{nitrous acid}\]

\[\text{-O-CH}_2\text{C-N}_3\] azide

Conversion of carboxymethyl cellulose to the azide.

\[\text{PROTEIN} \xrightarrow{\text{pH 8-9}} \text{-O-CH}_2\text{C-PROTEIN}\]
f) The Efficiency of Protein Immobilisation and Retention of Immunological Activity

The degree and efficiency of covalent bonding of proteins to cellulose and other insoluble carriers is dependent upon the concentration of the protein and the physical and chemical properties of both the protein and the carrier. The amount of protein coupled is restricted by the number of active groups on the carrier until the surface area of the latter becomes a limiting factor. By reprecipitation of aminocellulose from an ammoniacal copper solution, Gurvich et al (38) obtained a cellulose preparation with a much reduced particle size and consequently greater surface area. This treatment greatly increased the capacity of the aminocellulose for protein. For some applications, such as the 'batchwise'
extraction of protein from a small volume or the processing of large volumes through columns, it may be desirable to have a high protein: carrier ratio, provided that the protein retains a high percentage of its original capacity for antigen or antibody.

Most proteins, provided that they are of sufficiently high molecular weight, have more than one antigenic site. Glucagon, for example, with a molecular weight of 3485 and 29 amino acid residues, has been shown to have two antigenic sites (78). Some antigenic sites may be masked by the process of immobilisation, but there is a good chance that others will still be available to react with antibody. An antiserum raised to such an antigen will contain a heterogeneous antibody population with antibodies specific for all the antigenic determinants.

The antigen binding sites of an antibody are localised at the N-terminal ends of the heavy and light chains. If immobilisation involves covalent linking with amino acid residues in this region of the molecule the antigen binding sites may be masked with a complete loss of the ability to bind antigen.

The ease with which protein can be coupled to a carrier may be an important consideration. The conditions should be kept as mild as possible as regards pH, ionic strength and the use of organic solvents so as to minimise the chances of denaturation. Typical carriers which enable these conditions to be fulfilled are bromoacetylcellulose and cellulose carbonate, both of which have the added advantage that they can be stored and used without the need for further activation. Such properties would prove attractive if immunoabsorbents had to be prepared quickly.
The reactivities of various functional groups at different pHs' has been considered in the previous section. The importance of the choice of coupling pH has been demonstrated for insulin and insulin antibodies. Cuatrecasas (79) found that reaction of insulin with cyanogen bromide-activated Sepharose at pH 6.5 favoured coupling via the terminal amino group of the B chain (phenylalanine), while reaction at pH 9.0 lead to coupling via the ε-amino group of the B29 lysine residue. Both preparations adsorbed antibodies. Sheep antiporcine insulin antibodies coupled to Sepharose at pH 6.5 bound 80% of their theoretical capacity for insulin. However, when the coupling reaction was carried out at pH 9.5 only 7% of this capacity was available (25). This last figure is in good agreement with the findings of Bolton and Hunter (80) who recovered 6.2% of the original antigen binding capacity as measured with iodinated insulin. At pH 9.5 covalent coupling is likely to occur through the lysine residues. The considerable loss in activity would seem to indicate that either the lysine residues concerned are located in or near the active site, or that the extent of covalent coupling to the carrier is such that it introduces unfavourable steric effects. At the lower pH the reaction with lysine is not favoured.

Cellulose carbonate-IgG immunoadsorbents prepared under weakly acid or neutral conditions contained greater amounts of protein than those prepared at alkaline pH (43). An immunoadsorbent prepared at pH 6.0 retained 38% of the binding capacity of the antigen in free solution as opposed to 45% for an immunoadsorbent prepared at pH 9.0. The immobilisation of IgM on cellulose carbonate did not show a pH optimum between 6 and 10. The products obtained between pH 6 and 8 retained all their capacity for antibody, but this was reduced by more than half for the antigen immobilised at pH 10. These results again point to the dangers to be encountered if one generalises when considering immunoadsorbents. It is important to allow for the
specificities imposed on a system by the antisera, the antigen and the conditions of immobilisation.

Hill (81) found that on repeated re-use, a human serum albumin (HSA) immunoadsorbent bound less antibody, but that a higher percentage of this antibody was recovered on elution. The following hypothesis was put forward to explain these results. If an immunoadsorbent has a high antigen density then antibodies will bond through all their available antigen binding sites. At low antigen densities only a small proportion of these sites will be involved and consequently the antibody is eluted more readily. In the case of the HSA-immunoadsorbent only 53.3% of the antibody was eluted, whilst the remaining 46.7% blocked antigenic sites on the immunoadsorbent, so effectively reducing the antigen density. Subsequent re-use of the immunoadsorbent revealed a decreased capacity for antibody, but increasing amounts of this were recovered on elution. In the fifth adsorption 13.6% of the added antibody was bound of which 79.3% was recovered on elution. These figures compare with 19.5% and 53.3% respectively on the first cycle. A gradual diminution in the capacity of rabbit IgG coupled to cellulose carbonate was observed by Catty et al (43) on re-use of the immunoadsorbent.

An interesting study reported by Nicklin and Smith (82) concerned the recovery of protein antigens from disulphide linked immunoadsorbents derived from non-avid sera. They concluded from a study of human serum albumin, ovalbumin and lysozyme systems that the non-avid sera adsorbed and released antigen 3 to 4 times more efficiently than avid sera. A non-avid serum contained a higher proportion of antibody with a low binding affinity for antigen than did an avid one. The elution conditions necessary to recover antigen from non-avid antibody immunoadsorbents
were less severe than those required with avid antibody immunoadsorbents. Re-use of the non-avid antibody preparations produced recoveries of the order of 80 to 100% of the freshly adsorbed antigen. This was in keeping with the theory that antibodies with high binding affinities were blocked by antigen when the immunoadsorbent was used for the first time. These antibodies could then no longer influence the adsorption and subsequent elution of antigen.

The most widely used method of estimating the amount of protein coupled to an insoluble carrier involves measuring the difference between the initial amount of protein added and that found in the washings. This has its disadvantages in that it involves large volumes of solution which have to be concentrated in order to make accurate determinations of their protein content. More accurate results should be achieved by making direct measurements of the protein in the immunoadsorbent. Kjeldhal (28, 61) and Dumas (56) nitrogen determinations, isotopically labelled proteins (62) and amino acid analysis have been used in this approach. Methods employing specific properties of a protein may be applicable, as for example the estimation of iron as a measurement of the coupling of myoglobin (27). The activities of immobilised antigens and antibodies are measured by their respective capacities for antibody and antigen relative to those in solution, and also in terms of the percentage of adsorbed protein recovered by elution.

Immunoadsorbents are usually stored in suspension at 4°C in a suitable buffer containing a bacteriostat such as sodium azide.
Aims

The project has been undertaken with the object of comparing anti-human immunoglobulin G (IgG) immunoadsorbents prepared from a wide range of cellulose derivatives. The various antibody immobilisation procedures will be compared so as to find which is the most economical in respect of the amount of antibody immobilised and the percentage of total antibody activity retained. Particular methods of antibody immobilisation will be investigated to establish the effects of varying the extent of covalent-bonding between the protein and the cellulose and of introducing spacer arms between the two. The ease with which the antigen can be dissociated from the immunoadsorbent will also be considered. After ascertaining which methods of immobilisation are the most satisfactory, anti-human IgG immunoadsorbents will be prepared in order to study various protocols for IgG purification. The most satisfactory protocols will then be applied to the purification of IgD from both myeloma and 'normal' human sera. Solid-phase radioimmunoassays will be developed for the routine measurement of the immunoglobulins.
CHAPTER TWO

The Preparation, Characterisation and Comparison of Cellulose-Based Antibody-Immunoadsorbents
(a) Introduction

Sheep IgG, containing antibodies specific for the Fc region of human IgG, has been linked covalently to cellulose by a variety of methods. This variety has been achieved by employing several cellulose derivatives, different types and numbers of covalent bonds, and spacer arms between the support and the protein.

The amounts of sheep IgG coupled to the cellulose were measured with the aid of $^{125}$I-labelled IgG and compared for the different methods of preparation. The capacities of the antibody-immunoadsorbent for human IgG were measured in a radioimmunoassay system employing $^{125}$I-labelled human IgG and compared with the values for free antibody. The assay curves were used to deduce the properties of the immunoadsorbents. Subsequently, a study was made to ascertain the ease with which antigen could be recovered from the different immunoadsorbents by elution.

A less extensive study was made of antibody-immunoadsorbents to insulin.

The ideal immunoadsorbent was expected to meet the following criteria:

i) Linkage of the antibody to cellulose should be via covalent bonds.

ii) The immunoadsorbent should be highly insoluble.

iii) The specific antigen should bind to the antibody-immunoadsorbent under conditions in which no other protein is retained.

iv) A high percentage of the adsorbed protein should be recoverable by elution.

v) The immunoadsorbent should be stable with respect to time and eluant.

vi) The physical properties of the immunoadsorbent should lend itself to either column or batchwise purification procedures or radioimmunoassay.

vii) The preparation should be economical, a large proportion of the antibody being incorporated in the immunoadsorbent, and the immunological properties of the antibody should not be affected by immobilization.
(b) Materials and Methods

1. Materials

The chemicals used were Analar grade wherever possible and were purchased from B.D.H. Chemicals, Poole, Dorset, with the following exceptions. Ethylene diamine, 1,5-diaminopentane, 3,3'-diaminodipropylamine, N-hydroxysuccinimide and 1-cyclohexyl-3-(2-morpholonoethyl) carbodiimide metho-p-toluene sulphonate were purchased from Ralph Emmanuel, Wembley, Middlesex. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, papain and bovine serum albumin were purchased from Sigma Ltd., Kingston-Upon-Thames, Surrey. Agarose was obtained from L'industrie Biologique Francaise SA, 35 a49, Quai du Moulin de Cage, Gennevilliers, Seine, France, and Ionagar No. 2 from Oxoid Ltd., London, S.E.1. Cotton wool was a product of Boots Ltd., Nottingham. Whatman CF11 and CC31 celluloses were purchased from W.R. Balston, Maidstone, Kent, as were DEAE-cellulose and CM cellulose. CM-cellulose hydrazide was obtained from Miles Seravac (Pty) Ltd., Maidenhead, Berks. Sephadex, Sepharose and Dextran blue were supplied by Pharmacia Fine Chemicals Ltd., Ealing, London.

Antisera were prepared at The Wellcome Research Laboratories, Beckenham, Kent.

Polystyrene tubes with caps (GL3) were obtained from Greyward Laboratory Disposables, Bedford.
Buffers

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<td><strong>Na$_2$HPO$_4$$\cdot$2H$_2$O</strong></td>
<td>10.68 g.</td>
</tr>
<tr>
<td></td>
<td><strong>NaCl</strong></td>
<td>8.5 g.</td>
</tr>
<tr>
<td></td>
<td><strong>E.D.T.A.</strong></td>
<td>3.72 g.</td>
</tr>
<tr>
<td></td>
<td><strong>NaN$_3$</strong></td>
<td>1 g.</td>
</tr>
<tr>
<td></td>
<td><strong>B.S.A.</strong></td>
<td>5 g.</td>
</tr>
<tr>
<td></td>
<td><strong>to 1 litre with distilled water pH 7.4</strong></td>
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<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
<th>Amounts</th>
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<tbody>
<tr>
<td>B2</td>
<td><strong>H$_3$BO$_3$</strong> (boric acid)</td>
<td>5.5 g.</td>
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<tr>
<td></td>
<td><strong>Na$_2$B$_4$O$_7$</strong> (sodium tetraborate)</td>
<td>4.5 g.</td>
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<tr>
<td></td>
<td><strong>NaCl</strong></td>
<td>8.5 g.</td>
</tr>
<tr>
<td></td>
<td><strong>E.D.T.A.</strong></td>
<td>3.72 g.</td>
</tr>
<tr>
<td></td>
<td><strong>NaN$_3$</strong></td>
<td>1 g.</td>
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<tr>
<td></td>
<td><strong>B.S.A.</strong></td>
<td>5 g.</td>
</tr>
<tr>
<td></td>
<td><strong>to 1 litre with distilled water pH 8.4</strong></td>
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Phosphate Buffered Saline (PBS) pH 7.66

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<tbody>
<tr>
<td><strong>Na$_2$HPO$_4$</strong></td>
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<td><strong>NaH$_2$PO$_4$</strong></td>
<td>12.80 g.</td>
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<td><strong>NaCl</strong></td>
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<table>
<thead>
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</thead>
<tbody>
<tr>
<td><strong>to 10 litres with distilled water</strong></td>
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Borate Buffer pH 8.75, 0.25M

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<tr>
<td><strong>H$_3$BO$_3$</strong></td>
<td>12.37 g.</td>
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<td><strong>Na$_2$B$_4$O$_7$</strong></td>
<td>10.20 g.</td>
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<table>
<thead>
<tr>
<th>Components</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>to 1 litre with distilled water.</strong></td>
<td></td>
</tr>
</tbody>
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Phosphate/Citrate pH 5.5

<table>
<thead>
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<th>Components</th>
<th>Amounts</th>
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<tbody>
<tr>
<td><strong>Na$_2$HPO$_4$$\cdot$2H$_2$O</strong></td>
<td>20.11 g.</td>
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<tr>
<td><strong>Citric acid (C$_6$H$_8$O$_7$$\cdot$H$_2$O)</strong></td>
<td>9.14 g.</td>
</tr>
</tbody>
</table>
**Washing of Immunoadsorbents**

Following the protein coupling reaction, remaining uncoupled protein was removed by washing with various buffers. The washing procedure was performed on a glass sinter fitted to a Buchner vacuum flask. Aliquots (100 ml) of the buffers were used in the following sequence:- phosphate-buffered saline (PBS) pH 7.66 three times; glycine 50mM - hydrochloric acid pH 2.8; PBS; phosphate buffered sodium thiocyanate 3M pH 6.0; and PBS three times.

**Freeze Drying**

Samples of selected immunoadsorbents were freeze-dried in order to ascertain the effect of this process on the stability of the antibody. Before freezing the immunoadsorbents were suspended in 0.05 M borate buffer pH 8.4 containing 0.5% BSA, 0.9% sodium chloride and 5% sucrose.

**Synthesis of 3-nitrobenzyl alcohol**

Sodium borohydride (3.8 g) was dissolved in 40 ml of 0.2 N sodium hydroxide and was added to a continuously stirred suspension of m-nitrobenzaldehyde (31 g) in 200 ml of methanol. The temperature was maintained between 20 and 25°C by occasional cooling in ice water. The methanol was evaporated under reduced pressure followed by the addition of 200 ml of distilled water to the residual gum. The upper oil fraction was extracted four times with diethyl ether and the combined extracts dried over anhydrous sodium sulphate. The solution was filtered and evaporated under reduced pressure, and the residual oil distilled in vacuo.

b.p. 112-115°C| 0.05mm Hg. yield 28.1 g.
Synthesis of 3-nitrobenzylchloromethyl ether (84, 85)

Dry hydrogen chloride was passed into a vigorously stirred mixture of m-nitrobenzyl alcohol (16 g) and 36% formaldehyde (13.3 g) in benzene (200 ml) for four hours. The mixture was kept at room temperature overnight, and the benzene layer separated and washed with water. After drying over sodium sulphate the benzene was evaporated under reduced pressure and the residue distilled in vacuo. A residue of a higher b.p. remained.

b.p. 112°C | 0.07 mm Hg. yield 14.7 g.

Synthesis of N-(3-nitrobenzyloxymethyl) pyridinium chloride monohydrate

3-nitrobenzylchloromethyl ether (14.7 g) was added dropwise to stirred dry pyridine (37 ml) over 30 minutes. A white crystalline solid began to precipitate. After a further 20 minutes the mixture was cooled in ice, and the solid filtered and washed with petroleum ether (80-100°C). The product was dried over phosphorous pentoxide in vacuo.

m.p. 98-100°C yield 19.2 g

Preparation of N-(3-aminobenzyloxymethyl) cellulose

N-(3-aminobenzyloxymethyl) cellulose was prepared either from cotton wool, as described by Gurvich et al (38) with minor modifications (28, 86) or from microcrystalline cellulose. When using the microcrystalline cellulose the initial step of treatment with ethanol was omitted, as was the reprecipitation of the final product from a cuprammonium solution. Various quantities of 3-nitrobenzyloxymethyl-pyridinium chloride monohydrate, between 0.25 and 7.0 g per 100 g of cellulose, were used to treat the cellulose in order to obtain different degrees of substitution. The
degree of substitution was estimated by measuring the nitrogen content of the dried product.

**Coupling of antibody to N-(3-aminobenzylloxymethyl)cellulose**

The aminocellulose was diazotised using the method described by Gurvich et al. (38) and Miles and Hales (86). The aminocellulose (2 g) was suspended in 20 ml of 1 N hydrochloric acid and stirred at 0°C. A ten-fold excess (relative to the amino groups) of sodium nitrite was added, and stirring continued for 30 minutes. Solid urea was added until the starch-iodide paper test was negative, indicating that excess nitrous acid had been neutralised. The diazocellulose was washed three times in ice cold distilled water and twice in 0.1 M borate buffer pH 8.75. After the final wash the diazocellulose was resuspended in 6 ml of the borate buffer containing the immunoglobulin. The reaction mixture was stirred gently for 36 hours at 4°C. The suspension was centrifuged and the resulting pellet resuspended in fresh borate buffer. Remaining diazo groups were inactivated by the addition of β-napthol or tyrosine and stirred for a further 18 hours at 4°C. The resulting immunoadsorbent was washed thoroughly and stored either freeze-dried or in phosphate buffered saline (PBS) at 4°C.

**Coupling of antibody to p-aminobenzyl cellulose**

p-aminobenzyl cellulose (BDH) was washed with 50% ethanol and distilled water until the washings were colourless. Nitrogen estimation then showed that the product contained 0.08 mequiv. NH₂ per gram of aminocellulose compared with 0.11 mequiv. claimed by the manufacturer. Diazotisation, coupling of the antibody and blocking of unreacted diazo-groups were achieved by the same procedures as with N-(3-aminobenzylloxymethyl) cellulose.
Coupling of immunoglobulins to carboxymethyl cellulose hydrazide

Carboxymethyl cellulose hydrazide (CMC-hydrazide) contained 600 μequiv. of hydrazide groups per gram. Immunoglobulin was coupled to CMC-hydrazide by the Curtis azide method as described by Mitz and Summaria (54), but with minor modifications.

One gram of CMC-hydrazide was suspended in 20 ml of 1 N hydrochloric acid and stirred at 0°C. After the cellulose derivative had swollen, 0.7 g of solid sodium nitrite was added and stirring continued for a further 20 minutes. The resulting CMC azide was washed twice with 30 ml of ice cold 1 M sodium chloride and twice with 30 ml of ice cold distilled water. The pellet was resuspended in 10 ml of 0.1 M borate buffer pH 8.75 at 0°C, containing the immunoglobulin. The suspension was stirred at 0°C for six hours followed by the addition of 0.2 g of glycine which reacted with any remaining azide groups. The suspension was then stirred overnight at 0°C. The preparation was washed as described previously, and the resulting immunoadsorbent was stored in PBS at 4°C.

Preparation of 2-amino-4,6-dichloro-s-triazine

The method was that of Thurston et al. (87) with some modifications.

Cyanuric chloride (18.4 g) was dissolved in a mixture of dioxan (100 ml) and dimethoxyethane (15 ml) in a 250 ml beaker equipped with stirrer, thermometer and gas inlet tube. The beaker was then cooled in ice on a balance pan. The solution was maintained at 4°C while a stream of ammonia was passed into it. 3.4 g of ammonia was absorbed in 75 minutes, during which time a solid was precipitated. Excess ammonia was flushed from the mixture by the passage of nitrogen for five minutes. The mixture was heated to 95°C in order to dissolve the 2-amino-4,6-dichloro-s-triazine while the ammonium chloride remained insoluble and could therefore be filtered from the hot solution. The filtrate was cooled to 3°C, and
the product crystallised as fine colourless needles. The solution was filtered, the crystals collected and dried in vacuo.

\[ \text{m.p. } 235-236^\circ \text{C} \quad \text{yield } 8.5 \text{ g.} \]

**Coupling of immunoglobulin to aminochloro-s-triazinyl cellulose**

Aminochloro-s-triazinyl cellulose was prepared as described by Kay and Lilly (57). Different degrees of substitution were achieved by varying the quantity of 2-amino-4,6-dichloro-s-triazine between 20 and 300 mg per gram of cellulose. The degree of substitution was estimated by measuring the nitrogen content of the products. The cellulose derivative was suspended in a solution of immunoglobulin in 0.2 M borate buffer pH 8.75. The coupling reaction was allowed to proceed at room temperature for six hours, and for a further 16 hours at 4°C. The reaction mixture was centrifuged at 2,500 g for five minutes. The resulting pellet was suspended in 0.2 M glycine and stirred at room temperature for six hours in order to block any unreacted triazinyl groups. The immunoadsorbent was then washed using the procedure previously described in order to remove any non-covalently bound protein.

**Coupling of immunoglobulin to carboxymethyl cellulose in the presence of carbodiimides (50, 61, 62).**

Carboxymethyl cellulose (CMC) was suspended in 2N hydrochloric acid and stirred at room temperature for 30 minutes to ensure complete conversion to the acid form. The CMC was then washed on a Buchner funnel with distilled water until the washings were of neutral pH. Two grams of the treated CMC was added to a stirred solution of immunoglobulin in 8 ml of 0.15 M phosphate/citrate buffer pH 5.5 to give a thick slurry. \( \text{N,N'} - \text{dicyclohexyl carbodiimide} \) (600 mg) was dissolved in
1 ml of tetrahydrofuran and 1 ml of water was added forming an emulsion. This emulsion was then added to the CMC/protein mixture, and the slurry stirred at 4°C for three days. Alternatively, 1-ethyl-3-((3-dimethylaminopropyl) carbodiimide hydrochloride or 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulphonate dissolved in 2 ml of distilled water were used. The immunoadsorbent was washed following the procedure described previously.

Cyanogen bromide activation of cellulose (3, 88, 89)

The entire procedure was conducted in a well ventilated fume-cupboard. A 10% w/v solution of cyanogen bromide in distilled water was prepared, 4 ml of this was added to a stirred suspension of cellulose (2 g of cellulose in 20 ml of distilled water). The pH of this mixture was immediately adjusted to 11.0 by the addition of 2 N sodium hydroxide, and the pH maintained by further additions of alkali until it remained constant after eight to ten minutes. The activated cellulose was transferred to a Buchner funnel and quickly washed with 250 ml of ice-cold distilled water followed by 50 ml of ice-cold 0.5 M bicarbonate buffer pH 9.0. The activated cellulose was suspended in the minimum volume of 0.5 M bicarbonate buffer pH 9.0, containing the immunoglobulin, and the mixture was stirred at 4°C for 16 hours. The resulting immunoadsorbent was collected by centrifugation and resuspended in either 100 mM 3-aminopropan-1-ol in 0.25 M sodium bicarbonate buffer pH 9.0 or 100 mM glycine pH 9.0 and allowed to stand for 16 hours at 4°C. Remaining unreacted groups on the activated cellulose were blocked by the 3-aminopropan-1-ol or glycine.
**Preparation of ω-aminoalkyl cellulose**

Cellulose was activated with cyanogen bromide as previously described. The cellulose: cyanogen bromide ratio was varied from 20:1 to 3:1. The activated cellulose was washed with ice cold water. 2 g of this cellulose was then added to 10 ml of a solution containing 6 m moles of the appropriate diamine previously titrated to pH 10.0 with 5 N hydrochloric acid. The mixture was stirred for 16 hr. at 4°C prior to washing with distilled water. The diamines used in these experiments were ethylene diamine, 1,5-diaminopentane, and 3,3'-diamino-bis-propylamine.

**Coupling of immunoglobulin to aminocellulose**

a) **Carbodiimide method (25, 61)**

The method employed was that previously described for coupling immunoglobulin to CM-cellulose.

b) **Glutaraldehyde method (60, 63)**

Two grams of aminocellulose were suspended in 8.0 ml of 0.2 M phosphate buffer pH 7.0. To this suspension was added 8.0 ml of 25% glutaraldehyde adjusted to pH 7.0. The mixture was incubated at room temperature for 18 hr and then filtered using a Buchner funnel. Still using the Buchner funnel the cellulose was washed with 21 of 50 mM phosphate buffered 1 M saline, pH 7.0, and 3 litres of distilled water, in order to remove the excess glutaraldehyde. The activated cellulose was suspended in 6.0 ml of 0.2 M phosphate buffer pH 7.0 containing the protein for coupling. The suspension was stirred at room temperature for 4 hr and then for a further 44 hr at 4°C. The resulting immunoadsorbent was collected by centrifugation and resuspended in either 100 mM glycine, pH 7.0, or 100 mM 3-amino-propan-1-ol, pH 7.0, and stirred for a further 16 hr. The preparation was washed and stored at 4°C in PBS.
Preparation of and Coupling to Sulphydrylcellulose

Cellulose was activated by the cyanogen bromide method, and 2g of this was reacted with 600 mg N-acetylhomocysteinethiolactone in 10 ml 1M sodium bicarbonate buffer pH 9.5. After stirring for 24hr at 4°C the suspension was filtered on a Büchner funnel and washed with 2 l of PBS. Immunoglobulin was coupled by the carbodiimide method previously described for coupling to CM-cellulose.

Preparation of Bromoacetylcelluloses

a) Bromoacetamidoalkylcellulose

O-Bromoacetyl-N-hydroxysuccinimide was prepared as described by Cuatrecasas (25) and Cuatrecasas et al. (90). One mmole each of bromoacetic acid and N-hydroxysuccinimide was dissolved in 10 ml dioxan. One mmole of N,N'-dicyclohexylcarbodiimide was added and the mixture stirred for 1 hr, during which time a heavy precipitate of dicyclohexylurea was formed. The precipitate was removed by filtration and the filtrate added to 2 g of ω-aminocellulose in 8 ml of 100 mM phosphate buffer pH 7.5 at 4°C. The mixture was stirred for 16 hr and the cellulose washed with 1 litre of distilled water. A small sample was dried over phosphorus pentoxide for bromine estimation by the method of Schoniger (91).

b) Bromoacetylcellulose (39, 56)

A solution of 33.3 g of bromoacetic acid in 20 ml of dioxan was added to 10 g of dry powdered cellulose (CC31) in a 250 ml round bottomed flask. The flask was stoppered and the mixture stirred at room temperature for 24 hr. A sodium hydroxide trap was connected to the flask following the addition of 25 ml of bromoacetyl bromide, and after 6 hr the mixture was poured into 2 litres of vigorously stirred
deionised water. A sample was dried for bromine analysis.

Different degrees of substitution were achieved by varying the quantities and relative proportions of bromoacetyl bromide and bromoacetic acid.

Protein coupling was effected in 0.2M borate buffer pH 8.75. In some experiments coupling was preceded by physical adsorption of the protein to the carrier in 0.15M phosphate-citrate buffer pH 5.4. The preparation was stirred in 50mM 2-aminoethanol pH 8.75 for 6 hr in order to react any remaining bromoacetyl groups prior to washing.

Periodate Oxidation of Cellulose and Coupling of Immunoglobulin (70)

Powdered cellulose (CC31) was stirred in suspension in 10 or 100mM solutions of sodium periodate at room temperature for 24 hr. The oxidised cellulose was washed exhaustively with distilled water followed by the coupling buffer, and then reacted with immunoglobulin at 4°C for 48 hr. Remaining aldehyde groups were blocked by reaction with 50mM 3-aminopropan-1-ol pH 7.0 for 6 hr prior to washing of the immunoadsorbent.

Coupling of Immunoglobulin to Cellulose by the Oxirane Method (69)

A suspension of 2g of powdered cellulose (CC31) in 10ml of distilled water was mixed with an equal volume of 1M sodium hydroxide. The mixture was made 10% with respect to 1-chloro-2,3-epoxypropane and stirred in a round bottomed flask for 2 hr at 60°C. The product was washed exhaustively with distilled water and then 0.5M sodium bicarbonate pH 9.0. Immunoglobulin was added in the minimum volume of the same buffer necessary to give a stirrable suspension. The coupling reaction was allowed to proceed for 48 hr at 4°C. The product was stirred in 50mM 2-aminoethanol buffered with 100mM tris-HCl pH 7.8 for 16 hr, and then washed.
N-Hydroxysuccinimide Ester of Cellulose (92)

Carboxyl cellulose, prepared by reacting 6-aminohexanoic acid with the imidocarbonate of cellulose was treated with 100mM N,N'-dicyclohexylcarbodiimide and 100mM N-hydroxysuccinimide in dioxan. After 70 min at room temperature the derivative was washed with dioxan and methanol to remove dicyclohexylurea. Immunoglobulin was coupled to the cellulose ester in 0.2M sodium bicarbonate buffer pH 9.0, the reaction being left for 16 hr at 4°C. Remaining succinimide groups were reacted with 3-aminopropan-1-ol at the same pH before washing the immunoadsorbent.

Mixed Anhydride Derivative of CM-Cellulose

Two g of CM-cellulose were stirred in 2N HCl for 30 min and then washed with distilled water until the washings were neutral. The CM-cellulose was dried and mixed with 1.4 ml triethylamine and 0.5 ml ethyl chloroformate in 12 ml dioxan at room temperature. After 1 hr the product was filtered and washed with dioxan and distilled water before adding it to a solution of the immunoglobulin in 100mM sodium phosphate buffer pH 7.0 at 4°C. After 48 hr the product was transferred to either 100mM glycine or 100mM 3-aminopropan-1-ol pH 7.0 and stirred for 16 hr. The resulting immunoadsorbent was washed and stored at 4°C.

Carboxycellulose

Powdered cellulose (7.5 g) was reacted with cyanogen bromide (500 mg/g cellulose). After washing the resulting imidocarbonate derivative with 2 l of distilled water it was suspended in 50 ml of water containing 9.83 g 6-aminohexanoic acid (75 mmole) at pH 9.0. The mixture was stirred at room temperature for 18 hr and the derivative washed with distilled water.
Alternatively, aminoalkyl cellulose was reacted with succinic anhydride (25) and washed with 0.1 N sodium hydroxide for 30 min (92) and then distilled water.

Cross-linking Proteins with Glutaraldehyde

Protein polymers were prepared with glutaraldehyde as described by Avrameas and Ternynck (2). Serum was dialysed against 150 mM sodium chloride at 4°C for 16 hr, and then adjusted to pH 4.4 by the addition of 1N HCl. One-tenth of the volume of 1M acetate buffer pH 4.4 was then added. Solutions of immunoglobulins or immunoglobulin fragments were adjusted to pH 7.0 and buffered with 1M sodium phosphate buffer. The protein solution was stirred vigorously while three-tenths the volume of 2.5% glutaraldehyde was added dropwise. The mixture began to gel after 8 to 10 min and was kept at room temperature overnight. The gel was dispersed by repeatedly forcing it through a syringe and then washed as were the cellulose immunoadsorbents.

Protein Estimations

The concentration of purified human IgG was estimated from the extinction at 280 nm, assuming the specific extinction coefficient ($E_{280}^{1%}$) to be 13.8. Otherwise, protein concentrations were determined by a modified Biuret method (93).

The amount of protein covalently coupled to cellulose was determined by one of two methods:-

(i) Samples of the immunoadsorbent were allowed to stand at room temperature for 16 hr in 0.5M sodium hydroxide. The mixture was centrifuged at 2000g for 15 min, and the supernatant assayed by an automated Folin-Lowry procedure (94, 95) (Fig. 2.1). The reagents used were as follows:– 0.5 N sodium hydroxide, copper
Technicon autoanalyser manifold for the measurement of protein by the Folin-Lowry technique.
tartrate (0.1% cupric sulphate plus 0.2% sodium potassium tartrate), Folin-Ciocalteau reagent diluted 1 in 3 with distilled water and bicarbonate buffer (84 g sodium bicarbonate plus 25 g sodium hydroxide in 1 litre).

(ii) Trace labelling. The immunoglobulin-cellulose coupling reaction was carried out in the presence of immunoglobulin trace-labelled with $^{125}$I. The amount of labelled protein added was compared with the amount in the product by counting samples of equal volume taken from the initial reaction mixture and a suspension of the washed immunoadsorbent. The results were corrected for $t_1$. 

Analysis of Serum Proteins and Antisera:–

(i) Measurement of Serum IgG by Radial Immunodiffusion (96)

A 2% solution of agarose was prepared by dissolving 2 g of agarose in 0.05M sodium veronal buffer pH 8.6 at 100°C. The solution was cooled to 54°C, and 10 ml was added to 9.8 ml 0.05M sodium-veronal buffer, pH 8.6, and 0.2 ml of the antiserum at the same temperature. After mixing, 12 ml of the solution was pipetted on to a 3½ in square glass slide and left to solidify at room temperature in a moist box. A 2 mm stainless steel tube connected to a vacuum line was used to cut wells in the gel. The wells were filled to the brim with dilutions of a standard containing from 10 to 100 mg IgG per 100 ml, or dilutions of unknowns. Drawn-out capillary tubes fitted with rubber bulbs were used for this purpose. Precipitin rings were allowed to develop for 30 hr, and their diameters were then measured. The square of the diameters were plotted as a function of the log$_{10}$ of the concentration of standard antigen.

(ii) Quantitative Precipitin Test

Various known amounts of antigen dissolved in 0.9 ml PBS were added to 0.1 ml of the antibody preparation. Control tubes contained either
PBS instead of the antigen solution or non-immune serum instead of the antiserum. All determinations were made in triplicate. The tubes were incubated for 72 hr at 4°C, and the precipitates collected by centrifugation at 2000 g for 20 min. They were washed twice by resuspension in PBS at 4°C, and then dissolved in 0.2 N sodium hydroxide. The extinctions were measured at 280 nm.

Immunoelectrophoresis

Immunoelectrophoresis was carried out after the method of Grabar and Williams (97, 98) in barbitone-buffered 1% agar. Plates were prepared by pouring 12 ml molten agar on to 3½ in square glass slides. The electrophoresis was performed in Shandon tanks using a constant current of 25mA per plate.

Double Diffusion Test

This was performed as described by Ouchterlony (99). Plates were prepared in 9 cm plastic petri dishes with a 1% solution of Ionagar in PBS containing 0.1% sodium azide as preservative.

Purification of Human IgG

Human IgG was purified from 25 ml of a human serum pool. Serum was made 45% saturated with respect to ammonium sulphate by the dropwise addition of saturated ammonium sulphate to the stirred serum. The mixture was allowed to stand overnight at 4°C. The precipitate was collected by centrifugation at 2000g for 20 min and washed twice by resuspension in 45% ammonium sulphate. The precipitate was dissolved in the minimum volume of PBS and dialysed for 30 hr against three changes of 1 litre of sodium phosphate buffer 10mM, pH 7.66. The dialysate was applied to a column of DEAE-cellulose (DE32) 30 x 2 cm equilibrated with the same buffer. IgG was eluted from the column and dialysed overnight against sodium phosphate buffer 100mM- sodium
chloride 150 mM, pH 7.66. The solution was concentrated to approximately 30 mg per ml by pressure dialysis using a PM-30 membrane. The IgG was further purified by gel filtration on a Sephadex G-200 column, 120 x 2.5 cm.

Preparation of Fab Fragment (100, 101, 102, 103)

Human IgG (200 mg in 10 ml) was dialysed overnight at 4°C against 0.1 M acetate buffer pH 6.0. The solution was then made 10 mM with respect to cysteine and 20 mM with respect to ethylenediaminetetracetic acid (EDTA) before the addition of 2 mg of papain. The mixture was incubated for 16 hr at 37°C, and then dialysed against three changes of 10 mM acetate buffer pH 7.0 over 24 hr. The dialysate was applied to a 2 x 30 cm column of DEAE-cellulose (DE32) and eluted with a linear gradient of acetate buffer pH 7.0 from 10 to 350 mM. The digest was resolved into two major peaks, the first of which represented the Fab fragment.

Antibody for Immunoadsorbent Preparations

Sheep anti-human IgG (Fc) was rendered specific for the Fc region by immunoadsorption with protein polymers prepared from the Fab fragment of IgG with glutaraldehyde. The adsorptions were performed at room temperature for 1 hr and the antisera recovered by filtration. The immunoadsorbents were regenerated by treatment with 3 M sodium thiocyanate pH 6.0. Antisera were judged to be mono-specific by immunodiffusion and immunoelectrophoresis.

The antiserum was dialysed against PBS pH 7.66 overnight. Three and a half volumes of 0.4% Rivanol (2-ethoxy-6,9-di-aminoacridine lactate) were added dropwise to the antiserum with vigorous stirring. The resulting
precipitate was removed by filtration, and excess Rivanol was removed from the filtrate by dialysis against repeated changes of PBS. The immunoglobulins were precipitated by the addition of an equal volume of saturated ammonium sulphate. After standing at 4°C overnight the precipitate was collected by centrifugation, washed twice by resuspension in 50% ammonium sulphate and dissolved in PBS. The solution was dialysed against three changes of PBS over 36 hr, passed through an 0.22 μμ Millipore filter and stored at 4°C.

Iodination Methods

Human IgG and sheep and rabbit immunoglobulins were iodinated by the chloramine T method (104) using modifications of the procedure described by Sonoda and Schlamowitz (105).

Method (i): A small magnetic stirring bar was placed in a 2 ml glass bottle to which was added 25 μl 0.5M phosphate buffer pH 7.0, 1mCi Na125I (476 pmoles) and 25 μl of a 4 mg per ml solution of the protein (100 μg) in 0.05M phosphate buffer pH 7.0. The mixture was stirred in an ice bath and 50 μl of a 0.5 mg per ml solution of chloramine T (88 nmoles) was added. After 60 sec excess chloramine T was neutralised by the addition of 50 μl of 0.25M sodium metabisulphite. Two min later 50 μl 1.0M potassium iodide and 100 μl 15% BSA were added. The contents of the reaction vessel were transferred to the top of a Sephadex G15 column, and labelled immunoglobulin was eluted with PBS containing 1% BSA. The labelled fraction was diluted to 20 ml with the same buffer, and dialysed against three changes of PBS (1 litre) at 4°C. The 'label' was divided into aliquots and stored at -20°C.

Method (ii): The reaction was conducted in a 25 ml glass bottle equipped with a magnetic stirring bar. One ml 0.5M sodium phosphate buffer pH 7.0, 500 μCi of Na125I and 50 μl of a 4 mg per ml solution
of human IgG (200 μg) were added. The mixture was stirred vigorously, and 5 ml of freshly prepared chloramine T (690 nmoles) were added dropwise over 5 min. The reaction was allowed to proceed for 30 min before excess chloramine T was neutralised by the addition of 1.2 ml of sodium metabisulphite (667μM). After a further 2 min 0.5 ml of potassium iodide solution (1.0M) and 1 ml 15% BSA were added. The contents of the reaction vessel were transferred to a column of Amberlite IRA-400 (Cl) with a bed volume of 8 ml. The iodinated IgG was eluted with PBS containing 1% BSA.

Measurement of Immunoadsorbent Activity

(i) Immunoadsorbent Titration

Immunoadsorbent suspensions were diluted to the same protein concentration per ml. Serial dilutions of the suspensions were incubated at room temperature with fixed quantities of 125I-IgG and IgG standard in a borate buffer containing 0.5% BSA in polystyrene tubes. The tubes were tumbled throughout the incubation period, and then centrifuged at 2000 g for 10 min. The supernatants were decanted or aspirated and the immunoadsorbent samples washed by resuspension in the borate-BSA buffer. After centrifugation the supernatants were discarded and the tubes counted in an automatic γ-counter (LKB-Wallac or Tracerlab).

Typical assay: 200 μl 0.05M borate buffer pH 8.4
- 0.5% with respect to BSA.
100 μl dilution of the immunoadsorbent
100 μl IgG standard or human serum dilution or buffer.
50 μl 125I-IgG

450 μl

Incubation time 16 hr.
(ii) Immunoadsorbent suspensions were diluted to the same protein concentration per ml. Equal aliquots of the suspensions were incubated at room temperature with fixed quantities of $^{125}$I-IgG and various dilutions of either human serum or an IgG standard in borate buffered BSA in polystyrene tubes. The assay was continued as in (i) above.

Controls were run for both assays as follows:

(a) Omitting the immunoadsorbent.

(b) Employing a labelled preparation other than $^{125}$I-human IgG, e.g. sheep or rabbit $^{125}$I-IgG.

**Elution of IgG (antigen)**

The recovery of adsorbed human IgG from anti-IgG immunoadsorbents was measured with trace labelled IgG. Aliquots of immunoadsorbent suspensions were incubated at room temperature with fixed amounts of $^{125}$I trace-labelled IgG in borate buffer BSA in polystyrene tubes. The tubes were tumbled throughout the incubation period and then centrifuged at 2000 g for 10 min. The supernatants were aspirated and the immunoadsorbents washed by resuspension in assay buffer. The tubes were again centrifuged and aspirated. The eluting fluids were added and the tubes tumbled for a fixed time before centrifugation at 2000 g for 10 min. The supernatants were aspirated and the tubes counted in a γ-counter. The efficiency of elution was measured as the difference between the total amount of IgG adsorbed and that remaining after elution.

The eluting fluids employed were:
- glycine 50mM - hydrochloric acid pH 2.8
- glycine 50mM - hydrochloric acid pH 2.0
- acetic acid 1.0M pH 2.3
- propionic acid 1.0M pH 2.5
- propionic acid 0.1M pH 3.0
- citric acid 100mM - sodium chloride 154mM - glycine 133mM pH 2.8.
sodium thiocyanate 3.0M - 50mM sodium phosphate pH 6.0
sodium thiocyanate 3.0M - 50mM tris-HCl pH 9.0
magnesium chloride 2.5M
urea 8.0M - sodium chloride 154mM - phosphate 10mM pH 7.6
manganese sulphate 3.0M

(c) Results
Antisera

Antisera raised to the Fc fragment of human IgG were found to contain some antibody activity towards the other immunoglobulins and the Fab fragment. The antisera were rendered specific for the Fc fragment of IgG by immunoadsorption with the appropriate glutaraldehyde polymerised proteins. Immunoglobulin fractions were purified from each of the sheep anti human IgG (Fc) sera designated 51, 55 and 57. The protein and antibody concentrations of these preparations are given in Table 2.1. The precipitin curve obtained for antibody from sheep 57 is shown in Fig. 2.2, and an immunoelectrophoresis pattern in Fig. 2.3.

<table>
<thead>
<tr>
<th>Sheep</th>
<th>Immunoglobulin preparation mg protein/ml</th>
<th>Antibody concentration mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>57.0</td>
<td>7.07</td>
</tr>
<tr>
<td>55</td>
<td>68.0</td>
<td>7.52</td>
</tr>
<tr>
<td>57</td>
<td>72.0</td>
<td>14.40</td>
</tr>
</tbody>
</table>
Fig. 2.2 Precipitin curve for antibody from sheep 57 (AS 57). Upper curve represents total protein in the precipitate and the lower curve the antibody protein in the precipitate.

μl human IgG solution (3.4 μg/ml)

Fig. 2.3 Immunoelectrophoresis patterns of whole human serum against (in the troughs from top to bottom) sheep anti whole human serum, sheep anti whole human serum, sheep anti human IgG (Fc) (AS 51) and sheep anti-human IgG (Fc) (AS 57).
Iodine-Labelled Immunoglobulins

In method (i) described previously, IgG and Na$^{125}$I were employed in a molar ratio of approximately 4:3. Two experiments with sheep IgG gave substitution levels of 0.23 and 0.25 I atom per mole of IgG. In method (ii) the molar ratio was approximately 5:25:1, and with sheep IgG this gave substitution levels of about 0.02 atom I per mole of IgG. These results represent incorporation of 25-33% of the label by method (i) and 60-75% by method (ii).

Human IgG iodinated according to method (ii) was mixed with an aliquot of unlabelled IgG and the mixture subjected to chromatography on a Sephadex G-25 column (20 x 0.5 cm). Fractions were collected and the radioactivity measured in a γ-counter (Fig. 2.4). An equal volume of 15% trichloracetic acid (TCA) was added to each fraction and after 10 min the precipitated protein was collected by centrifugation at 2000 g for 10 min. The supernatants were aspirated and the precipitates washed twice by resuspension in 7.5% TCA before measurement of radioactivity. The first peak emerging from the column in the void volume represented IgG. The second peak was accounted for by free $^{125}$I (Fig. 2.5). The Amberlite resin had removed most of the free label, and that which remained was removed by dialysis. A small amount of iodine was presumed to have passed through the column physically adsorbed to the immunoglobulin. The iodinated IgG co-chromatographed with unlabelled IgG on a Sephadex G-150 column (90 x 2.5 cm), indicating that the molecule remained intact.

Samples of iodinated human IgG prepared by each method were incubated with aliquots of sheep anti human IgG as described for the quantitative precipitin test. Radioactivity in the precipitates and supernatants was measured. At least 97% of the radioactivity was associated with the
Fig. 2.4. Gel filtration of a sample taken from the iodination mixture after the addition of sodium metabisulphite and potassium iodide. Solid line represents radioactivity emerging from the column and the broken line the radioactivity precipitated by TCA. Column: Sephadex G-25, 20.0 x 0.5 cm, equilibrated in phosphate buffered saline.

Fig. 2.5. Gel filtration of a sample taken from the iodination mixture (solid line) and a sample after passage down a column of Amberlite IRA 400 (Cl\textsuperscript{-}). The small amount of free iodine remaining was removed by dialysis.
specific precipitate in the equivalence zone (i.e. at concentrations of the antiserum giving a maximum precipitation of the antigen).

**Preparation of Immunoadsorbents**

Pilot experiments were conducted to test the conditions chosen for each immunoadsorbent preparation using 200 mg of the cellulose derivative and 10 mg of sheep immunoglobulin. Wherever necessary, published procedures were modified as set down in section 2.b. In several cases techniques which had hitherto been employed only for the immobilisation of enzymes were used successfully in the preparation of immunoadsorbents.

The amount of immunoglobulin linked to cellulose was measured by both an automated Folin-Lowry technique and a trace labelling technique. A typical autoanalyser trace for the Folin-Lowry assay is shown in Fig. 2.6. The 150 µg/ml standards were duplicated to demonstrate the lack of cross-contamination between samples. The appropriate cellulose derivatives were run as blanks, and in a few cases, notably with preparations involving N-acetylhomocysteine thiolactone, there was marked interference with the assay. Two experiments were conducted to compare measurement of protein content by the two techniques, and the results are combined in Table 2.2. Subsequent experiments were standardised on results obtained with the trace labelling technique because of its practical advantages. The interference with the Folin-Lowry assay by certain cellulose derivatives increased the possibility of error.

The validity of the trace labelling technique was tested with two bromoacetylcellulose preparations containing 1.29 and 0.89 meq bromide per g. Four different preparations of $^{125}$I-sheep IgG were used, and experiments were duplicated and run in parallel (Table 2.3).
Fig. 2.6

Typical Autoanalyser trace obtained using the manifold illustrated in Fig. 2.1.
(Protein estimation by Folin-Lowry method).
Table 2.2
A comparison of the amounts of protein coupled to cellulose carriers as measured by an automated Folin-Lowry procedure and a trace labelling technique.

<table>
<thead>
<tr>
<th>Cellulose derivative</th>
<th>Protein incorporated (mg) per g cellulose derivative</th>
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<tbody>
<tr>
<td></td>
<td>Trace labelling method</td>
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<tr>
<td>Triazinyl</td>
<td>13.2</td>
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<tr>
<td></td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>20.5</td>
</tr>
<tr>
<td>Diazotized m-amino-benzoyloxymethyl</td>
<td>18.6</td>
</tr>
<tr>
<td></td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>52.5</td>
</tr>
<tr>
<td></td>
<td>60.0</td>
</tr>
<tr>
<td>Diazotized p-aminobenzyl</td>
<td>23.5</td>
</tr>
<tr>
<td></td>
<td>12.1</td>
</tr>
<tr>
<td>Bromoacetyl</td>
<td>44.3</td>
</tr>
<tr>
<td></td>
<td>96.8</td>
</tr>
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<td></td>
<td>89.4</td>
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<tr>
<td>Imidocarbonate</td>
<td>23.0</td>
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<td></td>
<td>21.5</td>
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<tr>
<td>Aminoethyl</td>
<td>14.6</td>
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<td>27.9</td>
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<tr>
<td>Aminopentyl</td>
<td>36.3</td>
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<td></td>
<td>26.0</td>
</tr>
<tr>
<td>Aminodipropylamine</td>
<td>26.6</td>
</tr>
<tr>
<td></td>
<td>18.7</td>
</tr>
<tr>
<td>Sulphydryl (N-acetylhomocysteinethiolactone)</td>
<td>30.4</td>
</tr>
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</tr>
</tbody>
</table>

** Uncorrected value.
Table 2.3
Experiment to test the validity of the trace labelling technique

<table>
<thead>
<tr>
<th>Label</th>
<th>% Counts Coupled</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>BAC 1.29 meq Br/g</td>
</tr>
<tr>
<td>A*</td>
<td>66.6</td>
</tr>
<tr>
<td>B*</td>
<td>64.7</td>
</tr>
<tr>
<td>C+</td>
<td>69.2</td>
</tr>
<tr>
<td>D+</td>
<td>66.3</td>
</tr>
<tr>
<td>Mean</td>
<td>66.7 ± 1.61</td>
</tr>
</tbody>
</table>

* Iodinated by Method (i)
+ Iodinated by Method (ii)
Wherever possible the degree of substitution of active groups on the carrier was varied, and, by using cyanogen bromide-activated cellulose, aliphatic diamines of various chain lengths were attached to the carrier in order to investigate the effects of these variations on antibody activity (Table 2.4). Other variations investigated were the coupling pH and the choice of coupling reagent. For example, antibodies were coupled to amino-celluloses either by using water-soluble or-insoluble carbodiimides or by first activating the carrier with glutaraldehyde. Results of some of the immunoadsorbent preparations are shown in Table 2.5.

Samples of 3-aminobenzyloxymethylcellulose containing 67 µeq NH₂ per g were activated by diazotisation and reacted with sheep immunoglobulin. When 110 mg of protein was used the capacities of 1 g quantities of the reprecipitated and untreated preparations were similar - 52.5 and 60 mg respectively. In a second experiment the effective protein concentration was doubled. This increased the amount of protein coupled to the untreated diazotised cellulose from 52.5 to 61 mg/g. On the other hand the amount bound by the reprecipitated derivative was more than doubled from 60 to 129.8 mg/g. Therefore the amount of protein coupled was limited by the amount of protein added and the surface area of the cellulose as previously shown by Gurvich et al (38). Further, by increasing the number of 3-aminobenzyloxymethyl groups the capacity of the derivative was also increased.

Immunoglobulin was coupled via a diazo bond to 4-aminobenzylcellulose. The capacity of this derivative was less than half that of the corresponding 3-aminobenzyloxymethylcellulose. Again reprecipitation increased the capacity of the derivative for protein.
Table 2.4 Variation of the degree of substitution of active groups in cellulose derivatives

<table>
<thead>
<tr>
<th>N- (3-aminobenzoyloxymethyl) pyridinium chloride monohydrate (mg per 10 g)</th>
<th>μeq N₂ groups per g product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) 50</td>
<td>18</td>
</tr>
<tr>
<td>2) 250</td>
<td>67</td>
</tr>
<tr>
<td>3) 500</td>
<td>121</td>
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<td>4) 700</td>
<td>160</td>
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</table>

<table>
<thead>
<tr>
<th>2-amino-4,6-dichloro-s-triazine (mg per g)</th>
<th>% N in product</th>
</tr>
</thead>
<tbody>
<tr>
<td>5) 8</td>
<td>0.05</td>
</tr>
<tr>
<td>6) 80</td>
<td>0.16</td>
</tr>
<tr>
<td>7) 400</td>
<td>0.42</td>
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Bromoacetylcellulose Preparations

<table>
<thead>
<tr>
<th>Cellulose (g)</th>
<th>Bromoacetic acid (g)</th>
<th>Bromoacetyl bromide (ml)</th>
<th>Br in product meq per g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>8) 10</td>
<td>33.3</td>
<td>2.5</td>
<td>1.67</td>
</tr>
<tr>
<td>9) 3</td>
<td>1.33</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>10) 3</td>
<td>0.67</td>
<td>0.5</td>
<td>0.83</td>
</tr>
<tr>
<td>11) 3</td>
<td>0.53</td>
<td>0.4</td>
<td>0.73</td>
</tr>
<tr>
<td>12) 3</td>
<td>0.44</td>
<td>0.33</td>
<td>0.49</td>
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</table>

Employing the quantities as in 8), substitution levels ranged from 10.3 to 13.4% or 1.29 to 1.67 meq Br per gram of bromoacetyl-cellulose in nine replicate experiments.

Bromoacetamidoalkylcellulose

<table>
<thead>
<tr>
<th>CNBr per g. cellulose (mg)</th>
<th>Diamine</th>
<th>Br in product meq per g</th>
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<tr>
<td>13) 20</td>
<td>ED</td>
<td>0.090</td>
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<tr>
<td>14) 20</td>
<td>DP</td>
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<tr>
<td>15) 20</td>
<td>DADP</td>
<td>0.036</td>
</tr>
<tr>
<td>16) 80</td>
<td>ED</td>
<td>0.17</td>
</tr>
<tr>
<td>17) 80</td>
<td>DP</td>
<td>0.18</td>
</tr>
<tr>
<td>18) 80</td>
<td>DADP</td>
<td>0.18</td>
</tr>
<tr>
<td>19) 200</td>
<td>ED</td>
<td>0.27</td>
</tr>
<tr>
<td>20) 200</td>
<td>DP</td>
<td>0.23</td>
</tr>
<tr>
<td>21) 200</td>
<td>DADP</td>
<td>0.25</td>
</tr>
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<td>Cellulose Derivative</td>
<td>Designation in Table 2.4</td>
<td>Reaction Conditions</td>
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<tr>
<td>Diazotised N-(3-aminobenzyl-oxymethyl)</td>
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<td>Aminodipropylamine</td>
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<td>Sulphhydril</td>
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Table 2.5 continued

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<td>73</td>
<td>18.0</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td>9.0</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td>13.5</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td>14.5</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td>6.2</td>
<td>1.7</td>
</tr>
<tr>
<td>Carboxyethyl</td>
<td></td>
<td>73</td>
<td>60.1</td>
<td>43.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>73</td>
<td>75.5</td>
<td>55.1</td>
</tr>
</tbody>
</table>

Key: CMC - carboxymethyl cellulose
dCC - N,N'-dicyclohexylcarbodiimide
EDC - 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
CMCM - 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulphonate.

Table 2.5 The amounts of immunoglobulin immobilised by covalent bonding in various preparations of sheep anti-human IgG immunoadsorbents. In all cases 1 g quantities of the cellulose derivative were used. Results represent the means of triplicates. Information listed under 'reaction conditions' qualifies that given in the Materials and Methods section, p.28 et seq.
CM-cellulose hydrazide was converted to the azide by reaction with nitrous acid according to the Curtis rearrangement reaction. The method had the disadvantage that it was not reproducible.

The physical adsorption of immunoglobulin by bromoacetylcellulose at pH 4.6 prior to covalent coupling at pH 8.75 (39) was found to be unnecessary with bromoacetylcellulose preparations containing in excess of 1 meq Br per g. However, with bromoacetyl and bromoacetamidoalkyl celluloses containing lower levels of bromine the inclusion of a physical adsorption step improved the level of protein coupling. Phosphate-citrate buffer between pH 3.8 and 5.2 caused precipitation of the sheep immunoglobulin. When the protein solution was dialysed against pH 5.0 buffer before addition of bromoacetylcellulose the physical adsorption step was not as successful as that achieved by adding the protein to the cellulose derivative buffered at pH 5.0 (Table 2.6,p.63).

Bromoacetyl and bromoacetamidoalkyl cellulose derivatives showed an increased capacity for protein with greater degrees of bromoacetylation. Furthermore, the capacity of pentyl derivatives was greater than that of ethyl derivatives, a factor which may be attributable to the longer aliphatic chain. However, the bromoacetylated aminodipropylamine cellulose, which has an even longer side chain than the pentyl derivative, had a capacity only marginally greater than that of the ethyl derivative.

The preparation of bromoacetylcellulose involved a reprecipitation step resulting in a preparation with a very small particle size. This property makes the derivative unsuitable for some applications such as use in columns. The preparation of bromoacetamidoalkyl cellulose derivatives does not involve a reprecipitation and consequently the physical properties of the starting material may be retained.
The method of synthesis of bromoacetamidoalkyl cellulose described by Cuatrecasas (25) involves reaction of the O-bromoacetyl-N-hydroxysuccinimide ester with aminoalkyl cellulose at pH 7.5. A possible side reaction not mentioned by Cuatrecasas involves reaction of the bromoacetyl groups with the amino groups on the carrier, resulting in an N-hydroxysuccinimide cellulose. This may warrant investigation.

The degree of substitution of aminochloro-s-triazine in cellulose influenced the capacity of the derivative for protein, increased substitution leading to increased capacity. A 5.5 fold increase in the amount of protein added led to the incorporation of 20.5 mg of protein as opposed to 15.5 mg, a difference of only 32%. This again points to the carrier as the limiting factor and not the protein.

In view of its popularity the cyanogen bromide technique proved to be somewhat disappointing as regards the capacity of the imidocarbonate derivative to couple protein. Cellulose activated with 200 mg CNBr per gram bound only 11.1 and 21.5 mg or 15.2 and 19.5 % of the added protein per gram of cellulose (2 expts.). The activated cellulose was more efficient in coupling sheep immunoglobulin at pH 6.0 than at pH 8.9.

N,N'-Dicyclohexylcarbodiimide was the most satisfactory diimide for coupling immunoglobulin to CM-cellulose in terms of the amount of protein coupled, but the product was invariably granular and intractable. This may have resulted from cross-linking of the protein since the reaction involves a condensation between amino and carboxyl groups. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, a water-soluble diimide, was used to prepare an immunoadsorbent which retained the physical properties of the starting material. The same reagent was used to couple immunoglobulin to carboxycellulose prepared either by reacting 6-amino-
hexanoic acid with cyanogen bromide activated cellulose or by reaction of aminoalkylcellulose with succinic anhydride, and to aminoalkylcelluloses.

Aldehyde groups were introduced on to aminoalkylcellulose by reacting the amino groups with glutaraldehyde, a bifunctional aldehyde. Proteins were successfully coupled to this derivative through their amino groups. The same reaction was also used to couple immunoglobulin to periodate-oxidised cellulose, but the amount of protein that could be coupled was far less than that achieved with the glutaraldehyde-activated aminocellulose.

The mixed anhydride technique with CM-cellulose and ethylchloroformate produced a very granular immunoabsorbent. This almost certainly resulted from the ability of the ethylchloroformate to cross-link the protein.

Table 2.7 lists various guinea pig anti insulin immunoabsorbents. The percentages of available antibody covalently bound to the carrier were of the same order as was achieved for sheep anti-human IgG.
### Table 2.6  
**Coupling of Immunoglobulin to Bromoacetylcellulose**

<table>
<thead>
<tr>
<th>pH</th>
<th>After dialysis</th>
<th>Physically adsorbed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.8</td>
<td>turbid</td>
<td>72</td>
</tr>
<tr>
<td>4.2</td>
<td>precipitate</td>
<td>88</td>
</tr>
<tr>
<td>4.6</td>
<td>precipitate</td>
<td>92</td>
</tr>
<tr>
<td>5.0</td>
<td>precipitate</td>
<td>91</td>
</tr>
<tr>
<td>5.4</td>
<td>clear</td>
<td>100</td>
</tr>
<tr>
<td>5.8</td>
<td>clear</td>
<td>96</td>
</tr>
<tr>
<td>6.6</td>
<td>clear</td>
<td>98</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cellulose Derivative</th>
<th>Coupling conditions</th>
<th>Counts Bound %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromoacetyl (1.0 meq Br/g)</td>
<td>pH 6.0</td>
<td>36.7</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>pH 9.0</td>
<td>47.2</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>(1.33 meq Br/g)</td>
<td>85.1</td>
</tr>
<tr>
<td>chloro-s-triazine (0.21% N)</td>
<td>-</td>
<td>69.4</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>(0.50% N)</td>
<td>80.3</td>
</tr>
<tr>
<td>chloro-s-triazine CM-cellulose (0.21% N)</td>
<td>-</td>
<td>11.4</td>
</tr>
<tr>
<td>Aminoethyl</td>
<td>EDC</td>
<td>26.6</td>
</tr>
<tr>
<td>Carboxymethyl</td>
<td>EDC</td>
<td>52.7</td>
</tr>
<tr>
<td>Bromoacetamidoethyl (0.27 meq Br/g)</td>
<td>-</td>
<td>24.0</td>
</tr>
<tr>
<td>Bromoacetamidopentyl (0.23 meq Br/g)</td>
<td>-</td>
<td>46.5</td>
</tr>
<tr>
<td>Bromoacetamidodipropylamine (0.25 meq Br/g)</td>
<td>-</td>
<td>30.0</td>
</tr>
</tbody>
</table>

### Table 2.7  
**Guinea pig anti-insulin immunoadsorbents prepared by reaction of cellulose derivatives with 0.5 ml guinea pig anti-bovine insulin and ¹²⁵I trace labelled guinea pig immunoglobulin.** Counts bound represent the means of triplicates.
Time Dependence of Immunoadsorption

The time dependence of immunoadsorption was investigated for immunoadsorbents prepared with antibodies to the Fc region of human IgG, porcine insulin and human placental lactogen (HPL).

Immunoadsorbents prepared from bromoacetylcellulose and diazotised N(3-aminobenzylxoxymethyl)cellulose and two different antisera to human IgG were incubated with aliquots of labelled IgG and a dilution of human serum equivalent to that necessary to inhibit 50% binding of the labelled material. Incubations were carried out for different periods of time, and terminated by centrifuging the assay tubes and washing the immunoadsorbent with borate buffered saline. The extent of binding of the IgG was determined by measuring the bound radioactivity in a γ-counter (Fig. 2.7). Binding of the antigen by immunoadsorbents prepared with antibodies from sheep 57 was essentially complete within 3 hr, whereas the preparations with antibody from sheep 51 bound only 80% of their total capacity for antigen in the first 5 hr. Controls were run from which the immunoadsorbents were omitted, and it was found that a constant background count was achieved within the first 30 min. representing < 2.5% of the total counts bound. The time required to achieve maximum binding of antigen is dependent upon the avidities of the antibodies and any influence different carriers may have on the reaction can only be assessed when antibodies from the same animal are considered. A comparison of several different antibody immunoadsorbents prepared with AS 57 revealed no significant difference in the time required to bind equivalent amounts of antigen. However, the method of immobilisation did appear to influence the avidity of AS 51 as demonstrated by the difference between binding curves obtained with bromoacetyl- and diazotised N(3-aminobenzylxoxymethyl)cellulose based immunoadsorbents (Fig. 2.7). Time curves for the
binding of HPL and porcine insulin by their appropriate immunoadsorbents are shown in Fig. 2.8. Both immunoadsorbents were prepared with bromoacetylcellulose.

Fig. 2.7  Time dependence of the binding of IgG by anti-IgG immunoadsorbents. Solid symbols - AS51 and open symbols - AS57. ○ and ◆ - immunoadsorbents prepared from diazotised N-3-amino-benzylloxymethyl cellulose. □ and □ - immunoadsorbents prepared from bromoacetyl cellulose.

Fig. 2.8  Time dependence of the binding of insulin ○ and human placental lactogen ◆ by appropriate immunoadsorbents. (Insulin antiserum - AS243/1).
**pH Optimum for Immunoadsorption**

The immunoadsorbent $B_{50}$ values were determined at pH 8.4 in 0.05 M borate-phosphate buffer containing 0.5% BSA. Aliquots of the immunoadsorbents were incubated at room temperature for 16 hr with $^{125}$I-IgG and dilutions of IgG in a range of borate-phosphate buffers of different pH. Over the pH range 6.0 to 9.0 the antigen binding capacities of the preparations were constant within the limits of experimental error. Ionic groups on the carrier did not appear to affect the pH optima of the reactions, but the possible significance of such groups is discussed later in relation to non-specific adsorption and overall capacity.

**Non-specific Binding of Antigen**

In the assessment of immunoadsorbents non-specific binding of protein was routinely monitored with iodinated sheep IgG and porcine insulin.

**a) Buffers for Immunoadsorption**

Early experiments were conducted using a 0.05M phosphate buffer pH 7.4 containing 0.5% BSA, 0.15M sodium chloride and 0.01M EDTA. Triplicate measurements frequently showed a variation greater than 5% of their mean values, and as such were considered unsatisfactory. Experiments were conducted to compare the relative merits of borate and phosphate buffers. BSA was used routinely to minimise non-specific binding to the polystyrene tubes used for the assays.

Borate and borate-phosphate buffers gave consistently lower values for the binding of labelled IgG than were obtained with phosphate buffer (Table 2.8).

The interaction of borate ions with the 2' and 3' hydroxyl groups in the hexose ring may contribute to this result. However, in blocking the hydroxyl groups on the cellulose the borate ion introduces two hydroxyl groups of its own.
Table 2.8 The effect of buffer ions on the antigen binding capacity of anti-IgG immunoadsorbents

<table>
<thead>
<tr>
<th>Cellulose Derivative</th>
<th>Counts bound (cpm) + SEM</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphate buffer</td>
<td>Borate buffer</td>
<td></td>
</tr>
<tr>
<td>N-3-aminobenzyloxymethyl cellulose</td>
<td>2446 ± 167</td>
<td>2075 ± 69</td>
<td></td>
</tr>
<tr>
<td>4-aminobenzyl cellulose</td>
<td>1049 ± 52</td>
<td>901 ± 34</td>
<td></td>
</tr>
<tr>
<td>Bromoacetyl cellulose (pH 6.0)</td>
<td>1907 ± 70</td>
<td>1707 ± 68</td>
<td></td>
</tr>
<tr>
<td>Bromoacetyl cellulose (pH 8.9)</td>
<td>1768 ± 81</td>
<td>1609 ± 89</td>
<td></td>
</tr>
<tr>
<td>Chlorotriazinyl cellulose</td>
<td>1817 ± 104</td>
<td>1406 ± 53</td>
<td></td>
</tr>
<tr>
<td>Aminoethyl cellulose (glutaraldehyde-coupled)</td>
<td>2393 ± 142</td>
<td>2299 ± 88</td>
<td></td>
</tr>
</tbody>
</table>

Figures represent the mean values (n = 6) of the counts bound by equal aliquots of the immunoadsorbents in the presence of a 1 in $10^4$ dilution of a standard human serum sample.

The buffers used are designated B1 and B2 in Materials and Methods section of this chapter.

(SEM - standard error of the mean)
b) **Blocking of Unreacted Groups on the Carrier**

Blocking of unreacted groups was effected in most cases by reaction with glycine, BSA, 2-aminoethanol or 3-aminopropan-1-ol as detailed in Methods. Exceptions included reagents such as β-naphthol and tyrosine, sodium borohydride and ammonium ions employed for reaction with diazonium groups, aldehydes and chloro-s-triazinyl derivatives respectively.

Sheep IgG, containing antibodies specific for the Fc region of human IgG, was coupled to diazotised N-(3-aminobenzyloxyethyl) cellulose. The preparation was divided, and unreacted diazo groups were blocked by reaction with β-naphthol or tyrosine. Similar experiments were undertaken with immunoabsorbents prepared by the oxirane method and with cyanogen bromide activated cellulose. Glycine, BSA and 3-aminopropan-1-ol were used as blocking agents.

From Fig. 2.9 (a) it can be seen that the immunoabsorbents prepared from the diazotised derivative gave rise to similar assay curves except at lower concentrations of unlabelled antigen. Similar curves were obtained with two preparations employing the cellulose derivative containing 121 μEq NH₂ groups per gram. Because of the instability of diazonium groups it might have been reasonable to suppose that any unreacted groups would have decomposed making blocking unnecessary. However, on the addition of β-naphthol the derivative changed from a pale yellow colour to orange, indicating a reaction with an azo dye as the product.

The assay curves obtained with immunoabsorbents prepared by the oxirane method (Fig. 2.9 (b)) and treated with 3-aminopropan-1-ol or glycine are compatible with a failure on the part of the amino acid to block unreacted oxirane residues on the carrier. This conclusion was supported by the
finding that the glycine treated immunoadsorbent bound 33% more iodinated sheep IgG than the one treated with the amino alcohol. Differences in the amounts of labelled antigen bound were most marked at low concentrations of unlabelled antigen. Consequently erroneously high results would have been obtained at low antigen concentrations had the curve been used as a standard in a radioimmunoassay. This finding would explain the results of Polmar et al (121) who obtained apparently high values when measuring low levels of IgE, as compared with those obtained from a double antibody assay.

The results with cyanogen bromide activated cellulose were similar to those for the previous experiment: 3-aminopropan-1-ol proved to be more satisfactory than BSA or glycine (Fig. 2.10(a)).

The use of the reducing agent sodium borohydride for the blocking of aldehyde groups proved satisfactory. A comparison of the antigen binding capacities of blocked and unblocked immunoadsorbents prepared from glutaraldehyde activated aminoethyl and aminopentyl celluloses is made in Fig. 2.10(b). Again failure to block unreacted groups on the carrier leads to the incorporation of high levels of iodinated antigen at low concentrations of unlabelled antigen. Although no direct evidence was obtained against the use of sodium borohydride it was felt that its strong reducing properties could be detrimental to the protein, and for this reason it was not used routinely.

The introduction of ionisable groups on to the surface of the carrier during the blocking process may influence the antibody configuration and hence its capacity for antigen. Consequently aminoalcohols should be used in preference to amino acids.
Fig. 2.9
A comparison of the effectiveness of various blocking agents.

(a) Immunoadsorbent: sheep anti-human IgG(Fc)/diazotised N-(3-aminobenzylxoyxymethyl)cellulose.
Blocking agents: β-naphthol (o) and tyrosine (e).

(b) Immunoadsorbent: sheep anti-human IgG(Fc)/epichlorohydrin activated cellulose (oxirane).
Blocking agents: glycine (•) and 3-aminopropan-1-ol (▲).

Fig. 2.10
A comparison of the effectiveness of various blocking agents.

(a) Immunoadsorbent: sheep anti-human IgG(Fc)/cyanogen bromide activated cellulose.
Blocking agents: BSA (■), 3-aminopropan-1-ol (▲) and glycine (○).

(b) Immunoadsorbent: sheep anti-human IgG(Fc)/(i) aminoethyl cellulose (□ and ■) and (ii) aminopentyl cellulose (○ and ○). Both derivatives activated by glutaraldehyde.
Blocking agent: sodium borohydride (□ and ○).
No blocking agent - (■ and ○).
The Influence of the Degree of Substitution of Active Groups on the Carrier

Cellulose derivatives which could easily be prepared with different degrees of substitution included N-(3-aminobenzyloxymethyl) cellulose, bromoacetyl cellulose, 3-amino-5-chlorotriazinyl cellulose and aminoalkyl cellulose (Table 2.4 p.57). In all cases a reduction in the number of active groups lead to a reduction in the amount of antibody which could be covalently coupled (Table 2.5 p.58) Figs. 2.11, 13, 14, 15 and 16 (p.80 et seq.).

A set of titration curves for three immunoadsorbents based on N-(3-aminobenzyloxymethyl) celluloses indicated that titres* were similar with the higher levels of substitution and increased slightly when the number of covalent linkages was reduced (Table 2.9 p.79). The titre of the immunoadsorbent prepared with 4-aminobenzyl cellulose was less than the other three. Standard dose response curves obtained with these preparations are shown in Fig. 2.11. In order to assist interpretation of these curves theoretical curves were constructed (see Appendix to this chapter).

The smaller number of covalent bonds involved in immunoadsorbent 3 resulted in less damage to the antibody population, and consequently the titre of this preparation was slightly higher than those of the other two which employ a larger number of covalent linkages. The B/B₀ vs. dose response curve for 3 is more sensitive than the other two at low antigen dose (Fig. 2.11(b)) and this may be explained in terms of a heterogeneous antibody population. If high avidity antibody were more susceptible to damage by immobilisation than low avidity material, then the chances of damage may be made greater by increasing the number of

---

* (Titre - the dilution of immunoadsorbent required to bind 50% of the labelled antigen at zero dose of unlabelled material).
covalent bonds involved in coupling to the carrier. If this were the case then immunoadsorbent 3 would contain more undamaged high avidity antibody than 2 and 1. Assuming that the low avidity antibody is not saturated at low dose, then the dose response curve obtained for preparation 3 would be the most sensitive at low dose. Such is the case in Fig. 2.11(b). The packing density of the antibody is very unlikely to influence the activity of these preparations because of the small quantities of protein involved. The results of elution experiments suggest that 3 has a lower overall avidity than 2 (Fig. 2.12), a finding not in keeping with conclusions drawn from the dose response curve.

Fig. 2.13 shows the $B/B_0$ vs. log dose response curves obtained for two adsorbents with higher protein contents. Also, AS 57 was employed as opposed to AS 55 in the previous experiments. The higher degree of activation of the carrier resulted in immobilisation of a larger amount of protein, but this was paralleled by a considerable loss of antibody activity and a resultant drop in titre.

It was difficult to make a direct comparison with the preparation based on 4-aminobenzyl cellulose because of differences in chemical structure and the fact that this derivative had a comparatively large particle size. However, immobilisation resulted in considerably more antibody damage than was experienced with the other preparations as reflected in the titres.

Meaningful interpretation of Scatchard plots (Fig. 2.11(c)) was difficult because of the heterogenous nature of the antisera employed.

At pH 8.75, the pH used in the preparations of the immunoadsorbents discussed here, the weakly electrophilic diazonium ions react with the phenoxide ion of tyrosine. The other immunoadsorbents to be discussed in this section, ones based on bromoacetyl celluloses & aminochlorotriazinyl celluloses, all involve immobilisation of the protein via its amino groups.
Dose response curves for a series of bromoacetyl and 3-amino-5-chlorotriazinyl derivatives are shown in Figs. 2.14 and 2.15 respectively. The titres of the immunoadsorbents decreased with an increase in the number of potential covalent linkages between the carrier and protein. Higher levels of substitution of bromoacetyl or triazinyl groups on the carrier resulted in the immobilisation of larger amounts of protein and an increased antibody packing density. The dose response curves indicated that the immunoadsorbents with the highest protein contents were the least active. The results of elution experiments (Fig. 2.12) suggest that the most effectively immobilised antibodies have a higher overall avidity because smaller percentages of adsorbed antigen were recoverable by elution. There are several possible explanations for this.

High packing densities may result in more than one antibody reacting with different antigenic determinants on a single antigen molecule. The possibility of such reactions introduces a new phenomenon - 'immunoadsorbent avidity' (as opposed to antibody avidity).

Increasing the degree of substitution of active groups on the carrier increases the number of potential covalent linkages and also leads to larger amounts of protein being coupled. Consequently the three dimensional structure of the molecule will be more restricted and the conformational changes necessary for antigen binding become more difficult to achieve. High avidity antibody, where a tight lock-key fit is involved, is most likely to be affected. A relatively poor fit, which is in keeping with a low avidity antibody, is likely to require less conformational juggling on the part of the antibody. Restrictions may however destroy any small measure of recognition a low avidity antibody shows for an antigen.

By increasing the packing density of the antibody the amount of 'solid' required to introduce the same amount of antibody into an assay
system is reduced. The effective antibody concentration is therefore greater while the distribution of antigen in the liquid phase is unaltered. This will tend to push the antibody-antigen reaction to the right in the equation

\[ \text{Ab} + \text{Ag} \rightleftharpoons \text{Ab.Ag} \]

and so apparently increase the equilibrium constant.

The recoveries achieved on elution from anti-IgG triazinyl cellulose preparations were on the whole lower (Fig. 2.12) than for the other immunoadsorbents compared here. However, this was not consistent for all preparations of this type, a finding presumably attributable to variability of the cellulose derivative (e.g. Table 2.13 - equivalent recoveries of antigen were made from immunoadsorbents prepared from bromoacetyl cellulose and aminochlorotriazinyl cellulose when treated with glycine-HCl buffers).

The degree of substitution of active groups also influenced antibody activity when the protein was coupled to aminoalkyl celluloses prepared from cellulose activated with 50 and 300 mg quantities of cyanogen bromide per gram of cellulose. In the case of each of the three aminoalkyl celluloses tested, i.e. aminoethyl, aminopentyl and 3-aminodipropylamine, the antibody capacity or titre was less when coupled to the more highly substituted derivative (Fig. 2.16 & 2.17). Dose response curves plotted as the ratio \( \frac{B}{B_o} \) vs. log dose showed that immunoadsorbents prepared from ethyl and pentyl derivatives were more sensitive at low antigen dose when the potential number of covalent linkages between the carrier and protein was greater. Comparable dose response curves were obtained with the different 3-aminodipropylamine derivatives. These results were substantiated by the findings of elution experiments (Fig. 2.16, p. 88) where the difference in percentage recoveries between the two 3-aminodipropylamine derivatives was less than those between the other pairs of immunoadsorbents. This suggested that
immunoabsorbents containing a higher potential number of covalent bonds between the carrier and protein had a higher avidity. This cannot be explained in terms of antibody packing density because the protein contents of the different immunoabsorbents were not of the right order. The more effective immobilisation involving a greater number of covalent bonds is perhaps more damaging to low avidity antibody, destroying any small degree of recognition it showed for the antigen.

The three diamines employed in this study may have shown different reactivities towards the cellulose imidocarbonate resulting in different degrees of substitution. However, analysis revealed that the degrees of substitution of the three derivatives were comparable, being of the order of 50-65 \( \mu \text{eq.} \) \( \text{NH}_2 \) groups per gram. Had there been wide variation then the antibody activity could also have been influenced by the extent of covalent coupling as discussed previously.

One might reasonably expect longer spacer arms to make any active groups they carried more readily available for reaction with the protein, and as a result the protein would be more effectively immobilised with a greater chance of antigen binding sites being sterically hindered. Both pentyl and dipropylamine chains are flexible, the latter more so because it is longer and also contains a secondary amino group which is capable of spontaneous inversion, so imparting even greater flexibility to the chain. Furthermore, under the mild alkaline conditions employed for coupling the protein there is a possibility of hydrogen bonding involving the long electron pair of the secondary amine and hydroxyl groups on the carrier. If such bonding were to occur the effective length of the dipropylamine chain would be considerably reduced. Finally, the buffering action of this secondary amino group may affect the micro-environment of the spacer arm and so influence the reaction with the protein. It is this
last point which may account for the fact that the bromoacetamidodipropyl-
amine derivative coupled considerably less protein than the pentyl
derivative (Fig. 2.19, p. 95). The affect is not observed when glutaraldehyde
is used as a coupling reagent because this effectively increases the
length of the spacer arm by a further four carbon units. Protein cross-
linking conceals any such affect when carbodiimide is used as a coupling
agent.

The combination of longer spacer arm and high density results in
very effective immobilisation of the protein and loss of activity due
to steric hinderance and restriction on possible conformational changes.
In the case of shorter arms the active groups they carry find amino groups
in the protein less accessible with the result that the protein retains
more freedom. A similar result is achieved by using a smaller number of
spacer arms. Such a hypothesis explains the observations for glutaraldehyde-
coupled protein (Fig. 2.16 & 2.17, p. 95). Spacer arms are therefore only of
benefit when they are employed in relatively low numbers. Then, the arm
holds the antibody away from the surface of the carrier and so reduces the
steric effects the carrier may have upon the protein and the number of
covalent linkages is not sufficient to have too significant an effect upon
the antibody activity.

Whereas a high antibody:carrier ratio may be considered desirable from
the point of view of adsorption, the results presented here suggest that this
is not the case as far as elution is concerned. The packing density of
the antibody should be as high as can be achieved without introducing the
phenomenon of 'immunoadsorbent avidity'. Furthermore, the degree of
activation of the carrier should be as low as is practicable so as
to minimise the number of covalent linkages between the carrier and the
protein and cause as little damage to the antibody as is possible in the way of three dimensional flexibility and blocking of active sites.

Not only are the immobilisation conditions critical to the retention of biological activity, but so also is the choice of antiserum.

Table 2.9 The relationship between titre and the extent of covalent bonding between antibody and carrier. (Determined by the degree of substitution of active groups - amino groups - in the carrier).

<table>
<thead>
<tr>
<th>µeq. NH₂ groups/g</th>
<th>mg protein per g</th>
<th>Titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>121</td>
<td>11.1</td>
</tr>
<tr>
<td>2</td>
<td>67</td>
<td>9.2</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>4.3</td>
</tr>
</tbody>
</table>

1, 2 and 3 - N-(3-aminobenzylxoxymethyl) cellulose.
4 - 4-aminobenzyl cellulose.

* - the dilution of immunoadsorbent required to bind 50% of the labelled antigen at zero dose of unlabelled material. Expressed as an equivalent dilution of antiserum immunoglobulin preparation.
Fig. 2.11

(a) Dose response curves, $B$ vs. log dose. 1, 2 and 3, N-(3-amino benzylloxymethyl)cellulose; 4, 4-aminobenzyl cellulose.

1. - 121 μeq NH$_2$ groups/g, 11.1 mg protein/g.
2. - 67 μeq NH$_2$ groups/g, 9.2 mg protein/g.
3. - 18 μeq NH$_2$ groups/g, 4.0 mg protein/g.
4. - 80 μeq NH$_2$ groups/g, 4.3 mg protein/g.

(b) Dose response curve $B/B_0$ vs. dose. Data as in (a).

(c) Scatchard plot. Immunoadsorbents as in (a).

Immunoadsorbents have been diluted so as to have the same concentration of antibody protein per ml.

Prepared with immunoglobulin from AS 55.
x 0.67 μ moles IgG
Fig. 2.12

Percentage recoveries of antigen (IgG) with seven different eluting fluids. *Means of triplicates*.

(a) glycine 50mM pH 2.5
(b) sodium thiocyanate 3M phosphate buffered pH 6.0
(c) sodium thiocyanate 3M tris buffered pH 9.0.
(d) acetic acid 1M.
(e) propionic acid 1M.
(f) urea 8M tris buffered pH 7.0.
(g) magnesium chloride 2.5M.

(i) Immunoadsorbents 2, 3 and 4 (Fig. 2.11).

(ii) Immunoadsorbents prepared from bromoacetylcellulose.

1. 1.67 meq.Br/g cellulose - 39.8 mg protein/g.
2. 0.49 meq.Br/g cellulose - 12.1 mg protein/g.

(iii) Immunoadsorbents prepared from 3-amino-5-chlorotriazinyl cellulose.

1. 0.42% N - 18.1 mg protein/g.
2. 0.05% N - 6.7 mg protein/g.
Fig. 2.12

Percentage of adsorbed human IgG recovered by elution

(i)

(ii)

(iii)
Fig. 2.13
Dose response curves, $B/B_0$ vs. IgG dose, for two immunoabsorbents prepared from diazotised N-(3-aminobenzylxymethyl) cellulose and immunoglobulin from AS 57.

<table>
<thead>
<tr>
<th>meq. NH$_2$ groups per g cellulose</th>
<th>mg protein per g</th>
<th>Titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>121</td>
<td>33.9</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>11.4</td>
</tr>
</tbody>
</table>

* These figures are not comparable with those in Fig. 2.11 or Table 2.9.

---

Fig. 2.14
(a) Dose response curves, $B$ vs. IgG dose, for immunoabsorbents derived from bromoacetyl cellulose.

<table>
<thead>
<tr>
<th>meq. Br per g</th>
<th>mg protein per g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.49</td>
</tr>
<tr>
<td>2</td>
<td>0.73</td>
</tr>
<tr>
<td>3</td>
<td>1.67</td>
</tr>
</tbody>
</table>

(b) Dose response curves, $B/B_0$ vs. IgG dose. Immunoabsorbents as in (a).
Fig. 2.13

![Graph showing dose response curve with dose on the x-axis and \( \frac{B}{B_0} \) on the y-axis.](image)

- Curve 5
- Curve 6
Fig. 2.15

(a) Dose response curves, B vs. IgG dose, for immunoadsorbents prepared with 3-amino-5-chlorotriazinyl celluloses.

<table>
<thead>
<tr>
<th>%N in derivative</th>
<th>mg protein per g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>0.16</td>
</tr>
<tr>
<td>3</td>
<td>0.42</td>
</tr>
</tbody>
</table>

(b) Dose response curves, B/B₀ vs. IgG dose. Data as in (a).

Fig. 2.16

(a) Dose response curves for immunoadsorbents prepared from amidoalkyl cellulose derivatives with different chain lengths (glutaraldehyde activated).

Cellulose activated with 50 mg cyanogen bromide per g:

(i) o - aminoethyl cellulose 12.4 mg protein per g.
(ii) □ - aminopentyl cellulose 30.9 mg protein per g.
(iii) ▲ - aminodipropylamine cellulose 22.4 mg protein per g.

Cellulose activated with 300 mg cyanogen bromide per g:

(iv) o - aminoethyl 23.7 mg protein per g.
(v) □ - aminopentyl 22.1 mg protein per g.
(vi) ▲ - aminodipropylamine cellulose 15.9 mg protein per g.

Immunoadsorbents were diluted to the same protein concentration per ml.

(b) The percentages of adsorbed antigen (IgG) recovered by treatment of the immunoadsorbent with eluting agents. Other data as for Fig. 2.16(a).
Fig. 2.16 (a)

Fig. 2.16 (b)
The Use of Spacer Arms between the Carrier and Protein

Spacer arms were introduced on to the carrier by first reacting it with cyanogen bromide and then reacting the resulting imidocarbonate with 1,2-diaminoethane, 1,5-diaminopentane or 3,3'-diaminodipropylamine.

The effects of different chain lengths at two different levels of substitution of the carrier are shown in Figs. 2.16(p.88) and 2.17, protein having been coupled with glutaraldehyde. As mentioned previously, immunoadsorbent titre was less at the higher level of aminoalkyl substitution on the carrier, and decreased in the order ethyl > dipropylamine > pentyl at the higher level of substitution and dipropylamine > ethyl > pentyl at the lower levels.

In order to confirm that differences were attributable to variation in chain length as opposed to the method of coupling, experiments were conducted in which the protein was coupled to the carrier either by the carbodiimide method or after 'activating' the aminocellulose with either glutaradehyde or O-bromoacetyl-N-hydroxysuccinimide. The carbodiimide method resulted in immobilisation of a high percentage of the added protein, but the titre or capacity of the antibody was very low. The immunoadsorbent, although particulate, had a somewhat gelatinous appearance, and it was concluded that protein cross-linking had occurred as well as coupling to the carrier. This resulted in the blocking of a high proportion of the antigen binding sites on the antibody. Titres of those immunoadsorbents prepared by the 'glutaraldehyde method' were of the order dipropylamine > ethyl > pentyl (Fig. 2.18) and this order was also found to apply to the three bromoacetamido-alkyl derivatives (Fig. 2.19). In the latter case immunoadsorbents prepared from bromoacetyl cellulose were included for comparison and were found to have titres less than the ethyl derivative but greater than the pentyl derivative. Equilibrium constants were confirmed as being in the order
dipropylamine > ethyl > acetyl > pentyl by Steward-Petty plots (Appendix 1).

A decrease in antibody activity associated with the different spacer arm lengths in the order dipropylamine > ethyl > acetyl may be explained in terms of the reduced steric effects of the carrier on the antibody. However, why the pentyl derivative consistently had less activity than the ethyl derivative, and even the acetyl derivative in the case of bromo-acetyl cellulose, was not immediately obvious.

N'-Hydroxysuccinimide esters of cellulose were prepared from aminoethyl and aminodipropylamine cellulose. The shorter chain derivative coupled larger quantities of protein than did the longer chain preparation (Table 2.5,p.58) but the antibody coupled to the latter had a higher titre (Table 2.11,p.104). The lower level of protein coupling may be due to a competitive aminolysis reaction on the part of the secondary amine or the buffering influence of this group on the microenvironment of the spacer arm, thus influencing activity and specificity of the N-hydroxy-succinimide towards amino groups on the protein. As mentioned earlier (p.61) it is possible that the method of synthesis of bromoacetamidoalkyl cellulose results in incorporation of some N-hydroxysuccinimide groups through reaction of the bromine function with the amino groups on the carrier. These groups would be available to react with the protein along with the bromoacetamido groups.

Table 2.10 Titres of the immunoadsorbents described in Fig. 2.17

<table>
<thead>
<tr>
<th></th>
<th>Glutaraldehyde</th>
<th>Carbodiimide</th>
</tr>
</thead>
<tbody>
<tr>
<td>aminoethyl</td>
<td>1/730</td>
<td>1/180</td>
</tr>
<tr>
<td>aminopentyl</td>
<td>1/575</td>
<td>-</td>
</tr>
<tr>
<td>aminodipropylamine</td>
<td>1/955</td>
<td>1/70</td>
</tr>
</tbody>
</table>
Fig. 2.17
Titration curves for immunoadsorbents to human IgG. Aminoalkyl derivatives of different chain length and degree of substitution activated with glutaraldehyde:

Cellulose activated with 50 mg cyanogen bromide per gram.
- o - aminoethyl
- □ - aminopentyl
- ▲ - aminodipropylamine

Cellulose activated with 300 mg cyanogen bromide per gram.
- o - aminoethyl
- □ - aminopentyl
- ▲ - aminodipropylamine

Fig. 2.18
Titration curves for immunoadsorbents to human IgG. A comparison of activities retained after coupling the antibody to aminoalkyl cellulos es (i) with glutaraldehyde and (ii) in the presence of carbodiimide.

Immunoadsorbents were standardised to the same protein concentration per ml before dilutions were prepared.

<table>
<thead>
<tr>
<th>Coupling method</th>
<th>Glutaraldehyde</th>
<th>Carbodiimide</th>
</tr>
</thead>
<tbody>
<tr>
<td>aminoethyl</td>
<td>23.3 ▲</td>
<td>19.8 ▲</td>
</tr>
<tr>
<td>aminopentyl</td>
<td>15.6 □</td>
<td>20.3 ■</td>
</tr>
<tr>
<td>aminodipropylamine</td>
<td>17.7 ○</td>
<td>21.6 ●</td>
</tr>
</tbody>
</table>

mg protein per gram of cellulose.
Percentage counts bound

Immunoadsorbent dilution

Fig. 2.17

Fig. 2.18
Fig. 2.19

Dose response curves for a series of bromoacetamido derivatives of cellulose. (i) bromoacetyl; (ii) bromoacetamidoethyl; (iii) bromoacetamidopentyl and (iv) bromoacetamido-3-dipropylamine.

<table>
<thead>
<tr>
<th></th>
<th>meq Br₂</th>
<th>Protein mg/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>0.43</td>
<td>48.2</td>
</tr>
<tr>
<td>(2)</td>
<td>0.17</td>
<td>12.5</td>
</tr>
<tr>
<td>(3)</td>
<td>0.18</td>
<td>49.5</td>
</tr>
<tr>
<td>(4)</td>
<td>0.18</td>
<td>18.1</td>
</tr>
</tbody>
</table>

(a) Counts bound (B) vs. dose.
(b) $B/B_0$ vs. dose.

Fig. 2.20

Dose response curves, B vs. dose, illustrating the effect of coupling pH upon activity of the immobilised antibody.

<table>
<thead>
<tr>
<th>Coupling pH</th>
<th>mg protein per g.</th>
<th>Titre**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Carboxyethyl cellulose</td>
<td>6.5</td>
<td>43.8</td>
</tr>
<tr>
<td>2. Carboxyethyl cellulose</td>
<td>8.75</td>
<td>55.1</td>
</tr>
<tr>
<td>3. Aminoethyl*</td>
<td>7.0</td>
<td>29.0</td>
</tr>
<tr>
<td>4. Aminoethyl</td>
<td>8.75</td>
<td>22.9</td>
</tr>
<tr>
<td>5. Aminodipropylamine*</td>
<td>7.0</td>
<td>28.7</td>
</tr>
<tr>
<td>6. Aminodipropylamine</td>
<td>8.75</td>
<td>14.8</td>
</tr>
</tbody>
</table>

* - activated with glutaraldehyde.
** - data obtained from titration curves.
Fig. 2.20

Counts bound vs. Dose (µg)

10^3 Counts bound

Dose (µg)

0.1 1.0 10.0 100.0 1000.0
Influence of pH on the Coupling Reaction

The reaction of immunoglobulin with diazotised 4-aminobenzyl cellulose and N-(3-aminobenzylxoxymethyl) cellulose was more satisfactory at pH 6.0 than at pH 8.75 in terms of the amount of protein immobilised (Table 2.5, p.5) but coupling antibody at the higher pH produced immunoadsorbents of higher titre, i.e. the molar ratio of antigen to immobilised antibody was increased (Table 2.11).

Glutaraldehyde aminoalkyl celluloses were reacted with immunoglobulin at pH 7.0 and 8.75. Larger amounts of the added protein were immobilised at the lower pH, but coupling at the higher pH produced an immunoadsorbent with greater capacity (Fig. 2.20, p.95). Dose response curves plotted in terms of the $B/B_0$ ratio showed that the avidities were not significantly different. Similar percentage amounts of antigen were recovered by elution from the two types of preparation.

Antibodies were coupled to carboxyethyl cellulose at either pH 6.5 or 8.75 in the presence of the water-soluble carbodiimide EDC. At the lower pH the product was invariably obtained in an aggregated form, and if mixing was inadequate during the coupling procedure large gelatinous lumps were formed. The product of the reaction at pH 8.75 was homogenous, but its gelatinous nature, attributable to its ion exchange properties, made it difficult to handle in the assay. The titre of this preparation was greater than that of the immunoadsorbent prepared at the lower pH (Fig. 2.20, p.95).

Similar results were obtained for the coupling of immunoglobulin to cyanogen bromide activated cellulose and bromoacetyl cellulose. Comparable percentages of adsorbed antigen were recovered by elution.
The advantage of coupling antibody to diazotised N-(3-aminobenzyl-oxymethyl) cellulose at pH 6.0 in terms of the amount of antibody coupled was outweighed by the loss of antibody activity. Coupling at pH 8.75 was much more satisfactory in this respect. The higher pH favours reaction with the phenoxide ring of tyrosine, which at lower pH exists as the phenol which is much too unreactive to be attacked by the weakly electrophilic diazonium ion. The imidazole group of histidine is active at the lower pH.

Glutaraldehyde-activated aminocelluloses, bromoacetyl cellulose and cellulose imidocarbonate all react with amino groups in the protein. Alkaline conditions around pH 9.0 favour reaction with ε-amino groups of lysine residues, whereas lower pH values favour reaction with N-terminal α-amino groups and histidine. The antigen-binding sites reside at the N-terminal ends of neighbouring heavy and light chains, and consequently any coupling reaction involving these groups will lead to blocking of the antigen binding site.
The Choice of Conditions for Protein Immobilisation

The methods of antibody immobilisation which produced immunoadsorbents conforming closely to the criteria for an 'ideal immunoadsorbent' defined at the beginning of this chapter were selected for further studies.

Antibody activity has been considered in terms of equivalent amounts of immobilised protein. As has been seen, the immobilisation of large amounts of antibody and the resultant high ratio of antibody to carrier frequently results in the loss of antibody activity. Even if there is no loss the percentage of adsorbed antigen recoverable by elution may be less because of 'immunoadsorbent avidity'. In view of these findings it has not been possible to satisfy all the criteria for an 'ideal immunoadsorbent'.

Titration curves for a series of immunoadsorbents prepared under optimum conditions on the same occasion are shown in Fig. 2.21. The highest titre (1/1900) was obtained by coupling antibodies to diazotised N-(3-aminobenzyloxymethyl) cellulose at pH 8.75. Glutaraldehyde-activated aminodipropylamine cellulose reacted with antibody to produce an immunoadsorbent with a similar titre, 1/1860. Immunoadsorbents prepared from aminochlorotriazinyl cellulose, bromoacetyl cellulose and glutaraldehyde-activated aminoethyl cellulose had similar titres ranging from 1/1200 down to 1/1070. The titres of preparations with diazotised 4-aminobenzyl cellulose and aminoethyl and carboxyethyl cellulose (carbodiimide) coupling method - (DCC) were considerably lower, that with carboxymethyl cellulose being so low that it could not be measured. The titre of the AS 51 immunoglobulin preparation was estimated at 1/2800 by the double antibody method. A comparison of the relative immunoadsorbent activities can be drawn from the titres included in Table 2.11. The highest titres were obtained
Titration curves of immunoadsorbents prepared under previously determined optimum conditions.

In sequence from right to left the derivatives used to prepare the immunoadsorbents were:

1. diazotised N-(3-aminobenzyloxymethyl)cellulose
2. aminodipropylamine cellulose (glutaraldehyde activated)
3. aminooethyl cellulose (glutaraldehyde activated)
4. aminochlorotriazinylcellulose
5. bromoacetylcellulose
6. aminooethyl cellulose (N,N'-dicyclohexylcarbodiimide)
7. diazotised 4-aminobenzyl cellulose
8. carboxymethyl cellulose (N,N'-dicyclohexylcarbodiimide)

(Series 1 in Table 2.11)
Table 2.11

Titres of different immunoadsorbent preparations determined from immunoadsorbent titration curves. Series 1, 2, 3 and 4 represent different groups of immunoadsorbent preparations. Titres in each series were determined on the same occasion with reference to the same standard and are directly comparable.
<table>
<thead>
<tr>
<th>Cellulose derivative</th>
<th>Series 1</th>
<th>Series 2</th>
<th>Series 3</th>
<th>Series 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-(3-aminobenzoyloxy)methyl</td>
<td>1/1900</td>
<td>-</td>
<td>-</td>
<td>1/1750</td>
</tr>
<tr>
<td>4-aminobenzyl</td>
<td>1/182</td>
<td>1/300</td>
<td>-</td>
<td>1/300</td>
</tr>
<tr>
<td>Aminoethyl (i)</td>
<td>1/1070</td>
<td>1/1500</td>
<td>-</td>
<td>1/1340</td>
</tr>
<tr>
<td>Aminopentyl (i)</td>
<td>-</td>
<td>1/860</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aminodipropylamine (i)</td>
<td>1/1860</td>
<td>1/1850</td>
<td>-</td>
<td>1/1910</td>
</tr>
<tr>
<td>3-amino-5-chlorotriazinyl</td>
<td>1/1200</td>
<td>1/1400</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bromoacetyl</td>
<td>1/1150</td>
<td>1/1120</td>
<td>1/1105</td>
<td>1/1320</td>
</tr>
<tr>
<td>Bromoacetamidoethyl</td>
<td>-</td>
<td>1/1350</td>
<td>1/1300</td>
<td>-</td>
</tr>
<tr>
<td>Bromoacetamidopentyl</td>
<td>-</td>
<td>-</td>
<td>1/500</td>
<td>-</td>
</tr>
<tr>
<td>Bromoacetamidodipropylamine</td>
<td>-</td>
<td>1/1520</td>
<td>1/1550</td>
<td>-</td>
</tr>
<tr>
<td>Aminoethyl (ii)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/670</td>
</tr>
<tr>
<td>Aminopentyl (ii)</td>
<td>-</td>
<td>very low</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aminodipropylamine (ii)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/750</td>
</tr>
<tr>
<td>Carboxymethyl (iii)</td>
<td>very low</td>
<td>very low</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N-hydroxysuccinimide ester (aminoethyl)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/1200</td>
</tr>
<tr>
<td>N-hydroxysuccinimide ester (aminodipropylamine)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/750</td>
</tr>
<tr>
<td>Carboxymethyl hydrazide</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/1040</td>
</tr>
<tr>
<td>Carboxyethyl (ii)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/950</td>
</tr>
<tr>
<td>Aminoethyl (iii)</td>
<td>1/320</td>
<td>1/300</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aminodipropylamine (iii)</td>
<td>-</td>
<td>No binding</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Imidocarbonate (iv)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/1240</td>
</tr>
<tr>
<td>Oxirane</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/920</td>
</tr>
<tr>
<td>Periodate oxidised (v)</td>
<td>-</td>
<td>&lt; 1/250</td>
<td>-</td>
<td>1/2200</td>
</tr>
</tbody>
</table>

(i) Activated with glutaraldehyde
(ii) Coupled with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
(iii) Coupled with N,N'-dicyclohexylcarbodiimide
(iv) Coupled at pH 8.9.
(v) Oxidised with 0.1 M periodate.
consistently with immunoadsorbents prepared from diazotised N-(3-aminobenzyloxymethyl) and glutaraldehyde-activated aminodipropylamine derivatives. A titre of 1/2200 achieved with antibody coupled to periodate-oxidised cellulose could not be reproduced.

The success of the method involving coupling to diazotised N-(3-aminobenzyloxymethyl) cellulose must lie with the involvement of tyrosine residues and the spacer arm furnished by the benzyloxylethyl group. Coupling via tyrosine residues is not in itself responsible for the retention of a greater percentage of the antibody activity, as is evident from the relatively poor results with diazotised 4-aminobenzyl cellulose. The majority of the other coupling procedures involved protein amino groups. The glutaraldehyde coupling method in conjunction with a dipropylamine spacer arm provided the next most satisfactory immunoadsorbent, and the bromoacetamido derivative gave almost equally satisfactory results. The carbodiimide method proved to be very effective as a means of immobilising protein, but the efficiency as regards retention of activity depended upon the carbodiimide and the carrier. The water-soluble carbodiimide (EDC) was much more satisfactory than DCC which had to be used in a water tetrahydrofuran emulsion. Coupling via amino groups on the carrier and carboxyl groups on the protein was considerably less satisfactory than coupling the protein via its amino groups to a carboxylated carrier. This may point to the greater involvement of carboxyl groups in and around the antigen binding sites of the antibody. Loss of antibody activity in these preparations may be explained in part by cross-linking of the protein.
Elution of Antigen (IgG) from Specific Immunoadsorbents

The relative efficiencies of a variety of different eluting agents were determined, and the results are presented in Figs. 2.12 (p.83), 2.16 (p.88) and Tables 2.12 and 2.13. The findings clearly indicate that the relative efficacies of different eluting agents vary from one immunoadsorbent to another and depend upon the nature of the carrier, the method of coupling and the avidity of the antibody.

The largest recoveries of IgG were generally made under conditions of low pH with 50 mM glycine-HCl buffers or organic acids such as acetic and propionic. The percentage recovery of IgG was proportional to the pH of the eluting acid and, with the possible exception of citrate, was independent of the anionic species. On the other hand, the efficacies of electrolyte solutions are dependent upon the valency of the cation, the ionic species, the electrolyte concentration and the immunoadsorbent. Furthermore, electrolyte solutions were found to be more efficient eluting agents when employed in batch-type purification procedures as opposed to column procedures.

The eluting power of tris-buffered 8 M urea was shown to be comparable to that of 3 M sodium thiocyanate (Fig. 2.12, p.83), but its use as an eluting agent was not entertained beyond a preliminary investigation because of its protein denaturing properties.
Table 2.12

Percentage recoveries of human IgG from sheep anti-human IgG(Fc) immunoadsorbents as measured by the recovery of $^{125}$I-IgG.

<table>
<thead>
<tr>
<th>Cellulose derivative</th>
<th>% recoveries of adsorbed IgG*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glycine-HCl pH2.5</td>
</tr>
<tr>
<td>Series 1. Table 2.11</td>
<td></td>
</tr>
<tr>
<td>Diazotised N- (3-amino-benzylloxyethyl)</td>
<td>53.1</td>
</tr>
<tr>
<td>Bromoacetyl</td>
<td>46.6</td>
</tr>
<tr>
<td>Aminolchlorotriazinyl</td>
<td>59.0</td>
</tr>
<tr>
<td>Aminodipropylamine$^1$</td>
<td>71.0</td>
</tr>
<tr>
<td>Carboxymethyl</td>
<td>20.2</td>
</tr>
<tr>
<td>Diazotised 4-aminobenzyl</td>
<td>52.0</td>
</tr>
<tr>
<td>Series 4. Table 2.11</td>
<td>pH 2.0</td>
</tr>
<tr>
<td>Diazotised N- (3-amino-benzylloxyethyl)</td>
<td>58.7</td>
</tr>
<tr>
<td>Aminoethyl$^1$</td>
<td>60.8</td>
</tr>
<tr>
<td>Aminodipropylamine$^1$</td>
<td>67.0</td>
</tr>
<tr>
<td>Bromoacetyl</td>
<td>71.4</td>
</tr>
<tr>
<td>N-hydroxysuccinimide ester (aminoethyl)</td>
<td>65.0</td>
</tr>
<tr>
<td>N-hydroxysuccinimide ester (aminodipropylamine)</td>
<td>78.6</td>
</tr>
<tr>
<td>Carboxymethyl hydrazide</td>
<td>87.8</td>
</tr>
<tr>
<td>Carboxyethyl$^2$</td>
<td>77.0</td>
</tr>
<tr>
<td>Aminoethyl$^2$</td>
<td>82.7</td>
</tr>
<tr>
<td>Aminodipropylamine$^2$</td>
<td>83.3</td>
</tr>
<tr>
<td>Imidocarbonate $^3$</td>
<td>77.9</td>
</tr>
<tr>
<td>Oxirane</td>
<td>69.1</td>
</tr>
<tr>
<td>Diazotised 4-aminobenzyl</td>
<td>82.5</td>
</tr>
</tbody>
</table>

$^1$ Activated with glutaraldehyde.

$^2$ Coupled with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

$^3$ Coupled at pH 8.9.

* Mean values of three determinations.
Table 2.13
Comparisons of various eluting agents

<table>
<thead>
<tr>
<th>Cellulose derivative</th>
<th>% recoveries of adsorbed IgG</th>
<th>Eluting agents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glycine-HCl pH2.8</td>
<td>pH2.0</td>
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<tr>
<td></td>
<td>Acetic Acid 1M</td>
<td>Propionic Acid 1M</td>
</tr>
<tr>
<td></td>
<td>NaSCN 3M</td>
<td>MgCl₂ 3M</td>
</tr>
<tr>
<td>Diazotised N-(3-amino-benzyloxymethyl)</td>
<td>60.8 68.8</td>
<td>62.3 62.7</td>
</tr>
<tr>
<td>Bromoacetyl</td>
<td>64.0 64.9</td>
<td>63.2 62.1</td>
</tr>
<tr>
<td>Imidocarbonate</td>
<td>69.8 -</td>
<td>68.7 -</td>
</tr>
<tr>
<td>Aminoethyl¹</td>
<td>65.9 71.9</td>
<td>66.0 60.2</td>
</tr>
<tr>
<td>Aminodipropylamine¹</td>
<td>65.6 73.2</td>
<td>66.6 60.0</td>
</tr>
<tr>
<td>Amino-chlorotriazinyl</td>
<td>78.7 75.7</td>
<td>42.7 44.7</td>
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</table>

<table>
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<tr>
<th></th>
<th>Propionic Acid 0.1 M</th>
<th>NaSCN 3M</th>
<th>MgCl₂ 3M</th>
<th>Citric acid: NaCl:glycine pH2.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazotised N-(3-amino-benzyloxymethyl)</td>
<td>60.1 65.4</td>
<td>74.7</td>
<td>60.2</td>
<td>63.9 **</td>
</tr>
<tr>
<td>Bromoacetyl</td>
<td>61.8 72.2</td>
<td>61.2</td>
<td>29.7</td>
<td>47.8</td>
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<td>Aminodipropylamine¹</td>
<td>69.8 80.0</td>
<td>67.4</td>
<td>36.8</td>
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<td>Aminodipropylamine²</td>
<td>65.3 78.7</td>
<td>69.9</td>
<td>37.5</td>
<td>56.5</td>
</tr>
</tbody>
</table>

¹ Activated with glutaraldehyde
² Coupled with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

* Mean values of three determinations.
** Mean values of six determinations.
Anti-bovine Insulin Immunoadsorbents

Fig. 2.22 shows a series of log dose response curves obtained with bovine insulin and seven different immunoadsorbent preparations. The highest titres were obtained when the antibody was coupled to either diazotised N-(3-aminobenzylloxymethyl)cellulose or bromoacetamidodipropylamine cellulose. The bromoacetamidoethyl derivative was only slightly less efficient and the pentyl derivative considerably less efficient. As has already been pointed out (Table 2.7, p. 63) a pentyl derivative bound more of the available guinea pig immunoglobulin (46.5%) than did the ethyl (24.0%) and dipropylamine (30.0%) derivatives. This predisposition on the part of the pentyl derivative to react more readily with the protein may point to a more effective immobilisation resulting in blocking of a higher proportion of antigen binding sites and a resultant loss of capacity. This order of effectiveness of the spacer arms is the same as that found for anti-IgG immunoadsorbents. Bromoacetyl- and aminochlorotriazinyl-cellulose based immunoadsorbents were less efficient, and the carbodiimide method of coupling to aminoethyl cellulose, employing N,N-dicyclohexylcarbodiimide, resulted in a gross loss of antibody activity. These results are of the same order as those obtained with anti-IgG immunoadsorbents. When plotted as the B/Bo ratio, the dose response curves were comparable.
Fig. 2.22 Log dose response curves obtained with guinea-pig anti-insulin immunoadsorbents prepared from the following cellulose derivatives: - ◊ - diazotised N-(3-aminobenzylxoxymethyl), △ - bromoacetamidodipropylamine, □ - bromoacetamidoethyl, ○ - bromoacetamidopentyl, ▲ - aminochlorotriazinyl, ■ - bromoacetyl and ● - aminooethyl.
Stability of Immunoadsorbents on Storage

Immunoadsorbents for protein purification were routinely stored in PBS, containing 0.1% sodium azide, at 4°C. Before use the preservative was removed by washing. For radioimmunoassay purposes freeze-dried material had its advantages in that it could be stored easily in small aliquots and reconstituted immediately before use.

In early freeze-drying experiments the cellulose-based immunoadsorbents were diluted to a concentration of 80 mg solid per ml PBS, quick frozen in a dry ice/cellulosolve mixture and freeze dried. The products were invariably granular and attempts to reconstitute them failed to yield a uniform suspension even on sonication. The results were only slightly improved by making the buffer 0.5% with respect to BSA. The suspensions were then diluted 20-fold before freezing and the end result was a fine powder. However, when reconstituted this did not regain the same small particle size and smooth suspension characteristic of the starting material. The procedure was again improved, this time by adding sucrose (5%) to the buffer. After freeze-drying the material remained as a cake with a volume similar to that of the initial suspension. On adding water the immunoadsorbent was reconstituted in a form indistinguishable from a fresh suspension.

The results of an assay conducted with aliquots of immunoadsorbents which had been freeze dried in a buffer containing 0.5% BSA, but no sucrose, or stored in suspension at 4°C, are shown in Fig. 2.23. The freeze-dried material was reconstituted with distilled water to its original volume. Freeze drying caused a 20% decrease in the number of available antibody sites but the avidity of the immobilised antibody was unchanged.
The effect of freeze-drying on the activity of anti-human IgG (Fc) immunoadsorbents prepared from bromoacetyl cellulose (○ and ●) and triazinyl cellulose (□ and ■). Preparations were stored at +4°C for six weeks (○ and □) or freeze dried ( ● and ■).

Fig. 2.24

Stability of sheep anti-human IgG (Fc) immunoadsorbents.

(i) Triazinyl cellulose based immunoadsorbents.

○ Freeze-dried, neat
○ Freeze-dried, neat + 5% sucrose
□ Freeze-dried, 1:12 dilution
□ Freeze-dried, 1:12 dilution + 5% sucrose
▼ Stored at 4°C.

(ii) Bromoacetyl cellulose based immunoadsorbents.

▲ Freeze-dried, neat.
○ Freeze-dried, neat + 5% sucrose

(iii) Diazotised N-(3-aminobenzylxoxymethyl)cellulose based immunoadsorbents.

▼ Freeze-dried, neat + 5% sucrose.
Fig. 2.24

Counts bound per min

Dose (µg)

Fig. 2.24

Counts bound per minute

Dose (µg)
Antigen binding curves obtained with three different immunoadsorbents freeze dried under different conditions are shown in Fig. 2.24. The binding curves for sheep antihuman IgG(Fc) coupled to aminochlorotriazinyl cellulose did not differ appreciably for material kept at 4°C or freeze dried in the presence of sucrose. Freeze drying in the absence of sucrose resulted in a loss in antibody activity which was more marked in the case of the neat suspension than in that of the 1:12 dilution. Sucrose was also shown to stabilise freeze-dried samples of immunoadsorbents prepared with bromoacetylcellulose and N-(3-aminobenzylxoxymethyl)cellulose. Similar results were obtained with anti-insulin immunoadsorbents.

From these experiments it was concluded that immunoadsorbents for radioimmunoassay were best freeze-dried in buffer containing 0.5% BSA and 5% sucrose. The immunoadsorbent concentration should not exceed 80 mg/ml. If a stabilising agent such as sucrose cannot be used suspensions should be diluted, preferably to a concentration of less than 5 mg/ml, before freeze drying.

**Standardisation of IgG Preparations and Sera** (cf. p. 43, Purification of IgG)

Human IgG purified from pooled serum by ammonium sulphate precipitation, DEAE-cellulose chromatography and Sephadex G-200 chromatography was standardised on the basis of absorbance at 280 nm and single radial diffusion analysis (SRD). Serum IgG levels were also measured by SRD. The results were expressed in a semi-logarithmic plot - (diameter of precipitin ring)$^2$ vs. log antigen concentration. Fig. 2.25 shows a typical plot and the precipitin rings obtained in 1% agarose gel containing 3% anti human IgG(Fc).
Fig. 2.25

Dilution of MRC Standard

\[ (\text{Diameter})^2 \text{ vs. Dilution of MRC Standard} \]
Conclusion

The results presented here clearly indicate that to believe in the existence of the 'ideal immunoadsorbent' is sheer utopianism. Compromise is inevitable.

Of the seven criteria listed at the outset of this comparative study (p.27) it has been possible to achieve four with some degree of consistency. These are: immobilisation by covalent coupling to a carrier; insolubility; stability with respect to time and eluting agent; and suitability of the physical form of the immunoadsorbent for a particular application. The accomplishment of two of the remaining three criteria, namely the specific binding of antigen to antibody without non-specific binding of other proteins, and the recovery of a large percentage of this specifically bound antigen by elution, depends very much on the compromise reached over the seventh and final criterion, which embodies all that goes to make for the efficient utilisation of the antibody. The conditions of the immobilisation process are likely to occasion the immunoadsorbent falling into one of two categories, and a choice has to be made between these. Importance is attached either to the immunoadsorbent having a high capacity for antigen, necessitating a high protein: carrier ratio, or to the antibody retaining a high percentage of its native activity, the amount of antibody coupled being a secondary consideration.

None of the methods of immunoadsorbent preparation investigated has resulted in retention of all the native antibody activity. The very nature of the immobilisation process makes it inevitable that activity will be lost due to chemical modification and steric hinderance of the antibody-antigen reaction. The carriers to which antibody could be coupled with the smallest loss in activity were diazotised N-(3-aminobenzyl oxy)methyl cellulose and glutaraldehyde-activated aminodipropylamine cellulose. Slightly less satisfactory in this respect were bromoacetamidodipropylamine cellulose and
glutaraldehyde-activated aminoethyl cellulose, with bromoacet: cellulose, 3-amino-5-chlorotriazinyl cellulose and bromoacetamidoethyl cellulose offering poorer alternatives.

High protein:carrier ratios were best achieved by coupling the antibody to diazotised N-(3-aminobenzyloxymethyl) cellulose, preferably the precipitated microcrystalline form, to bromoacetyl cellulose, or to glutaraldehyde-activated aminocellulose. These three coupling methods also have the added advantage of giving retention of relatively high percentages of the native antibody activity. High levels of protein immobilisation were achieved by reaction with diazotised 4-aminobenzyl cellulose and with carbodiimide-mediated condensation reactions with amino or carboxyl celluloscs, but both these methods led to gross losses of antibody activity. One problem of a high protein:carrier ratio is that of "immunoadsorbent avidity" referred to earlier. The high antibody density favours multiple reactions between one multivalent antigen and several antibodies. The energy of dissociation of such complexes is greater than that of a single antibody-antigen complex, and consequently elution of antigen from the antibody-immunoadsorbent is more difficult to achieve. This is also the case when there is a large number of covalent bonds between the antibody and the carrier. The three-dimensional structure of the protein is frozen. The advantages to be gained from incorporating spacer arms between antibody and carrier, in terms of reduced steric hindrance, are negated if large numbers of arms are involved.

On the basis of the findings reported here, coupling to diazotised N-(3-aminobenzyloxymethyl) cellulose at pH 8.75 and glutaraldehyde-activated aminodipropylamine cellulose are the methods of choice.

The 'off the shelf' concept for activated carriers is particularly attractive to the laboratory involved in the routine preparation of immunoadsorbents. Of the immunoadsorbents investigated here only five can be
prepared directly without the need to activate the carrier. The cellulose derivatives involved are bromoacetyl, 3-amino-5-chlorotriazinyl, imidocarbonate, carboxy and amino. In the cases of the last two derivatives coupling is achieved in the present of a carbodiimide. The bromoacetyl and triazinyl derivatives are stored wet and the others dry. The bromoacetyl derivatives are to be recommended as 'off the shelf' reagents. Bromoacetyl cellulose itself may be preferred to the bromoacetamido derivatives, for although it causes greater losses in antibody activity these are more than outweighed by its larger capacity for coupling antibody. If a dried activated cellulose is more convenient the choice is limited to cellulose imidocarbonate.

The method most frequently reported in the literature is in fact that involving formation of an imidocarbonate by cyanogen bromide activation of the cellulose, followed immediately by reaction with the protein. Few reports admit to drying the activated cellulose and coupling protein at a later date. The results presented here suggest that the method is by no means the most efficient for immobilising antibody. Its success may stem from the fact that it may be used equally well for the activation of cellulose, beaded agarose (Sepharose), or beaded dextran (Sephadex). The method of activation widely advocated in the past has been made hazardous because of the problems presented by the cyanogen bromide which is a dangerous poison. However, the introduction of an improved activation procedure by March, Parikh and Cuatrecasas (213) using acetonitrile as a solvent for the cyanogen bromide and dispensing with the need to run a continuous titration, augurs well for the continued popularity of this method. One wonders how many people would continue to use it if they realised how wasteful it is of antibody.
The effect of immobilisation of an antibody may be judged by three properties relating to the activity of the antibody. These are capacity, affinity and avidity. The capacity is easily defined in terms of the number of antigen binding sites on the antibody. The terms affinity and avidity both relate to the binding energy involved in a particular antibody-antigen reaction. Affinity describes the reaction between an antigencic determinant and the antibody, and any measurement of this requires that either the antigen is univalent or the antibody is specific for a single antigencic determinant. Different, but related, antigencic determinants may show lesser affinities for the antibody species, reflecting a lesser degree of recognition. Avidity is a particular property of the antibody and is a measure of the tightness of fit to the antigen. For the reaction -

\[
\text{Ab} + \text{Ag} \rightleftharpoons \frac{k_1}{k_2} \text{AbAg}
\]

- the avidity is defined as the equilibrium constant \( K \), and

\[
K = \frac{k_1}{k_2} = \frac{|\text{AbAg}|}{|\text{Ab}| |\text{Ag}|}
\]

where \(|\text{AbAg}|\), \(|\text{Ag}|\) and \(|\text{Ab}|\) are the molar concentrations of antibody-antigen complex, free antigen and free antibody respectively, and \( k_1 \) and \( k_2 \) are the association and dissociation rates.

The Interpretation of Radioimmunoassay Data

Standard dose response curves can be most enlightening with regard to the relative properties of different immunoadsorbents. However, the pitfalls to be encountered in the interpretation of these curves are numerous as revealed by Ekins (106), and in the past researchers have been all too ready to draw unjustifiable conclusions. The mathematical theory
of radioimmunoassay has been examined by several workers, in particular Feldman and Rodbard (107), Berson and Yalow (108) and Ekins (109).

The data obtained from a radioimmunoassay may be expressed in a variety of graphical forms. Plotting the data in terms of the number of counts bound \((B)\) or one of the ratios \(B/B_0\), \(B/F\) or \(F/B\) against antigen dose on either an arithmetic or a logarithmic scale yields standard dose-response curves. (\(B\) - counts bound; \(B_0\) - counts bound at zero dose; \(T\) - total counts added; \(F\) - free or unbound counts).

Estimations of the equilibrium constant by application of the Michaelis-Menton equation have been made by Odell et al. (110) and (111). A Michaelis-Menton hyperbola was constructed from data obtained by incubating equal aliquots of antibody with increasing concentrations of antigen. The equilibrium constant was defined as the reciprocal of the free antigen concentration, expressed as a molar quantity, at 50% saturation of the antibody binding sites. The constant is an average value for the whole antibody population, and is influenced most by the fraction with the highest \(K_{Ab}\) value.

An 'index of avidity' has been defined by Celada et al. (112) and Steward and Petty (113). The volume of antiserum necessary to bind 50% of available antigen at a range of antigen concentrations was determined. A semilog plot of this value vs. antigen concentration gave a straight line with a gradient equal to the affinity index.

A standard dose response curve provides the necessary data for the construction of a Scatchard plot. This was first applied to radioimmunoassay by Berson and Yalow (114) and Ekins (115) and has since been described by several authors including Feldman and Rodbard (107). The plot is defined by the equation \(R = K(q - B)\), where \(R\) is the ratio of free to bound antigen,
K the equilibrium constant and q the concentration of antibody binding sites. A linear relationship is only found if there is a homogeneous antibody population.

The relative equilibrium constants of antisera containing heterogeneous antibody populations directed against several antigenic determinants of an antigen may be calculated by the method described by Steward and Petty (113, 116). A plot of $1/b$ vs. $1/c$ from the equation

$$\frac{1}{b} = \frac{1}{K} \cdot \frac{1}{c} \cdot \frac{1}{Ab} + \frac{1}{Ab},$$

derived from the Law of Mass Action and the Langmuir Isotherm, gives a measure of antibody concentration ($Ab$). ($b$ and $c$ represent bound and free antigen respectively and $K$ the equilibrium constant). The value of $K$ was determined graphically by a logarithmic transformation of the Sips equation. Deviation from linearity of the plot $1/b$ vs. $1/c$ was explained in terms of disproportionate binding of antigen in conditions of relative antibody and antigen excess (91). Differences in relative equilibrium constants were reflected in a change of slope of the curve, this being greatest at low values of $K$. The intercept with the ordinate represented the reciprocal of antibody concentration.

Theoretical dose-response curves have been plotted by substituting values for the equilibrium constant ($K$), and concentrations of antibody ($q$) and labelled ($p^*$) and unlabelled ($p$) antigen in the equation

$$\left(\frac{B}{p}\right)^2 + \frac{B}{p}(1 + Kp + Kp^* - Kq) - Kq = 0$$

(117, 107).

The equation satisfies all dose-response relationships for a single antibody-antigen system.

Berson and Yalow (114) considered the case of one antigen reacting with two antibody sites with different avidities and in different concentrations, but did not differentiate between labelled and unlabelled antigen;

$$R = K_{11}(q_1 - b_1) + K_{12}(q_2 - b_2).$$
(R = B/F ratio; K - equilibrium constant; q - concentration of antibody sites; b - amount of bound antigen). It is difficult to generate theoretical dose response curves because the quantities of antigen bound $b_1$ and $b_2$, by the different antibody sites are not independent (119). More recently Feldman et al. (120), Feldman and Rodbard (107) and Feldman (119) have examined the mathematical theory of the reaction of any number of antigens with any number of antibodies.

Figures A1, A2 and A3 illustrate the effects of varying the concentration of antibody for a given value of K. The plot of counts bound (B) vs. dose on an arithmetic scale leads to a raising and steepening of the curve with increasing antibody concentration, characteristics which also apply to the B vs. log dose curve. A plot of $B/B_0$ scales the curves to the same initial height and then they are virtually superimposable under the conditions chosen, i.e., 3 to 30-fold excess of label over antibody binding sites.

Figures A4, A5 and A6 show the effects of varying the equilibrium constant (K). Again plots of B vs. dose on both arithmetic and logarithmic scales are characterised by a raising and a steepening of the curve with greater values of K. A set of parallel curves is obtained by plotting the ratio $B/B_0$ vs. log dose.

Figures A7, A8 and A9 have been included for comparison, and show the effect of varying the amount of 'label' relative to the amount of unlabelled antigen.
The differences in saturation levels of the antibody have important implications for the dose response curves (118). There are essentially three types of competitive binding assay, distinguishable in terms of the relative concentrations of antibody and labelled antigen. The first type employs only a trace amount of label, and an antibody dilution is chosen such that 33% of the label is bound in the absence of unlabelled antigen:

\[ p^* \rightarrow 0 \text{ and } q = \frac{0.5}{K} \]

The second type of assay conforms to Ekins' Rule (117):

\[ p^* = \frac{4}{K} \text{ and } q = \frac{3}{K} \]

Under these conditions the B/P ratio will always be equal to one at zero dose.

The third type of assay is typified by high antibody concentrations and excess labelled antigen. This method is particularly suitable when low specific activities are employed.

The effects of varying K and antibody concentration are also demonstrated by Scatchard plots. Increasing antibody concentration shifts the plot to the right, the equilibrium constant, and hence the slope of the curve, being unchanged. If on the other hand curves are constructed for equal concentrations of antibody with increasing values of the equilibrium constant, then a progressive steepening of the curve is observed. The intercept with the abscissa remains the same. In practice it is inconceivable that an antiserum will contain a single population of antibodies, and consequently Scatchard plots will not produce straight lines. The effective equilibrium constant is given by a tangent to the curve (95), and this changes with antigen dose. If only two distinct antigen populations are present, values of the equilibrium constants are obtained from asymptotes drawn to the curve close to the x and y intercepts. The positions of the asymptotes are shifted, without altering the gradient, such that the sums of the intercepts with one axis are equal to the intercept of the curve with that axis. The slope of
the asymptote equals \(-K\), and its intercept with the abscissa, \(q\)
(concentration of antibody sites).

Figs. A1 - A9 (on the following pages).
Theoretical dose response curves demonstrating the effects of
varying concentration of antibody, equilibrium constant and
concentration of labelled antigen. See text, p. 120.
Counts bound

Fig. A3

q varied

Dose, log scale

K 1
p* 3
q varied

q 1.0
q 0.5
q 0.1
Fig. A4

Counts bound

Dose, log scale.

Figs. A4, A5

$p^* 3$
$q 1$
K varied

$B/B_0$

Dose, log scale.
Fig. A6

Counts bound

K varied

p* 3
q 1
K varied

Dose
Fig. A7

K 1
q 1
p* varied

Counts bound

Dose

p* = 9
p* = 6
p* = 3

Fig. A8

K 1
q 1
p* varied

B/B_o

Dose, log scale.
Fig. A9

Counts bound

- 2 - 1 0 1 2

Dose, log scale.

K = 1
q = 1
p* varied

p* = 6
p* = 9
p* = 3
CHAPTER THREE

Solid-Phase Radioimmunoassay
Radioimmunoassay

Introduction

Radioimmunoassay combines a high degree of sensitivity with specificity and precision for the microdetermination of proteins, peptides, steroids and other substances in unfractionated mixtures such as serum and urine. The assay originally conceived by Yalow and Berson (122) for the measurement of insulin levels in plasma depends upon competition between labelled and unlabelled antigen (insulin) for antigen binding sites on specific antibodies. The separation of antibody-bound antigen and free antigen permits the determination of the amounts of labelled antigen in the two fractions. The ratio of antibody-bound labelled antigen to free labelled antigen ($B/F$) is a function of the concentration of the specific antibody, the kinetics of the antibody-antigen reaction and, most importantly, the concentrations of labelled and unlabelled antigen. The $B/F$ ratio decreases progressively with increasing concentrations of unlabelled material. Standard curves are constructed for a known range of antigen concentrations with constant amounts of antibody and labelled antigen, and the concentrations of unknowns are determined by interpolation.

One of the many problems associated with the development of radioimmunoassays has been that of separating bound from free antigen. Methods making use of antibodies in an insoluble form offer the advantages of simplicity and completeness of separation.

Some techniques for the separation of bound and free antigen employ specific physico-chemical properties of the antigen. For example, insulin is adsorbed on to cellulose paper, and so when the assay mixture is electrophoresed on this medium the free antigen remains adsorbed to the paper at the origin whilst antibody-bound insulin migrates towards the anode (122).
The method is of limited practical value because of the difficulties involved in processing large numbers of paper strips and in handling large volumes. Electrophoresis on cellulose acetate exploits the different mobilities of the antigen and antigen-antibody complex, but has similar disadvantages to electrophoresis on paper (123). Solvent extraction techniques employing ammonium sulphate (124) or ethanol: sodium chloride (125, 126) do not place any restriction on the assay volume but tend to be very exacting. The ethanol:sodium chloride extraction in particular is very sensitive to temperature. The immunoassay of insulin using dextran-coated charcoal to separate free and bound labelled hormone, was reported by Herbert et al. (127). The technique has since been applied to the assay of several hormones and digoxin (128). The value of the dextran coating has been questioned (129) in the light of evidence presented suggesting that the dextran does not exhibit a molecular sieving action, which would exclude antibody-antigen complexes from the charcoal, but simply reduces the number of sites available for adsorption. Both free and bound antigen were shown to be adsorbed by the charcoal, the former to a far greater extent. Dextran coating was found only to be effective at low protein concentrations. These conclusions were reached using a human thyrotropin assay system. Other adsorption methods involve the use of talc (130) or ion-exchange resins (131). Adsorption methods are particularly sensitive to variations in total protein concentration and non-specific effects encountered when measuring antigen levels in plasma (132).

Probably the most widely used radioimmunoassay procedure is the double antibody method first employed for the determination of insulin levels (133). The primary reaction is between antibody and labelled and unlabelled antigen. A second antiserum raised against immunoglobulin of
the species employed to raise the first antibody is then added to precipitate the bound antigen. The technique gives a complete separation of bound and free antigen (134), but the need for a second incubation prolongs the assay by one to two days.

The first report of a radioimmunoassay employing an insoluble antibody derivative was that of Hales and Randle (135). Guinea pig antiserum was precipitated with rabbit anti guinea pig γ-globulin, and the precipitate was used as the solid phase in a radioimmunoassay. Gurvich and Drizlikh (136) developed a method of detecting radioactively labelled antigen with antigen covalently linked to cellulose. The principle of the method lay with the fact that bivalent antibody reacted with the immobilized antigen through one combining site leaving the second site free to react with soluble homologous antigen. Catt et al. (137) and Wide and Porath (138) coupled antibody to the solid-phase, the former by physical adsorption to polystyrene tubes and the latter by covalent bonding to isothiocyanotophenoxyhydroxypropyl-Sephadex. Specific antibodies to insulin, growth hormone (GH), human chorionic gonadotrophin (HCG), luteinising hormone (LH) and IgG were successfully coupled to Sephadex and used for the assay of the appropriate antigens. Solid phase radioimmunoassays for numerous other antigens have since been developed with the solid phase variously taking the form of polymerised antibodies (139), antibodies physically adsorbed on to polystyrene tubes (140) or thin discs (141) and antibody covalently coupled to cellulose particles (142), paper discs (143), Sephadex (138) and Sepharose (80).

The solid phase radioimmunoassay systems mentioned so far have all been indirect techniques employing competition between antigen and radioactively labelled antigen for binding sites on an immobilised antibody. The uptake of labelled antigen varies inversely with the concentration of
unlabelled antigen in the assay mixture. Such assays do not achieve maximum sensitivity for four reasons. Firstly, only a fraction of the unknown reacts with the antibody as a result of competition with the radioactively labelled derivative. Secondly, the iodination procedure may affect the antigen in such a way that its reaction with antibody shows a different equilibrium constant. Thirdly, the equilibrium of the antibody antigen reaction may be affected by the low antibody concentration so that the amount of complex formed is limited. Finally, the indirect systems do not make allowance for possible cycling steps which would increase the yield of complex from the unknown (144). The sandwich and immunoradiometric techniques are both direct methods of assay and come close to overcoming these problems. However, the former may be affected by iodination damage to antigen employed in the measurement of antibody.

The 'sandwich technique' requires that the antigen has at least two antigenic sites. An excess of the immunoabsorbent is incubated with the antigen, and then washed. Immunoabsorbent purified antibodies labelled with a radioactive isotope are added to the immunoabsorbent. These antibodies react with the bound antigen; the amount of activity bound is directly proportional to the amount of antigen present. This direct technique is particularly sensitive since binding of a small amount of radioactivity is indicative of the presence of antigen. HCG, LH and immunoglobulin E (IgE) have been measured using this technique. The method proved to be ten times more sensitive than the competitive inhibition system for the detection of IgE (145). A similar sandwich technique is used for the assay of antibodies. The antibodies are incubated with insolubilised antigen. The immunoabsorbent is then washed prior to the addition of radioactively labelled antigen. The uptake of antigen gives a direct measure of the antibodies present.
A specialised radioimmunoassay employing the sandwich technique was used for the detection and assay of reaginic antibodies (146). The antigens which elicit production of reaginic antibodies and which cause an allergic response are known as allergens. In the radioallergosorbent technique allergen is coupled to an insoluble support and reacted with reaginic antibody (IgE). The immunoadsorbent is washed and then incubated with radioactively labelled anti-IgE antibody. After a further wash, the radioactivity bound to the immunoadsorbent is counted and gives a direct measure of the reaginic antibody concentration.

The immunoradiometric technique introduced by Miles and Hales (4) for the assay of insulin has the advantage of being a "direct" method. Antibodies are isolated by immunadsorption on to an insolubilised antigen immunoadsorbent, and are radioactively labelled in situ in order to protect at least one antigen binding site. The labelled antibodies are eluted and an excess of these incubated with the antigen. "Free" antibody is removed by the addition of an antigen-immunoadsorbent. After centrifugation of the assay mixture to sediment the immunoadsorbent the remaining radioactivity in the supernatant is directly proportional to the amount of antigen present. Ideally univalent antibodies or antibody fragments should be used in order to avoid binding to free hormone and immunoadsorbent occurring simultaneously (147). The method is highly sensitive and should find general application.

The double antibody radioimmunoassay has been simplified by introducing the second antibody in the form of an immunoadsorbent (148). The conventional technique requires the introduction of two variables, namely carrier \( \gamma \)-globulin and the antiserum to \( \gamma \)-globulin (second antibody) after the initial incubation. Consequently it is necessary to optimise the assay conditions regularly. If the second antibody is introduced in an immobilised form there is no need for carrier \( \gamma \)-globulin and the amount of second
antibody required is consequently much less. Furthermore, provided the immobilised second antibody is in excess, the optimum assay conditions are less critical. In theory the antibody can be regenerated by treatment with a suitable eluent such as glycine-HCl buffer pH 2.0 and reused.

The first radioimmunoassay was developed for the measurement of insulin (122), and the assay of this hormone has since attracted more attention than that of any other protein. The first solid-phase assay was performed by Hales and Randle (135) using a precipitated antibody to human insulin. The quantitative determination of small amounts of immunoglobulin was undertaken by Mann et al. (149) who employed anti human immunoglobulin antibodies covalently linked to cellulose in radioimmunoassay systems. IgG, IgA, IgM, and κ and λ-light chains were measured using appropriate immunoadsorbents prepared with bromoacetylcellulose. The sensitivity of this test ranged from 1 μg for IgM to 10 ng for IgG and the κ light chain. Prior to this Gurvich and Drizlikh (136) reported the use of a sandwich technique employing anti-human IgG immunologically bound to human IgG, which in turn was coupled to cellulose. The uptake of 1131 labelled IgG was measured by a rather laborious technique, which was improved upon by Salmon et al. (150) using polystyrene isocyanate discs in preference to cellulose. Wide and Porath (138) mentioned the use of Sephadex-coupled antibodies for the measurement of IgG, and their method was subsequently used to assay IgND (now known to be IgE) (151, 152, 153, 154). McLaughlan et al. (154) advocated the use of cellulose carbonate (43). Carrel et al. (19) and Polmar et al. (121) assayed IgE after the method of Mann et al. (149), and both groups were able to detect the immunoglobulin at a level of 30 ng/ml. Polmar et al. (121) compared three radioimmunoassay techniques, double antibody, Sepharose-bound antibody and bromoacetylcellulose bound antibody, for the measurement of serum IgE. Using these methods it was possible to detect 2-4 ng/ml, 18 ng/ml and 30 ng IgE/ml respectively.
Comparable results were obtained when measuring elevated levels of IgE, but the immunoadsorbent methods were inaccurate insofar as they indicated the presence of higher levels of IgE than was actually the case at low concentrations of IgE. Consequently the double-antibody method was considered to be the superior technique.

Comparative assessments of double-antibody and solid-phase radio-immunoassays have also been made by Wide et al. (155), Bolton and Hunter (80) and Gardner et al. (156). In each of these cases the antiserum was immobilised by covalent coupling to a carrier by the cyanogen bromide method. In the assays of LH and FSH (155) the antibody-antigen reaction required longer to attain equilibrium when the antibody was immobilised. In order to keep the incubation time to a minimum without too great a loss in sensitivity the level of labelled hormone was increased so that the $B_0$ value was of the order of 10-15%. Non-specific binding of labelled material was reduced to approximately 0.1% by the use of 0.5% Tween 20 in the assay and wash buffers. Antibodies to steroids and small peptide hormones retained up to 90% of their activity when coupled to carriers (80), but those directed against larger peptide hormones retained only 10-15% and gave assay curves with reduced sensitivity. Losses in activity were put down to steric hindrance. Microcrystalline cellulose and Sephadex G25 Superfine were considered easier to handle than Sepharose because they packed down better on centrifugation. Furthermore, cellulose- and Sephadex-coupled antibodies yielded standard curves of higher precision than did Sepharose preparations.

Gardner et al. (156) considered that the main disadvantages of the solid-phase method were the large loss of antibody due to coupling, the need to mix tubes throughout the assay and a decreased apparent avidity leading to a reduced sensitivity. These conclusions were drawn from
experiments with a solid-phase assay system for human placental lactogen (HPL). Between 70 and 80% of the original antibody activity was lost and cellulose was found to couple less antibody than Sepharose 4B. The latter observation was thought to be because of there being fewer accessible activated sites and a smaller particle size. A diminished apparent equilibrium constant for the antibody-antigen reaction when the antibody was immobilised was explained in terms of the antibody being in discrete packets occupying a small volume rather than being freely available throughout the system. It was concluded that the solid-phase assay was to be preferred when antiserum was abundant and high sensitivity was not essential.

In the present study indirect solid-phase radioimmunoassays have been developed for the measurement of insulin, human placental lactogen (HPL), IgG and IgD. Double antibody assays were run in parallel except in the case of HPL.
Materials and Methods

Iodination of Insulin

Recrystallised porcine insulin was labelled with carrier-free Na\(^{125}\)I by the chloramine T method of Greenwood, Hunter and Glover (104). The iodination of 5 µg of insulin was achieved at pH 7.5 with ImC\(^{125}\)I. Purification of the iodinated protein was achieved by adsorption chromatography on Whatman CF11 cellulose powder. After washing unadsorbed material from the column with phosphate buffer (0.05M) the iodinated insulin was eluted with phosphate buffered 5.0% BSA. The fraction was diluted to 25 ml with buffered 0.5% BSA and stored in 1 ml aliquots at -20°C.

Iodination of IgG and IgD

This was achieved by the method described for IgG in Chapter 2 (Method i). Iodinated human placental lactogen was supplied by Wellcome Reagents Ltd., Beckenham, Kent.

Preparation of Immunoadsorbents

Methods previously described in Chapter 2 were employed to couple antibodies to cellulose. For the measurement of insulin and HPL, 'whole' antisera were coupled to the support. The purified globulin fractions of antisera were used to prepare immunoadsorbents for the assay of IgG and IgD. The cellulose derivatives employed were bromoacetylcellulose, bromoacetamidoalkylcellulose, aminochlorotriazinylcellulose, diazotised benzylxoymethyl cellulose and aminooalkylcellulose, coupling to aminooalkylcellulose being effected by the glutaraldehyde method.

Whatman CC31 microcrystalline cellulose was used in the preparation of all the immunoadsorbents used in solid-phase radioimmunoassay.
Assay Buffers: AB1 - boric acid 5.5 g
sodium tetraborate 4.5 g
sodium chloride 8.5 g
ethylene diamine tetra-acetic acid (sodium salt) 3.7 g
sodium azide 1.0 g
BSA 5.0 g
to 1 litre with distilled water pH 7.4 0.05M with respect to borate.

AB1T - as above plus 0.5% Tween 20.

AB2 - NaH2PO4·2H2O 2.34 g
Na2HPO4·2H2O 10.68 g
sodium chloride 8.5 g
ethylene diamine tetra-acetic acid (sodium salt) 3.7 g
sodium azide 1.0 g
BSA 5.0 g
to 1 litre with distilled water pH 7.4 0.05M with respect to phosphate.

AB2T - as above plus 0.5% Tween 20.
Performance of the Assay and Calculation of Results

Non-specifically bound radioactivity was estimated with assay tubes in which the specific antibodies were replaced by normal immunoglobulin. Appropriate controls were included in each assay.

All samples were run in triplicate and results were expressed as the means of triplicates. Where the coefficient of variation between triplicates exceeded 5% the result was discarded in order to exclude accidental drop-outs due to broken tubes, omission of label etc. This level was lifted to 8% for the HPL solid-phase radioimmunoassay.

The sensitivity of an assay system has been defined as the least amount of unlabelled antigen that can be detected with confidence, and precision the ability to achieve good replication both within and between assays.

Insulin Radioimmunoassays

Double antibody method

Titration of the second antibody and determination of the most satisfactory anti-insulin serum dilution were achieved in the same experiment. Dilutions of the guinea pig anti-bovine insulin serum (243/1) were incubated at 4°C for 72 hr with a dilution of normal guinea pig serum and a fixed amount of iodinated insulin. Dilutions of rabbit anti-guinea pig immunoglobulin were then added and the assay tubes incubated for a further 24 hr at 4°C. Details of the assay protocol were as follows:-
<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig anti-insulin serum dilution</td>
<td>100 μl</td>
</tr>
<tr>
<td>Assay buffer AB1</td>
<td>200 μl</td>
</tr>
<tr>
<td>1:100 dilution of normal guinea pig serum</td>
<td>100 μl</td>
</tr>
<tr>
<td>$^{125}$I-insulin (2 ng/ml)</td>
<td>100 μl</td>
</tr>
</tbody>
</table>

These four constituents were mixed and incubated at 4°C for 72 hr. The mixture was then incubated for a further 24 hrs following the addition of 200 μl of rabbit anti-guinea pig immunoglobulin (AS 180).

The precipitate was collected by centrifugation at 2000 g for 30 min. and washed once by resuspension in fresh assay buffers (AB1).

The same protocol was adopted for the insulin assay with the exception that 100 μl of the assay buffer was replaced by 100 μl of the insulin standard or unknown.

Typical second antibody titration curves are shown in Fig. 3.1. The rabbit anti-guinea pig immunoglobulin serum gave rise to significant pro and post-zones with maximum precipitation being achieved with a 1:8 dilution of the antiserum. Precipitation was shown to be unaffected by antigen free plasma when EDTA containing assay buffers were employed. The 1/40,000 dilution of the anti-insulin serum bound 50% of the added label and the 1/80,000 dilution 36%. The 1/80,000 dilution was used in the insulin assay to achieve maximum sensitivity. Insulin standards ranged from 62.5 pg/ml to 100 ng/ml (1.5 to 2500 μU/ml), the approximate working range of the resulting standard curve (Fig. 3.2) being from 0.2 to 10 ng (5 to 250 μU).

**Solid-Phase Method**

Immunoadsorbents were prepared by reacting 0.5 ml of whole guinea pig anti-insulin serum with the cellulose derivative. Iodinated guinea pig IgG was added to each reaction mixture to give a measure of the
Fig. 3.1 Optimisation of conditions for the assay of insulin by the double antibody method employing guinea pig anti-insulin (dilutions of 1/10000 to 1/80000) and rabbit anti-guinea pig globulin serum.
extent of coupling of the antibody. Results of some of the preparations have been summarised in Table 2.7. Preparations were diluted to the equivalent of an antiserum dilution of 1:10000.

Assay protocol: immunoadsorbent dilution 200 μl
assay buffer AB1 100 μl
insulin standard or unknown 100 μl
$^{125I}$-insulin 100 μl

500 μl*

Incubation 16 hr at room temperature with tumble mixing.

Immunoadsorbent was collected by centrifugation at 2000 g for 5 min. and washed twice by resuspension in fresh assay buffer (AB1).

* NB Volume was equal to that of the first incubation in the double antibody assay to permit a direct comparison to be made.

Standard curves obtained with various immunoadsorbent dilutions are shown in Fig. 3.3. The coefficient of variation between triplicates was less than 3%.

Covalent coupling of the insulin antibodies to bromoacetyl cellulose resulted in a considerable loss in antibody activity. Whereas a 1/80000 dilution of the uncoupled antiserum in the double antibody assay bound 36% of the $^{125I}$-insulin added, a dilution of between only 1/10000 and 1/20000 of the immobilised antibody was required to bind a comparable percentage of counts. Furthermore, immobilisation resulted in a considerable loss in sensitivity (Fig. 3.2 and 3.3) which reflects a loss in avidity. The method of antibody immobilisation has been bettered in terms of retention of antibody activity by introducing spacer arms between the carrier and the antibody (Fig. 2.2, p.108).
Fig. 3.2 Standard curve for the radioimmunoassay of bovine insulin using the double antibody method. A 1/80000 dilution of guinea pig antiserum 243/1 was used. This bound 36% of the labelled insulin in the absence of unlabelled insulin.

Fig. 3.3 Standard curves for the radioimmunoassay of bovine insulin using the indirect solid-phase method. Dilutions of the immunoadsorbent were calculated on the basis of 85.1% immobilisation of the antiserum. The antibody was coupled to bromoacetyl cellulose (Table 2.7). The results are directly comparable with those in Fig. 3.2.
The need for mixing the insulin assay incubation mixture was investigated by the same method as for the HPL assay described later. The results obtained with the two systems are presented together in Table 3.1.

**Human Placental Lactogen Radioimmunoassay**

**Solid-Phase Method**

Immunoadsorbents were prepared by reacting 1 ml of whole rabbit antiserum with 1 g of bromoacetyl cellulose.

A suitable working dilution of the immunoadsorbent was established by performing a titration with dilutions ranging from 1/100 to 1/3200 (Fig. 3.4).

Assay protocol: Assay buffer AB2 or standard 500 μl
125I-HPL (10 ng/ml) 100 μl
Immunoadsorbent dilution 100 μl incubated at 37°C on a tumble mixer for 2 or 4 hr. Immunoadsorbent collected by centrifugation at 2000 g for 5 min. and washed twice by resuspension in fresh assay buffer AB2T.

The 1/800 dilution of the immunoadsorbent, which bound 50% of the added counts, was selected for subsequent assays.

The assay for HPL was set up as follows, each sample or standard being assayed in triplicate.

Assay protocol: Assay buffer AB2 400 μl
Standard or unknown 20 μl
125I-HPL (10 ng/ml) 100 μl
Anti-HPL immunoadsorbent 1/800 100 μl
Fig. 3.4 Titration of immobilised anti HPL. ○ and □ - 2 hr incubation with label alone and label plus 500 ng of standard respectively. × and ● - 4 hr incubation with label alone and label plus 500 mg standard respectively.
Tubes were incubated at 37°C on a tumble-mixer for 4 hr. and then centrifuged at 2000 g for 10 min. The immunoadsorbent was washed twice by resuspension in 1.0 mL and AB2T. Standard curves obtained in two separate experiments are shown in Figs. 3.5 and 3.6. The results obtained with three dilutions of each of seven plasma samples are included in the legend to Fig. 3.6. The correlation shown between HPL concentrations measured in various plasma dilutions testifies for the validity of the assay. The coefficient of variation between triplicates was less than 6%.

Mixing During the Incubation

The need for mixing during the assay was investigated by comparing the results of 0.5 and 4 hr. incubations, both with and without mixing. The experiment was conducted for both HPL and insulin.

Mixing the tubes was found to have no significant effect upon the amount of hormone bound after 0.5 or 4 hr. incubations (Table 3.1). The very small particle size of the immunoadsorbents enabled them to stay in suspension long enough for binding of the hormones to occur. Earlier experiments (Fig. 28 p.68) showed that binding of the hormone was essentially complete within 1½ hr. (insulin) and 2½ to 3 hr. (HPL). For routine clinical applications the tumble mixing of tubes was not essential. However, for the purposes of the present study mixing was routinely employed to overcome problems presented by the variation in particle size between the different immunoadsorbents.
**Figs. 3.5 and 3.6**  Standard curves for solid-phase radioimmunoassay of HPL with anti HPL immobilised on bromoacetylcellulose. The "unknowns" are taken from standard curve 3.6.

<table>
<thead>
<tr>
<th>&quot;Unknowns&quot;</th>
<th>Dilutions of &quot;Unknowns&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/1</td>
</tr>
<tr>
<td>a</td>
<td>-</td>
</tr>
<tr>
<td>b</td>
<td>6.00</td>
</tr>
<tr>
<td>c</td>
<td>1.05</td>
</tr>
<tr>
<td>d</td>
<td>4.35</td>
</tr>
<tr>
<td>e</td>
<td>-</td>
</tr>
<tr>
<td>f</td>
<td>7.5</td>
</tr>
<tr>
<td>g</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Values represent μg/ml of HPL corrected for the dilution factors.
Table 3.1  The effect of mixing during the incubation stage
of solid-phase radioimmunoassays for insulin and HPL

(a)
Guinea pig anti-insulin serum (243/1) immobilised by reaction with
bromoacetyl cellulose. Immunoadsorbent dilution equivalent to a
1/10,000 dilution of the antiserum.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>(^{125}\text{I}-\text{insulin})</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr.</td>
<td>Alone</td>
</tr>
<tr>
<td></td>
<td>Counts bound (cpm), (n = 3)</td>
</tr>
<tr>
<td>Stood</td>
<td>0.5</td>
</tr>
<tr>
<td>Mixed</td>
<td>0.5</td>
</tr>
<tr>
<td>Stood</td>
<td>4.0</td>
</tr>
<tr>
<td>Mixed</td>
<td>4.0</td>
</tr>
</tbody>
</table>

(b)
Rabbit anti-HPL serum (HS 241) immobilised by reaction with bromoacetyl
cellulose

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>(^{125}\text{I}-\text{HPL})</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr.</td>
<td>Alone</td>
</tr>
<tr>
<td></td>
<td>Counts bound (cpm), (n = 3)</td>
</tr>
<tr>
<td>Stood</td>
<td>0.5</td>
</tr>
<tr>
<td>Mixed</td>
<td>0.5</td>
</tr>
<tr>
<td>Stood</td>
<td>4.0</td>
</tr>
<tr>
<td>Mixed</td>
<td>4.0</td>
</tr>
</tbody>
</table>
Radioimmunoassay of Human IgG

Double Antibody Method

Sheep anti-human IgG(Fc) was employed as first antibody, its precipitation, after incubation with the antigen, being effected with rabbit anti-sheep γ-globulin. A preliminary investigation revealed cross-reacting antibodies in the rabbit antisera. This activity was removed by adsorption with a glutaraldehyde polymerised human serum pool (Table 3.2).

Assay protocol:  

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay buffer AB1</td>
<td>200</td>
</tr>
<tr>
<td>Dilution of sheep serum (1/400)</td>
<td>100</td>
</tr>
<tr>
<td>Standard or unknown</td>
<td>100</td>
</tr>
<tr>
<td>125I-IgG</td>
<td>50</td>
</tr>
<tr>
<td>Dilution of sheep anti IgG(Fc) incubated at 4°C for 48 hr.</td>
<td>50</td>
</tr>
<tr>
<td>Dilution of rabbit anti-sheep γ-globulin incubated at 4°C for 24 hr.</td>
<td>100</td>
</tr>
</tbody>
</table>

Precipitate collected by centrifugation at 2000 g for 30 min. and washed once by resuspension in 500 μl AB1.

The standard curve obtained with a 1/2000 dilution of sheep anti-human IgG(Fc) (AS 57) is shown in Fig. 3.7. The sensitivity of the assay extended down to 10 ng/ml.

Solid-Phase Method

Cellulose based immunoadsorbents prepared by any of the methods described in Chapter 2 could be used for the radioimmunoassay of human IgG. Those prepared from bromoacetylcellulose, diazotised benzylooxymethyl cellulose and glutaraldehyde activated aminocellulose were used routinely.
Table 3.2

The removal of cross reacting antibodies with human IgG from rabbit anti-sheep γ-globulin by immunoadsorption.

<table>
<thead>
<tr>
<th>Dilution of rabbit anti-sheep γ-globulin (AS 322)</th>
<th>% counts precipitated Pre-adsorption</th>
<th>Postadsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2</td>
<td>89.0</td>
<td>-</td>
</tr>
<tr>
<td>1/4</td>
<td>85.7</td>
<td>55.5</td>
</tr>
<tr>
<td>1/8</td>
<td>85.5</td>
<td>56.4</td>
</tr>
<tr>
<td>1/12</td>
<td>85.9</td>
<td>-</td>
</tr>
<tr>
<td>1/16</td>
<td>85.1</td>
<td>47.3</td>
</tr>
<tr>
<td>1/24</td>
<td>81.6</td>
<td>-</td>
</tr>
<tr>
<td>1/32</td>
<td>77.6</td>
<td>36.4</td>
</tr>
<tr>
<td>1/48</td>
<td>63.1</td>
<td>29.1</td>
</tr>
<tr>
<td>1/64</td>
<td>48.0</td>
<td>23.3</td>
</tr>
<tr>
<td>0</td>
<td>2.7</td>
<td>1.3</td>
</tr>
<tr>
<td>1/12 (anti IgG(Fc) omitted)</td>
<td>61.0</td>
<td>4.5</td>
</tr>
</tbody>
</table>

A double antibody radioimmunoassay for IgG was necessary to enable a comparison to be made between antibody activity in the free and immobilised states.
**Fig. 3.7** Standard curve for double antibody radioimmunoassay of human IgG employing sheep antihuman IgG (Fc) at a dilution of 1/2000.

**Fig. 3.8** Standard curve for the solid-phase radioimmunoassay of human IgG employing sheep anti human IgG (Fc) immobilised on diazotised aminobenzylxoxymethyl cellulose (●) and bromoacetyl cellulose (○).
Assay protocol:-

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay buffer AB1</td>
<td>100µl</td>
</tr>
<tr>
<td>Standard or unknown</td>
<td>100µl</td>
</tr>
<tr>
<td>$^{125}$I-IgG</td>
<td>100µl</td>
</tr>
<tr>
<td>Immunoadsorbent suspension</td>
<td>100µl</td>
</tr>
</tbody>
</table>

Tubes were capped, mixed on a vortex mixer and then tumble-mixed for 16 hr at room temperature. The immunoadsorbent was collected by centrifugation at 2000 g for 5 min and washed thrice by resuspension in 1 ml ABIT. The sensitivity of the routine assay was 100 ng/ml, and it was possible to improve this to 10 ng/ml. However, such sensitivity was deemed unnecessary, and furthermore impractical, as it was achieved with a standard curve below the convenient working range of IgG concentrations. The coefficient of variation of triplicates was less than 3%.

Radioimmunoassay of IgD

Double-Antibody Method

The assay system employed rabbit anti-human IgD as the first antibody and goat anti-rabbit γ-globulin as the second antibody. The IgD antisera were raised according to the protocol outlined in Chapter 5, a primary immunisation being followed by three intramuscular booster injections. Anti light chain activity was removed by adsorbing the antisera with glutaraldehyde-polymerised Fab. Other cross-reacting antibodies were absorbed out with a pooled 'normal' human serum polymer. Controls were run with each assay and contained either pure IgG instead of IgD or $^{125}$I-IgG instead of $^{125}$I-IgD.

The working dilution of the second antibody was determined by titration with the following protocol:-
Assay buffer AB1 100 µl
Rabbit anti IgD (6) 1/1000 (GD21) 100 µl
Rabbit serum 1/100 100 µl
$^{125}$I-IgD 100 µl

Tubes were incubated at 4°C for 72 hr and then for a further 16 hr after addition of 100 µl of a dilution of goat anti-rabbit globulin (AS 357). The precipitate was collected by centrifugation at 2000 g for 30 min and washed once by resuspension in 700 µl AB1.

Maximum precipitation of the anti IgD/IgD complex was achieved with a 1/8 dilution of AS 357. The antiserum was judged to be specific for the rabbit protein in the assay system by its inability to precipitate $^{125}$I-IgD in the absence of anti-IgD. When $^{125}$I-IgG replaced the iodinated IgD in the assay system, less than 1.5% of the added counts (370 ex 25,000) were detected in the precipitate.

The protocol which was adopted for the assay of IgD, and which gave maximum sensitivity, was as follows:-

Standard or unknown 200 µl
Rabbit anti-human IgD (6) 1/3000 (GD 21) 100 µl
$^{125}$I-IgD 100 µl
Rabbit serum 1/100 100 µl

Tubes were incubated at room temperature for 24 hr and for a further 24 hr following the addition of 100 µl of a 1/8 dilution of AS 357. The precipitate was collected by centrifugation at 2000 g for 30 min, and washed once by resuspension in 700 µl AB1.

A typical standard curve obtained with this protocol is included in Fig. 3.10.
Solid-Phase Method

Immunoadsorbents were prepared by reacting 1.0 ml of rabbit anti-human IgD (6) with 1.0 g of bromoacetyl cellulose, diazotised benzyloxy-methyl cellulose or glutaraldehyde-activated amino cellulose. The relevant methods have been described in Chapter 2. The immunoadsorbents were suspended in buffer AB1, made up to 25 ml, and stored at 4°C.

The same protocol as was adopted for the first step of the double antibody technique was followed with the exception that the antiserum dilution was replaced by a dilution of the immunoadsorbent. Tubes were capped and tumble-mixed at room temperature for 24 hr, or 72 hr to achieve equilibrium. The solid phase was collected by centrifugation (2000 g, 5 min) and washed three times with AB1T. A fourth wash led to a further slight reduction in the number of background counts, but did not effect the sensitivity of the assay.

A 1/100 dilution of a bromoacetyl cellulose based immunoadsorbent bound 50% of the added \(^{125}\)I-IgD when the former was titrated. This was equivalent to between a 1/2500 and 1/3000 dilution of the original antiserum. A 1/4000 dilution of that antiserum bound an equal percentage of counts.

Standard curves obtained with 1/100 and 1/200 dilutions of the immunoadsorbent are shown in Fig. 3.9, and with both the solid-phase and double antibody techniques at equivalent antiserum concentrations in Fig.3.10. The smaller binding capacity of the immobilised antibody reflects the 'loss' of antigen binding sites by steric hindrance and blocking. The coefficient of variation of triplicates was of the same order as in the IgG assay, less than 3%.
The solid-phase assay was used routinely to assess the effectiveness of IgD purification procedures and for measuring the IgD levels in serum samples. The results of such measurements are included in Chapter 5.
Fig. 3.9 Standard curves for the solid-phase radioimmunoassay of IgD. Dilutions of the immunoadsorbent - • - 1/100; o - 1/200.

Fig. 3.10 Comparison of the standard curves obtained by the solid-phase ( • ) and double antibody ( o ) methods.
Conclusions

The majority of techniques for the quantitation of immunoglobulins are based on precipitation reactions. For most purposes the sensitivities of such methods are adequate, but with the discoveries of IgE and IgD, which occur in concentrations of 1 and 30 µg/ml or less in normal subjects, the advent of tissue culture studies with lymphocytes and an increasing interest in immunoglobulin sub-classes, the need for a more sensitive assay became acute. The double-antibody technique provides such an assay, but the problems associated with optimisation of this system and the length of time required to complete the assay leave room for improvement. Immobilisation of the antibody on a carrier such as cellulose leads to a reduction in the amount of work and time required to perform the assay. Furthermore, the solid-phase method is more economical than the double-antibody method insofar as there is no need for a 'second' antibody.

The one drawback with the indirect solid-phase method is the loss of antibody activity because of covalent coupling to the support. When antibodies to insulin were coupled to bromoacetylcellulose the activity was reduced by approximately 80%. Other coupling procedures, including those with cyanogen bromide activated cellulose and those involving the use of carbodiimide mediated condensation reactions, were even more detrimental to antibody activity. On the other hand a higher percentage of the original titre (up to 45%) was retained when coupling methods involving spacer arms between the carrier and insulin antibody were used. The results obtained with antibodies to human IgG were of a similar order, but the anti-IgD immunoadsorbents prepared with bromoacetyl cellulose and diazotised benzyloxymethyl cellulose retained relatively high percentages (up to 75%) of the original antibody titre. The insulin assay showed a
reduced sensitivity when compared with the double antibody method, but reductions were not so apparent in the cases of the immunoglobulin assays. Loss of actual avidity due to chemical modification of the antibody and/or loss of apparent avidity due to steric hindrance of the antibody-antigen reaction account for the loss in sensitivity. It is not possible to deduce which of these factors is the more important on the strength of results in the present study. However, it is interesting to note that the activities of the anti-immunoglobulin sera were less affected by coupling than was the insulin antiserum and the activity of anti-IgD less than that of anti-IgG. An explanation may be in the way the different sera were treated before coupling of the antibodies to the carrier rather than with the method of coupling. The insulin antiserum was not adsorbed and consequently contained antibodies with different specificities and a full spectrum of avidities. The anti-IgG serum was rendered specific for the Fc fragment by immuno-adsorption with immunoglobulin-deficient polymerised serum, appropriate immunoglobulin immunoadsorbents and an Fab immunoabsorbent. The anti-IgD serum was adsorbed with glutaraldehyde-polymerised "normal" human serum. The anti-IgG(Fc) serum is therefore specific for only a fragment of the whole IgG molecule, and contains a representative antibody population as regards affinities. The anti-IgD serum on the other hand is specific for the δ heavy chain and devoid of the high avidity antibodies present in the original serum. The immunoabsorbent prepared from "normal" human serum, which contains some IgD, will have removed the highest avidity antibodies. It was suggested earlier that immobilisation by covalent coupling to a carrier was more likely to affect high avidity antibodies, which are involved in a close fit with the antigen, than low avidity antibodies. The hypothesis was based on the argument that immobilisation
reduces the flexibility of the three dimensional structure of the antibody. Consequently the conformational changes required to achieve a close fit between antibody and antigen are denied. If the hypothesis is applied in this case, the anti-IgD, which has a lowered avidity, has less to lose by immobilisation than do the insulin and IgG antisera.

The sensitivity of the solid-phase radioimmunoassay for IgG was extended down to 10 ng but with some sacrifice of precision and of the working range of the assay. These results compared favourably with that of Mann et al. (149). With the solid-phase radioimmunoassay for IgD it was possible to detect the immunoglobulin at concentrations of 0.05 IU/ml (70 ng/ml) or 0.01 IU/assay tube (15 ng/assay tube). The sensitivity is dictated by the avidity of the antiserum and the availability of more avid antisera may make improvements in sensitivity possible. A similar assay technique applied to the measurement of IgE levels has achieved a sensitivity of 100 pg, a value which was improved upon with a solid phase sandwich technique using $^{125}$I-labelled antibodies to IgE (166, 167).

The coefficients of variation between triplicates obtained for the insulin and immunoglobulin assays were less than 3% and 4% respectively. Comparable figures could be achieved for the HPL assay but occasionally the coefficient of variation was as high as 8%. Had this high figure been obtained consistently the fault could have been attributed to a low avidity antiserum. The inclusion of 0.5% Tween 20 in the incubation buffer might have resulted in improved reproducibility and reduced non-specific adsorption, but because of the possibility of this detergent interfering with the antibody-antigen reaction it was considered advantageous to omit it until the final washing steps. The good precision obtained with the solid-phase radioimmunoassay method stems from its ability to achieve complete separation of bound and free antigen.
No evidence was found to suggest plasma concentrations were influencing the results as has been shown to be the case with other techniques for separation of free and bound antigen (168). Possible incubation damage caused by the action of plasma proteins on the label was not investigated, but the solid-phase assay lends itself to the avoidance of such a problem as has been suggested by Hunter (169). The plasma and immunoadsorbent are incubated together and the latter is then separated out and washed. The label is then added and the system left to equilibrate before washing and counting of the immunoadsorbent. In this way the plasma and label never come into contact and the possibility of plasma proteins damaging the label is avoided.

The solid-phase radioimmunoassay is particularly attractive because of both its practicality and economics. Possibly the most important consideration in most laboratories will be that of time. The double antibody method, which is the most widely used and the only one offering anything like the same degree of completeness of separation of free and bound antigen as with the solid-phase method, takes from 3 to 5 days to complete. The solid-phase assays for insulin and HPL reported here have been executed satisfactorily in one working day. Extra time is required for counting and processing of results, but when automatic counting and data processing facilities are available this can be achieved outside working hours.

One disadvantage of the solid-phase method is that of having to cap and tumble-mix the tubes. This is essential when maximum sensitivity is required, but for routine clinical applications it may be possible to omit these steps. Satisfactory results have been obtained in the insulin and HPL assays by mixing the assay mixture at the beginning of the incubation
period and then leaving it to stand. Providing that the particle size is small enough, and that the antibodies have a high avidity, the antibody-antigen will be essentially complete before sedimentation of the particles is sufficiently far advanced to influence the results. If the tumble-mixing procedure is to be omitted cellulose is the ideal carrier in preference to Sephadex, Sepharose or Biogel, all of which have a relatively large mass and separate out from suspension rapidly.

Good assay results have been obtained with anti-immunoglobulin immunoadsorbents prepared up to two years previously, after having been stored either in buffered 0.5% bovine serum albumin (AB1) at 4°C or freeze dried at 4°C. Anti-insulin immunoadsorbents were stored freeze-dried at 4°C and they too gave good results. This storage capability puts the immobilised antibody into the category of a "off the shelf reagent" which, for many laboratories, will be another point in its favour.

In conclusion, it must be stressed that the immobilisation of antibody invariably leads to a reduction in activity and that the assay in which it is used is usually less sensitive than the double antibody procedure for example. Good antisera for radioimmunoassay are particularly difficult to raise and it may be very difficult to justify wasting them in solid-phase radioimmunoassays. Certainly a large proportion of any development work should be carried out with model systems which employ less valuable antisera and antigens. Only when a system has been proven can one justify adopting it to save time and any other savings such as that of a 'second' antibody, and can only be regarded as offsetting the loss of antisera activity.
CHAPTER FOUR

Protein Purification by Immunoabsorption:
The Purification of Human IgG.
Protein Purification by Immunoadsorption

The purification of proteins by immunoadsorption involves the adsorption of the protein by specific antibodies immobilised on a solid support. The immunoadsorbent may be used in either a column or batch procedure.

Sepharose, Sephadex and beaded polyacrylamide based immunoadsorbents are ideal for use in columns which are prepared directly by pouring a slurry of the immunoadsorbent. Cellulose will form satisfactory columns, but it has been found necessary to mix reprecipitated microcrystalline cellulose with a solid diluent in order to improve its flow properties (157, 28). A coarse grade of cellulose may be used for this purpose (157), or better still Sephadex which has the added advantage of acting as a molecular sieve. Sephadex has been employed in conjunction with antibody immunoadsorbents prepared after the method of Gurvich (38) for the purification of serum immunoglobulins (28, 158), and also with disulphide linked antibodies (22, 23). Antibody polymers prepared with glutaraldehyde (2) and ethylene maleic anhydride (74) have been used alone in column procedures. Most reports advocate the use of a slow flow rate in order to expose the antibody for a maximum period of time. Alternatively, the antigen solution can be recycled through the column or the flow arrested once the antigen has entered the column (2). The last method has the disadvantage of exposing only a small part of the immunoadsorbent to the antigen, unless the antigen can be dispersed throughout the column.

Following the immunoadsorption step unbound protein is washed from the adsorbent, usually with phosphate or tris buffered saline. In the batch procedure this is achieved by centrifugation followed by resuspension.
of the immunoadsorbent pellet in fresh buffer. The process is repeated until no protein can be detected in the supernatant. Columns are washed through with buffer until the absorbance of the effluent at 280 nm is zero.

Elution of specifically adsorbed protein presents the greatest problem. The ideal eluting fluid alters the conformation of the proteins sufficiently to decrease the affinity of the antibody for its antigen thus allowing dissociation of the complex, but does not cause denaturation of the proteins.

Temynck and Avrameas (159) showed that a precipitate obtained with albumin and anti-albumin antibodies from hyperimmunised rabbits was not soluble in distilled water. However, if the same antibodies were adsorbed on to glutaraldehyde-polymerised albumin, 50% were recovered by washing with distilled water. They proposed that insolubilisation of the antigen decreased its affinity for the antibody, and consequently decreased the antigen-antibody association constant.

The integrity of the antigen-antibody complex is maintained by relatively weak bonds in the form of electrostatic interactions, hydrogen bonds, hydrophobic bonds and van der Waals forces. These may be broken by reagents such as urea, guanidine-HCl and chaotropic ions (thiocyanate, perchlorate and iodide) (160, 61). Where complete dissociation proves difficult disulphide bonds may be involved.

Dandliker et al. (160, 61) recovered active antibody from a BSA immunoadsorbent with chaotropic ions. A 65% recovery of precipitable antibody was achieved with 2M thiocyanate, but similar concentrations of perchlorate and iodide were less effective. It was suggested that
several factors could contribute to the success of the ions as eluting agents including electrostatic shielding, salting out, binding to the protein and influence on hydrophobic bond formation. Edgington (161) extended this work when investigating the dissociation and elution of fluoresceinated anti-human IgG from an IgG-BSA copolymer prepared by the ethyl chloroformate method (20). Thiocyanate proved to be a more efficient eluting agent than iodide. However, whereas the antibody recovered after thiocyanate elution retained only 40% of its precipitating capacity, that recovered after elution with iodide retained 89.3% of its original activity. The eluting powers of sodium chloride were particularly poor, causing dissociation of only 15% of the amount of protein released by thiocyanate and iodide with no apparent antibody activity. The dissolving power of bivalent cations was found to be considerably greater than that of monovalent cations in the order of $\text{Mg}^{2+} > \text{Ba}^{2+} > \text{Ca}^{2+} > \text{Sn}^{2+}$. The halides were most effective in the order iodide > bromide > chloride. However, at high concentrations of the eluting electrolyte the yield of antibody from the antigen immunoadsorbent was independent of both the type of ion employed and the pH of that solution. Stepwise increases in the electrolyte concentration permitted fractionation of the antibodies into various populations. Too low a pH or too high an ionic strength had irreversible effects on the antibody (160). The removal of chaotropic ions from the eluate can be achieved only by dialysis or desalting on a suitable grade of gel filtration medium.

Elution may be effected with a low pH achieved with hydrochloric acid or suitable organic acids. Antibodies to BSA, for example, were eluted from an antigen immunoadsorbent with 1% sodium chloride adjusted to pH 3.2 with hydrochloric acid (1), and human IgG has been eluted
from a specific rabbit antibody-immunoadsorbent by a similar solution at pH 2.3 (50). The hydrochloric acid is frequently used in a glycine buffer between pH 2.0 and 2.8. Such a buffer containing 0.2 M glycine was employed by Avrameas and Ternynck (63) to recover antibodies to human and rabbit IgG in yields of 91% from antigen immunoadsorbents prepared with beaded polyacrylamide. Complete recovery of homologous antigens was achieved with the same eluting fluid from anti-rabbit IgG and anti-rat IgG immunoadsorbents. The elutions were effected at 4°C. The same authors had previously reported (159) that elution of antibody was achieved sooner at 20°C than at 4°C whether glycine-hydrochloric acid buffers or an electrolyte solution was used. Maximum elution of BSA antibodies was achieved within 15 min. at 20°C with the acid buffer, whereas it took 90 min. at 4°C. The time taken to complete elution of specific antigen from an immunoadsorbent will depend upon antibody avidity and the equilibrium constant of the reactions, factors which will also dictate the severity of the elution conditions. Purification of antibodies to BSA, for example, was achieved using an ethylene maleic anhydride copolymer (74). Sequential treatment of the immunoadsorbent with glycine buffers of decreasing pH resulted in the elution of three distinct fractions. Antisera from animals that had received only two or three injections over a short period yielded the highest proportion of antibody at pH 3.0, with lesser amounts being eluted at pH 2.5 and 2.0. The first peak represented the antibodies of lowest avidity. In hyperimmune animals high affinity antibodies will predominate and consequently a lower pH will be required to elute them.

As has already been mentioned, Sephadex can be mixed with immunoadsorbents to improve the flow rates of columns. Sephadex G-200 was used in this manner in a human serum albumin (HSA) system (22, 162) and
the exclusion properties also gave partial resolution of the HSA and the acid eluent. Forty percent of the protein was recovered above pH 6.0 with 0.2 M glycine-HCl pH 2.2. The bed volume of the column was found to be critical with this grade of Sephadex since it influenced the pH profile and both the total amount of protein recovered and the amount recovered above pH 6.0. Subsequently (162) Sephadex G-15 was found to be better than the G-200 in that it packed easily into uniform columns with the immunoadsorbent and gave faster flow rates. Furthermore, the pH front was more sharply defined and a higher recovery of protein was achieved above pH 6.0, and in a smaller volume. The authors suggested that a shallow pH profile leads to a gradual protonation of the proteins, which causes partial dissociation of the antibody-antigen complex. Non-specific antigen-immunoadsorbent complexes are then formed which cannot be dissociated at low pH. On the other hand rapid protonation associated with a sharp pH profile produces complete dissociation of the antigen-antibody complex. Fluorescent-labelled human IgG was recovered in yields 70-90% at neutral pH after elution with 0.2 M glycine-HCl pH 2.2.

The relative abilities of 2 M potassium iodide, 8 M urea, 1 M propionic acid and 20 mM hydrochloric acid pH 1.8 to elute myoglobin antibodies from several different myoglobin-immunoadsorbents were studied by Boegman and Crumpton (27). Propionic acid and hydrochloric acid gave the highest protein recoveries, the former giving the higher recovery of precipitatable antibody.

Hill (81), bearing in mind the importance of hydrophobic bonding in the antibody-antigen reaction, investigated the effect of dioxan dissolved in weak organic acids on elution. The inclusion of 10%
Dioxane in 0.1 and 0.5 M solutions of acetic and propionic acids increased the effectiveness of the acids as eluents of antibody from bromoacetyl cellulose-HSA and Sepharose-HSA. Higher yields of pig lactate dehydrogenase antibodies were obtained by adding dioxane to citric acid used as eluent. Batch elution of most of the antibody was achieved in 10-15 min. at room temperature with 10% dioxane in 0.1 M acetic acid. Antibodies eluted in the presence of dioxane after 2 hr. at room temperature were of high avidity than those recovered in the absence of the dioxane. The organic solvent did not appear to have an adverse effect on the antibodies. It was suggested that the success of dioxane was due to its randomising the water structure in the hydrophobic regions of the antibody since it had been shown to reduce dipole-dipole interactions and hydrogen bonding in water (163). Hydrophobic bonds contribute considerably to the maintenance of antibody-antigen complexes, and disruption of these would therefore favour dissociation of the complex.

Antibody immunoadsorbents specific for IgG, IgA, IgM and K and L chains have been prepared by coupling the antibody to cyanogen bromide activated Sepharose 4B and used to remove immunoglobulins from serum (164). The adsorbed immunoglobulin was eluted with either 3 M thiocyanate or 0.01 M hydrochloric acid - 0.15 M sodium chloride. Sequential use of the immunoadsorbents allowed complete removal of the immunoglobulins from serum, and columns were used as many as 20 times over a period of 12 months. The authors suggested the possible application of the technique to the removal of IgD and IgE and possibly to the removal and isolation of IgG subgroups. Bennich and Johannson (165), Carrel et al. (19) and Aalberse et al. (158) applied the technique to the purification
of human polyclonal IgE. Immunoadsorbents were prepared with Sepharose 4B, cross-linked polyacrylamide and diazotised benzylxoymethyl cellulose respectively, and elution was effected with acid or 3.0-3.5 M thiocyanate (19, 165) and glycine-citrate buffer pH 2.8 (158). The cellulose based immunoadsorbent (158) was used in conjunction with Sephadex G25 in a manner similar to that described by Crooke et al. (162) for their Mk 3 column. Sephadex G25 was mixed with the immunoadsorbent, the former being in a 20 to 80-fold excess, in order to improve the flow rate of the column and resolve the protein and acid peaks emerging from the column. A batch procedure gave higher yields of IgE than did the column procedure, but the degree of contamination was greater. Glutaraldehyde-polymerised antibodies to IgE were also tested but were found to have a lower antigen binding capacity than the cellulose coupled antibodies, and the purity was even lower than that obtained with the batch procedure. The preparation obtained with the cellulose-based immunoadsorbent was found to have both a modified antigenic composition and a modified electrophoretic mobility, possibly due to denaturation, fragmentation or aggregation.

In the present study the purification of IgG by immunoadsorption has been used as a model for the purification of IgD from myeloma and 'normal' human sera. Cellulose-based immunoadsorbents were used and these were prepared by methods described in Chapter 2. Six possible purification procedures were investigated:—

1. Column (Immunoadsorbent alone)
2. Column (Immunoadsorbent mixed with Sephadex or Biogel)
3. Column (Immunoadsorbent packed on top of Sephadex or Biogel)
4. Batch.
5. Batch combined with use of Amicon filtration tubes.
6. A combination of both batch and column procedures.
Materials and Methods

All immunoadsorbents were prepared according to methods described in Chapter 2, the only variation being in the grade of cellulose used.

Phosphate-buffered saline (P.B.S.) pH 7.66 was prepared by dissolving 12.8 g Na_{2}HPO_{4}, 1.56 g NaH_{2}PO_{4}.2H_{2}O and 85.00 g NaCl in 10 litres of distilled water. Borate buffered saline (B.B.S.) pH 8.4 contained 4.50 g H_{3}BO_{3}, 5.50 g Na_{2}B_{4}O_{7} and 8.5 g NaCl in 1 litre. Phosphate buffer pH 7.66, 100 mM, contained 12.80 g Na_{2}HPO_{4} and 1.56 g NaH_{2}PO_{4}.2H_{2}O in 1 litre. Glycine-HCl buffers for elution were prepared by titrating 50 mM glycine to the desired pH with hydrochloric acid.

Antigen Purification Procedures

Method 1

The immunoadsorbent was packed into a glass column fitted with a teflon plunger supporting a plastic sinker, and equilibrated with B.B.S. The effluent buffer was monitored at 280 nm by a U.V. spectrophotometer fitted with a flow cell. An aliquot of serum was applied to the top of the column and washed through with B.B.S. In some experiments the serum was recycled through the column for a given time, or the flow was arrested before washing. Washing was judged to be complete when the adsorbance at 280 nm was zero. The B.B.S. was replaced by the eluting fluid, and fractions emerging from the column were collected into equal volumes of 100 mM phosphate buffer pH 7.66.

Method 2

The same protocol as in Method 1 was followed, with the exception that the immunoadsorbent was first mixed with preswollen Sephadex G-25.
Method 3

The immunoadsorbent was packed on top of a column of Sephadex G-25 or Biogel P.10. The bed volume of the gel-filtration medium was three times that of the immunoadsorbent.

Method 4

Batch Procedure.

A suitable amount of the immunoadsorbent was mixed with serum in a 25 ml screw-top universal bottle. The bottle was rotated on a Luckhams rocker-roller mixer for 30 min or more before the immunoadsorbent was collected by centrifugation in a bench centrifuge at 4°C. The immunoadsorbent was washed repeatedly by resuspension in fresh B.B.S. followed by centrifugation. Washings were checked for protein by absorbance at 280 nm, and once a steady state had been attained (usually 8 to 10 washes) the immunoadsorbent was mixed with the eluting fluid. The bottle was rotated on the Luckhams mixer for 5 min. or more before separation of the immunoadsorbent and eluate by centrifugation. The eluate was neutralised with alkali and dialysed against 1 litre of P.B.S. for 16 hr. before analysis.

Method 5

Modified Batch Procedure.

Method 4 was followed through to the immunoadsorption step. The suspension of the immunoadsorbent in serum was transferred to the upper chamber of an Amicon filtration tube fitted with a glass-fibre disc on top of a cellulose acetate membrane with an 8µ exclusion limit. Matched tubes were centrifuged at 1000 g for 3 min. The adsorbed serum passed through to the lower chamber leaving the immunoadsorbent on the filter in the upper chamber.

Non-specifically adsorbed protein was washed from the immunoadsorbent by introducing B.B.S. into the upper chamber and centrifuging. The
process was repeated until protein was absent from the washings. Elution was effected either by introducing the eluting fluid into the upper chamber with the immunoadsorbent and leaving it to filter through into an equal volume of 0.1 M phosphate buffer pH 7.66, or by suspending the immunoadsorbent in the eluting fluid.

Method 6

Combined Batch and Column Procedure.

Methods 4 and 3 were combined. The batch procedure was followed to the completion of the immunoadsorption step at which point the suspension was transferred on to the top of a column of Sephadex G-25 or Biogel P.10. The washing and elution procedures were carried out in the column.

Measurement of IgG and Total Protein

Protein-containing fractions of the eluate were pooled and dialysed against P.B.S. The dialysates were concentrated by pressure dialysis and the volume adjusted to equal that of the original serum volume used. The protein content was measured by the absorbance at 280 nm and IgG by radial immunodiffusion as described in Chapter 2. The purity was checked by immunodiffusion and immunoelectrophoresis and, when the results of these tests justified it, by disc gel electrophoresis and ultracentrifugation.

Results and Discussion

Whatman CC31 microcrystalline cellulose, which had proved to be very suitable for use in radioimmunoassay procedures, was found to be unsuitable for use alone in columns because of its very small particle size. Either flow rates were very slow or the columns clogged. More satisfactory results were obtained by preparing the immunoadsorbents with Whatman CF11 fibrous cellulose. The immunoadsorption step was
conducted in one of three ways: (i) the serum was loaded on to the column and run straight through; (ii) the flow was arrested once the serum had entered the immunoadsorbent and restored after a given time; (iii) the serum was recycled through the column. The highest recovery of IgG was obtained with the recycling procedure, but this preparation also contained considerable protein impurity (Table 4.1). The recovery of IgG after arresting the flow was slightly less and again there were considerable levels of protein impurity. After running the serum straight through the column the amounts of IgG recovered was even less, but this proved to be the cleanest of the three preparations (Table 4.1). From these results it was concluded that the shorter the time of exposure of the serum to the immunoadsorbent the smaller the amount of non-specific protein adsorption.

In an attempt to improve column flow rates and the efficiency of the washing procedure, columns were prepared from mixed slurries of the immunoadsorbent and Sephadex G-25 (Method 2). Differences between the densities of the immunoadsorbent particles and those of the gel made the packing of uniform columns difficult. The best results were obtained when thick slurries were poured with Sephadex in a three to four-fold excess. It was possible to use the Whatman CC31 based immunoadsorbents because the Sephadex prevented clogging and allowed the maintenance of satisfactory flow rates. Only partial resolution of the protein and acid front was achieved (Fig. 4.1). Two factors may contribute to this. Firstly, antigen is specifically adsorbed throughout the length of the column, and consequently antigen dissociated towards the end of the column will not be resolved from the acid front. The second possibility, which must be borne in mind, is that of readsoption of the antigen. Antigen dissociated from the immunoadsorbent at the top of the column passes ahead of the acid front as a result of
gel filtration. Once free of the acid conditions the antigen may be readsorbed by antibody further down the column. The protein front is therefore retarded by readsorption and fails to resolve fully from the acid front. Furthermore, if readsorption of the antigen occurs there is always the possibility that it will become involved in a reaction with antibody which shows a greater avidity for the antigen. Then, dissociation of the resulting antibody-antigen complex may require a 'stronger' eluting agent.

These problems were overcome by adopting the procedure described in Method 3. The results of a typical separation are presented in Fig. 4.2. Two protein peaks were eluted from the column, the first, and major peak, ahead of the acid front and the second peak with the acid front. The first peak contained 10.1 mg of IgG, and this was shown to be homogenous by ultracentrifugation (Fig. 4.3). Radioactivity was divided between the first and second peaks in the ratio 85:15. Similar results were obtained when 1M propionic acid was used as the eluting agent.

In the immunoadsorbent section of the column (Method 3) the eluted protein moves with or behind the acid front, and is only resolved from this on passing into the Sephadex G-25. There is therefore no danger of readsorption of the antigen. This may explain why the yield of IgG was greater than that achieved with the mixed bed column (Fig. 4.1).

Although column procedures gave satisfactory purification of IgG, batch procedures were also tested to ascertain the relative merits of the two approaches. Method 4 had one obvious disadvantage from the outset. After centrifuging and decanting the supernatant a considerable
amount of liquid remained trapped in the immunoadsorbent. This failure to separate the two phases completely prolonged the washing procedure required before specifically adsorbed protein could be eluted. The problem was easily overcome by the use of Amicon filtering centrifuge tubes (Method 5 and Fig. 4.4) which gave almost complete separation of the two phases and greatly facilitated the collection of washings and eluates. The eluates were collected directly into 0.1 M phosphate buffer, pH 7.66, in the lower chamber.

The results of some typical batchwise purifications are shown in Table 4.2. Immunoelectrophoresis revealed that IgG isolated from whole serum by Method 4 was heavily contaminated, a result corroborated by the discrepancy between theoretical and actual O.D.\textsubscript{280} values. Albumin appeared to be the major contaminant. Similar results were obtained with Method 5, although the amount of contamination was less. Ammonium sulphate precipitation of the immunoglobulin fraction of serum prior to immunoadsorption overcame the problem of albumin contamination, but, although immunoelectrophoresis with anti-whole human serum failed to show any obvious contamination of the IgG fraction, a discrepancy between theoretical and actual O.D.\textsubscript{280} values remained.

A reduction in the level of contamination, with a sacrifice in yield, was achieved by adopting a stepwise elution protocol (Table 4.2). After adsorption of IgG from unfractionated serum and washing of the immunoadsorbent at pH 7.66, the fraction eluted at pH 4.0 contained relatively more contaminating protein than that eluted at pH 2.0.

Methods 4 and 5 were used routinely for absorbing antisera to render them mono-specific.
The combination of batch and column procedures (Method 6) was used in the purification of IgD (Chapter 5). A comparison of column Method 3, batch/column Method 6 and batch Method 5 confirmed the findings for IgG.
Table 4.1

Recoveries of human IgG by Method 1 after varying the conditions of immunoadsorption

Antibody coupled to diazotised N-(3-aminobenzoyloxymethyl)cellulose (CF11)

Quantity of antibody immobilised: 8.4 mg (AS.55)

Elution with 50 mM glycine - HCl, pH 2.5

<table>
<thead>
<tr>
<th></th>
<th>Serum applied to column (ml)</th>
<th>IgG applied to column (mg) (1)</th>
<th>O.D. Units recovered by washing</th>
<th>IgG recovered by elution (mg) (2)</th>
<th>Theoretical O.D. Units attributable to IgG (2)</th>
<th>O.D. Units attributable to contaminants</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Serum run through</td>
<td>1.5</td>
<td>16.8</td>
<td>11.40</td>
<td>6.30</td>
<td>4.4</td>
<td>6.07</td>
</tr>
<tr>
<td>(b) Flow arrested for 30 min.</td>
<td>1.5</td>
<td>16.8</td>
<td>9.60</td>
<td>7.48</td>
<td>4.8</td>
<td>6.34</td>
</tr>
<tr>
<td>(c) Serum recycled for 30 min.</td>
<td>1.5</td>
<td>16.8</td>
<td>8.00</td>
<td>9.10</td>
<td>6.2</td>
<td>8.56</td>
</tr>
</tbody>
</table>

(1) Determined by radial immunodiffusion

(2) Assuming $E_{280nm}^1$ = 13.8 for human IgG.
Purification of human IgG by Method 2.

Antibody coupled to glutaraldehyde activated aminodipriopylamine cellulose.

Total protein coupled: 86 mg
Antibody coupled: 17 mg (AS 57)
Column dimensions: 20 x 1.5 cm.
Human Immunoglobulin added: 35 mg.
Eluting agent: 50 mM glycine - HCl, pH 2.0.
Yield: 7.1 mg (Determined by radial immunodiffusion).
Purification of human IgG by Method 3.

Antibody coupled to glutaraldehyde activated aminodipropylamine cellulose

Total protein coupled: 86 mg.
Antibody coupled: 17 mg (AS 57).
Bed volume Sephadex G 25: 30 ml.
Bed volume immunoadsorbant: 6 ml.
Column dimensions: 21 x 1.5 cm.
Human immunoglobulin added: 35 mg + trace labelled $^{125}$I-IgG.
Eluting agent: 50 mM glycine - HCl, pH 2.0.
Yield: 10.1 mg. (Determined by radial immunodiffusion).
Sedimentation pattern of human IgG purified by method 3.

Photographs taken at 8 min. intervals after reaching 59,780 r.p.m. at 20°C.

Protein dissolved in 0.05 M phosphate-buffered saline, pH 7.66.
Purification of human IgG from (a) pooled human serum diluted 1:1 with B.B.S. and (b) the ammonium sulphate precipitated fraction. IgG was eluted with 50 mM glycine-HCl.

<table>
<thead>
<tr>
<th>Serum added (ml)</th>
<th>IgG added (mg)(iii)</th>
<th>Elution pH</th>
<th>O.D. units recovered by elution (280 nm)</th>
<th>IgG recovered by elution (mg) (iii)</th>
<th>Theoretical O.D. units attributable to IgG(iv)</th>
<th>O.D. units attributable to contaminants</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) (i) 5</td>
<td>56</td>
<td>2.0</td>
<td>11.0</td>
<td>6.5</td>
<td>8.97</td>
<td>2.03</td>
</tr>
<tr>
<td>(b) (i) -</td>
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<td>7.4</td>
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<tr>
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<td>4.8</td>
<td>3.4</td>
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(i) Method 4.
(ii) Method 5.
(iii) Determined by single radial-immunodiffusion.
(iv) Assuming an extinction coefficient $E_{280\text{ nm}}^{1\%}$ 13.8 for human IgG.
Fig. 4.4

Diagrammatic representation of filtering centrifuge tube.

1. Cap
2. Plastic tube
3. Upper chamber/filter holder
4. O-ring
5. Glass fibre or cellulose acetate filter
6. Filter support screen
7. Wire-mesh under-support.
Conclusion

The five protocols applied here to the purification of IgG have proved successful to various degrees, column procedures on the whole giving the best results. The two methods of antibody immobilisation giving retention of the highest percentages of antigen-binding capacity (as reported in Chapter 2), namely covalent coupling to diazotised N-(3-aminobenzylxoxymethyl)cellulose and glutaraldehyde-activated aminodipropylamine cellulose, provided immunoadsorbents which performed admirably on a preparative scale. As mentioned previously the diazotised derivative has the apparent advantage of coupling via tyrosine residues and a spacer arm, whilst the glutaraldehyde activation of aminodipropylamine cellulose owes its success to the 14-atom spacer arm which results.

The most efficient conditions for immunoadsorption in columns were achieved by recycling the antigen solution. The method was only really satisfactory when the immunoadsorbent was used without a gel filtration column. Immunoadsorption of maximum amounts of antigen was achieved in batch procedures, but these methods also appeared to attract higher levels of non-specific protein binding. However, this may reflect less adequate washing of the immunoadsorbent than can be achieved in columns, and accentuates the importance of efficient washing prior to elution. The problem was resolved in part by using a stepwise wash/elution protocol with buffers of decreasing pH. Ideally the immunoabsorbent should be prepared from moderately high avidity antibodies so that washing with low acidity buffers does not result in loss of specifically adsorbed antigen prior to elution with a buffer of higher acidity. Ternynck and Avrameas (63) claimed that non-specific adsorption onto an immunoabsorbent prepared
from glutaraldehyde-activated Biogel was almost non-existent, and more recently (216) excellent results have been obtained with glutaraldehyde-activated aminohexyl-Sepharose-4-B, non-specific protein adsorption accounting for less than 0.2% in the case of IgA and even less for IgG. In the present study appreciable levels of non-specific adsorption were encountered with glutaraldehyde-activated aminodipropylamine cellulose. Cellulose may therefore be a less satisfactory carrier than are Biogel and Sepharose from this point of view.

Elution was effected with acid pH, glycine-HCl and propionic acid being the eluting agents employed routinely. Sodium thiocyanate (3M) was found to be a satisfactory eluting agent when employed in batch procedures, but did not come up to expectation when used in columns. This also was found to be the case in the purification of rabbit antibodies specific for HCG (I. Cayzer, personal communication). Antibodies were recovered from Sepharose-4-B-HCG with glycine-HCl pH 2.0, but not with sodium thiocyanate (3M) as judged by latex agglutination (Prepurex) and Ouchterlony immuno-diffusion.

Rapid removal or neutralisation of the eluting agent after dissociation of the immunoadsorbent-antigen complex is desirable in order that the chances of protein denaturation are minimised. The acid eluting agents used in columns were successfully resolved from the dissociated protein by gel filtration on Sephadex G-25. Resolution was achieved in the minimum time with the immunoadsorbent and gel filtration medium in series in the same column. This approach also circumvented the problem of readsorption of the antigen which apparently was encountered when the immunoadsorbent was mixed with the Sephadex. In the case of batch procedures, neutralisation and subsequent dialysis provided the
only effective means of removing the eluting agent.

Column techniques were generally superior to batch techniques in as far as post-immunoadsorption washing and elution were concerned. However, the efficiency of batchwise handling has been improved considerably by the use of Amicon filtration tubes.

Subject to the availability of pure antigen it may prove advantageous to prepare a pure antibody fraction from a suitable antiserum. If prepared by immunoadsorption the antibody will be pure with respect to both specificity and avidity. Immunoadsorbents with a high antibody:carrier ratio and medium to low avidity may then be prepared. The resultant need for smaller amounts of carrier and the presence of little or no non-antibody protein will reduce the potential risk of non-specific adsorption. These improvements have been implemented for the purification of IgD (Chapter 5), together with the use of a batch/column protocol (Method 6, Chapter 4).
CHAPTER FIVE

The Purification of IgD and anti-IgD, and
the Partial Characterisation of an IgD Myeloma
IMMUNOGLOBULIN D (IgD)

Introduction

Immunoglobulin D (IgD) was first discovered in the serum of a patient with multiple myeloma by Rowe and Fahey (170, 171). It constitutes a class of immunoglobulins distinct from immunoglobulins G, M, A and E.

The mean concentration of IgD in 'normal' human serum is generally accepted to be 30 µg/ml (171, 172), and it is this low concentration, together with a lack of physicochemical properties distinguishing it from other immunoglobulins, that have led to failures in attempts to purify it from serum. Consequently, the majority of studies have been carried out on myeloma IgD, with the exception of those reported by Bratcher et al. (173) for IgD from dysgammaglobulinaemic serum.

Rowe et al. (174) employed ion-exchange chromatography on DEAE-cellulose in conjunction with Sephadex G-200 gel filtration to effect purification of myeloma IgD. The serum was dialysed against 0.01 M phosphate buffer pH 8.0 and chromatographed on a DEAE-cellulose column with an eluting buffer gradient from 0.01 to 0.30 M. The IgD was eluted after transferrin and before α₂-macroglobulin. Sephadex G-200 chromatography was performed in 0.05 M tris pH 8.0, 0.2 M with respect to sodium chloride. One of the myeloma sera had very low levels of IgG and IgA, but even so the IgD preparation contained trace amounts of the other immunoglobulins. Initial attempts at purification yielded Fc-like fragments which were eluted from the Sephadex column immediately after the IgD. It was established that the protein underwent proteolysis, and the problem was partly resolved by working at 4°C and including the enzyme inhibitor ε-amino caproic acid at a concentration of 0.001 M. The
concentration of the inhibitor was increased to 0.01 M on completion of the purification. Griffiths and Gleich (175) noted that 5 mM ε-amino caproic acid inhibited the activation of plasminogen but did not effect plasmin activity. Leslie et al. (176) and Spiegelberg et al. (177) used similar procedures, but again the preparations contained small amounts (< 1%) of other immunoglobulins. Griffiths and Gleich (175) modified the procedure by introducing an initial dialysis against 0.02 M phosphate pH 5.4 containing 0.02 M EDTA. The resulting euglobulin precipitate was shown to contain the fifth component of complement which, if the dialysis step was omitted, contaminated the IgD preparations. Precipitation of the immunoglobulins was effected with 50% ammonium sulphate, and after desalting the purification was completed with DEAE-cellulose and Sephadex G-200 chromatography. Several of the preparations, although apparently homogeneous by physical techniques, were shown to contain trace amounts of IgG and α₂-macroglobulin by Ouchterlony analysis.

Bratcher et al. (173) took advantage of the solubility of IgD in 0.05 M zinc acetate at pH 6.5 and the absence of IgA from a dysgammaglobulinaemic serum. The purification also involved DEAE-cellulose and Sephadex chromatography and immunoadsorption. Any trace amounts of IgG and IgM were removed in a final purification step by immunoadsorption employing the appropriate antibodies covalently linked to p-aminobenzylcellulose. Saha et al. (178) also used immunoadsorbents prepared from bromoacetylcellulose to render their preparations homogenous. (See page 194).

Like IgG, IgD is composed of two heavy and two light chains linked together by disulphide bridges, one between the neighbouring heavy chains and one between each heavy chain and a light chain (177, 179). In the IgD myelomas studied light chains have predominated. The immunoglobulin differs markedly from IgG in that it contains 11-14% carbohydrate as
opposed to 3%, a figure more in keeping with the levels found in IgE and IgM. Hydration of the carbohydrate moieties of these glycoproteins is reflected in increased values of the frictional coefficient ratios, and consequently values obtained for IgE and IgD could be expected to be similar. Reported values of the frictional coefficient ratios are 1.80 (IgD), 1.60 (IgE) and 1.47 (IgG) (175). The molecular weight of the intact molecule has been reported variously between 166,000 and 200,000 daltons (174, 177, 178), and those of heavy and light chains as 60,000 and 23,000 daltons respectively (177). The sedimentation coefficients of various IgD myeloma proteins measured by different research groups vary considerably. Two IgD samples purified by Rowe et al (174) had values of 6.14 and 6.19 $S_{20,w}^0$, as compared with 6.64 $S_{20,w}^0$ for IgG, while values as high as 6.92 (173), 7.0 (177) and 7.2 $S_{20,w}^0$ (180) have been measured. The partial specific volume of IgD is 0.717 ml/g (173, 177) which is somewhat lower than that if IgG at 0.738 ml/g, and is indicative of a higher density. On chromatographing the immunoglobulins on Sephadex G-200, IgM is eluted first followed by IgD, IgE, IgA monomer and IgG (173), a sequence in keeping with their Stokes radii (175). The physical parameters of IgD together with its susceptibility to digestion by proteolytic enzymes (175) suggest that its structure is in some way unique amongst the immunoglobulins. The molecule is considered to be hydrated, elongated and flexible.

Myeloma IgD has been reported to migrate in the slow β-globulin region on zone electrophoresis (170) and was found by Bratcher et al (173) to have an isoelectric point of 5.85 and electrophoretic mobility of $2.52 \times 10^{-5}$ cm$^2$ volt$^{-1}$ sec$^{-1}$.

Amino acid analysis of IgD myeloma proteins has shown that one IgD differs considerably from another (178). The proline level was low
compared with other immunoglobulins, and the Bence-Jones protein excreted by one of the patients was unusual in that it contained three methionine residues as opposed to none in other Bence-Jones proteins.

The biological function of IgD is still a mystery, but antibody activity has been ascribed to the class in a few instances. Ritchie (181) demonstrated IgD antinuclear antibodies in a number of patients with rheumatic disorders, and these were also found by Watson et al. (182) in 12 out of 25 patients with systemic lupus erythematosus and Kantor et al. (183). IgD antithyroid antibody was also found in 1 out of 26 patients tested (183). A fluorescent labelled anti-rabbit globulin was used in conjunction with class specific anti Fc\(^\delta\) serum to detect the antibodies in the patients serum (182, 183). This technique was employed by Schmidt and Mueller-Eckhardt (184, 185) in a study of 82 patients with demonstrable antinuclear factor (ANF). Of these 32 were found to have IgD-ANF, cases with systemic lupus erythematosus, rheumatoid arthritis and chronic liver diseases being the most frequently involved.

Anti penicilloyl activity was found associated with IgD by Gleich et al. (186). The antibodies were detected in an antiglobulin test on benzyl penicillin coated erythrocytes. The antiglobulin reagent was blocked by pure IgD but not by other immunoglobulins. Heiner and Rose (187) conducted a study of antibody responses by radioimmunodiffusion and demonstrated antigen binding activity in the IgD fraction of four sera. Three of the patients were sensitive to cow's milk and had antibodies to bovine IgG while two of them also had antibodies to BSA. The fourth patient produced antibodies in response to a booster injection of diphtheria toxoid.

Devey et al. (188) found what appeared to be IgD antibody to insulin in three out of six diabetic patients investigated using a red blood cell
linked antigen-antiglobulin reaction.

Klapper and Mendenhall (189) investigated the concentration of IgD in pregnant women and found that 41% of the women screened at the time of delivery had high levels of circulating IgD. Other pregnant women and those using oral contraceptives showed lesser elevations in their IgD levels. IgD was not detected in 30 cord sera. However, the immunoglobulin was demonstrated in the sera of 7 out of 84 neonates tested by Evans et al. (190) and 4.5% of those screened by Leslie and Swate (191). Variations in the permeability of placentae could have accounted for IgD being present in the neonates.

A study of sarcoidosis and tuberculosis patients (192) led to the detection of IgD in the sera of 20% more tuberculosis patients and 20.7% fewer sarcoidosis patients than in the respective controls. Also the serum IgD concentration was found to be age dependent, high levels occurring predominantly in tuberculosis patients and low levels in middle-age sarcoidosis patients.

Pernis, Chiappino and Rowe (193) employed rhodamine-labelled anti IgG and fluorescein-labelled anti IgD to demonstrate separate populations of IgG and IgD secreting cells. Furthermore, the numbers of such cells were shown to be proportional to the circulating levels of the immunoglobulins.

van Boxel et al. (194) found that a high percentage (2.7%) of peripheral blood lymphocytes bear surface IgD. This figure represents 18% of lymphocytes with surface immunoglobulins. They suggested that either IgD or its Fc fragment is cytophilic for a subset of lymphocytes or that the lymphocytes are ones which fail to mature into immunoglobulin secreting cells. Piessens et al. (195) and Knapp et al. (196) obtained similar results, the latter demonstrating both IgD and IgM on the same lymphocyte surface, and Rowe et al. (197) demonstrated that c. 15% of peripheral lymphocytes in the
new-born contain IgD, the level of circulatory IgD being virtually undetectable. Rowe et al. (204) also reported that membrane-bound IgM and IgD with the same light-chain subtypes were demonstrable on the same peripheral lymphocytes in both neonates and adults. The same two immunoglobulins were found together on the cells of patients with chronic lymphocytic leukaemia (207), and on the surfaces of monoclonal B lymphocytes from the bone marrows of seven cases of Waldenstrom's macroglobulinaemia (199). van Boekel et al. (205) have shown that IgD is present in the membranes of lymphocytes grown in continuous culture in vitro, along with IgG, IgA and IgM, supporting the view that the immunoglobulins are produced in the cells on whose surfaces they occur. It has been suggested (204) that a switch from the synthesis of $\delta$ to $\mu$ chains accompanies maturation of the B lymphocytes. The more-mature members of each lymphocyte clone, the plasma cells, rarely carry IgD, even when the majority of lymphocytes in the bone marrow bear IgD on their surfaces. Further evidence for a switch from $\delta$ to $\mu$ chain synthesis during B lymphocyte differentiation came from studies on patients with immunodeficiency disorders (215). The immunodeficiency stemmed from a block in the B cell differentiation at a level between lymphocytes carrying both $\mu$ and $\delta$ chains and those carrying $\mu$ chains alone. It appears that B cells carrying both IgD and IgM have first to lose their surface IgD and become IgM producers before differentiating further into IgG and IgA bearing lymphocytes. The possibility of a switch from $\delta$ to $\mu$ chain production was not endorsed by Fu et al. (200). IgM and IgD occurring together on the leukaemic lymphocytes of one particular patient were found to have the same idiotypic specificity, a phenomenon observed over several months and therefore regarded as being a stable one. The concept of separate m-RNA species for $\delta$ and $\mu$ chains with identical $V_H$ regions was inferred.
Only one light-chain sub-type has been found on any one cell (205). In the case of IgD, λ light-chain producing cells predominate over κ producers in the spleen in the ratio of 5:1 (206), figures reflected in the serum levels in normal individuals and in the relative numbers among IgD myelomas. In 45 reported IgD myelomas (206), paraproteins with λ-chains outnumbered those with κ-chains by 37 to 8. In the cases of the other immunoglobulins the position is reversed, the ratio of κ to λ being close on 2:1.

The biological function of IgD may be related to its presence on cell surfaces, and the high proportion of IgD bearing lymphocytes could point to the involvement of the immunoglobulin in the induction of immunotolerance. The IgD molecules may function essentially as antigen receptors.

Kohler and Farr (208) have reported the occurrence of higher IgD levels in atopic (reaginic) sera than in non-atopic (non-reaginic) sera, and work by Hsieh and Chen (201) has shown that serum IgD is elevated in asthmatic children, suggesting a role in human atopy. However, there have been no reports so far of allergen-specific IgD class antibodies.

This summary of the work contributed thus far on IgD shows that the immunoglobulin may play an important role in the human immune system. The development of simple methods of purification of the immunoglobulin from both myeloma and normal sera together with sensitive assays for measurement of the protein and specific antibodies of the IgD class may help to solve part of the problem.
The applications of isotachophoresis (217) and isoelectric focusing (214) to the purification of human IgD myeloma proteins have recently been described in the literature. In both instances the success of the purification was judged by immunoelectrophoresis. After purification by isotachophoresis the electrophoresis mobility of the IgD was more cathodal, a fact attributed to loss of sialic acid residues.
Materials and Methods

(i) Cellulose Acetate Electrophoresis

Cellogram strips were floated in sodium barbitone buffer I-0.05 for 30 min. and then totally immersed in the buffer. The strips were then blotted lightly before use. Samples were applied to the strips with a Shandon 'comb type' applicator delivering 2 μl. The electrophoresis was run for 30 min. at 250 volts.

Proteins were stained by immersing the strips in either 0.2% ponceau S in 5% acetic acid or 0.1% coomassie brilliant blue R made up in 80 ml ethanol; 180 ml water; 30 g trichloracetic acid; 9 g sulphosalicylic acid (202). De-staining was effected with 5% acetic acid or a mixture of methanol, water and acetic acid in the proportions 250:650:80.

(ii) Isoelectric Focusing

Polyacrylamide was used to stabilise the ampholyte pH gradient and the gels were made up incorporating the protein sample and the ampholyte. A standard Shandon disc gel electrophoresis apparatus was used with glass tubes 75 mm long and 5 mm internal diameter. The electrofocusing was performed at 4°C. Gels were prepared by the method of Wrigley (203) with acrylamide and ampholyte concentrations of 5 and 2% respectively. Chemical polymerisation was achieved with ammonium persulphate. A maximum current of 1.5 mA per tube was maintained for 90 min. by increasing the voltage, and the voltage was then maintained for a further 3 hr. Protein bands were stained with coomassie brilliant blue R as described under 'cellulose acetate electrophoresis'. 
(iii) 'Gradipore' Electrophoresis

The apparatus was kindly loaned by Mr. Graham of Universal Scientific who also supplied the preformed polyacrylamide gel gradients.

The gel slabs were supplied containing borate-buffered saline, and these ions were removed by pre-running the gels in 0.05 M tris-glycine pH 8.3 for 2 hr. Samples were applied to the top of the gel through a comb template, and electrophoresis was commenced at 3 mA/cm with the template in place. After 30 min. the template was removed and electrophoresis continued for 24 hr. at constant current. Protein was stained with coomassie brilliant blue R or 0.7% Amido Black.

(iv) Analytical Ultracentrifugation

Ultracentrifugation studies were conducted in Spinco Model E centrifuges fitted with Schlieren and interference optics. An An-D rotor was used in conjunction with a 12 mm 2° sector cell at rotor speeds of 44,000 r.p.m. (electronic speed control) and 50,740 r.p.m. (mechanical speed control). Samples were made up in 50 mM phosphate buffer pH 8.0, 150 mM with respect to sodium chloride and containing 10 mM ε-amino caproic acid (Na₂HPO₄ 13.65g, NaH₂PO₄·2H₂O 0.65 g, NaCl 8.775 g and ε-aminocaproic acid 0.656 g to 1 litre with distilled water).

Sedimentation coefficients were determined for a number of serial dilutions of the protein and values extrapolated to infinite dilution. The viscosity of the buffer relative to distilled water was measured with a No. 0 Ostwald viscometer equilibrated in a water bath at 20°C. Fluid densities were measured with a 25 ml pycnometer. Values for partial specific volumes were taken from the literature.
Sedimentation coefficients were corrected to standard conditions using the following equation:

\[ S_{20,w}^{0} = S_{obs} \frac{\eta_i}{\eta_{20}} \frac{\eta_{sol}}{\eta_w} \frac{1 - \bar{v} C_{20,W}}{1 - \bar{v} C_{t \, sol}} \]

where \( \eta \) = viscosity, \( C \) = density and \( \bar{v} \) = partial specific volume.

(v) Reduction of Interchain Disulphide Bonds

A 1% solution of the immunoglobulin was mixed with an equal volume of 0.15 M tris-HCl pH 8.0 made 0.15 M with respect to sodium chloride and 0.002 M with respect to ethylene diamine tetraacetic acid. Dithiothreitol (DTT) was added to give a final concentration of 0.01 M, equivalent to a 150 molar excess, (assuming a molecular weight of 150,000 for immunoglobulins). The mixture was incubated for 2 hr. at 25\(^\circ\)C. Solid iodoacetamide was added to a final concentration of 0.04 M, and the reaction vessel was wrapped in foil and placed in the dark for 15 min. The reaction mixtures were then dialysed overnight against 0.01 M phosphate buffer pH 7.1, again in the dark. The dialysates were concentrated to 1% protein in Amicon B15 concentrator cells. This solution was made 1% with respect to DTT and sodium dodecyl sulphate (SDS), and 5% with respect to sucrose, and heated at 100\(^\circ\)C for 2 min. A sample containing the equivalent of 100 \( \mu \)g of the original protein was examined by electrophoresis.

(vi) Electrophoresis in Sodium Dodecyl Sulphate

Bromophenol blue was included as a marker in all samples, and these were run in a 5% polyacrylamide gel containing 0.1% SDS and buffered with 0.1M phosphate pH 7.1. The electrophoresis was run for 2 hr. at 90 volts.

5% Acrylamide gel:-
1.67 ml Acrylamide 30 g, \(\text{N,N'}\)-methylene bisacrylamide 0.8 g to 100 ml with distilled water.

2.00 ml 0.5 M phosphate buffer. \(\text{Na}_2\text{HPO}_4\) 40.50 g, \(\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}\) 29.65 g to 1 litre.

1.00 ml 1% SDS.

0.005 ml \(\text{N,N,N',N'}\)-tetramethylethylenediamine.

5.20 ml Distilled water.

0.10 ml 10% ammonium persulphate.

Electrode Buffer:- 200 ml phosphate buffer

100 ml 1% SDS

700 ml Distilled water.

The mobilities of proteins and peptides were determined by the method described by Weber and Osborne (218):

\[
\text{Mobility} = \frac{\text{Distance protein migrates}}{\text{Length of gel after destaining}} \times \frac{\text{Length of gel before destaining}}{\text{Distance dye migrates}}.
\]
(vii) IgD Myeloma Sera

Two myeloma sera were available in sufficient quantities to make purification of the immunoglobulin a viable proposition. A small amount of an IgD myeloma pool was also available, and this was used in the first instance for raising antisera in rabbits.

A preliminary study of the sera involved cellulose acetate and 'Gradipore' electrophoresis. The electrophoretic mobilities of the two myeloma proteins were different, that of S.T. being greater than that of M.V. (Fig. 5.1 (a)). A comparison with two other known myeloma sera designated 'red' and 'blue' showed that the IgD band in myeloma serum S.T. had a similar mobility (Fig. 5.1(b)). The different mobility of the IgD in serum M.V. may be explained by the fact that the serum had been kept at room temperature for several days during transit.

'Gradipore' electrophoresis clearly demonstrated the presence of the myeloma protein in the four sera tested (Fig. 5.1(c)).

(viii) Antisera to IgD

Two groups of three rabbits were immunized with 1 µl and 0.5 µl respectively of an IgD myeloma pool in 1 ml of saline/Freund's complete adjuvant in the proportion 1:2 by volume. Booster injections of the same amount of material were given at 4 week intervals for 12 weeks. Test bleeds were made 2 weeks after the final booster injection. The antisera were tested by immunoelectrophoresis with the IgD myeloma pool and were found to contain antibodies to the whole spectrum of serum proteins (Fig. 5.2). Adsorption of the antiserum with an immunoadsorbent prepared by glutaraldehyde polymerisation of a human serum pool rendered the antiserum specific for protein migrating to the
Fig. 5.1 (a)
Myeloma sera S.T. (top) and M.V. (bottom) run on cellulose acetate and stained with coomassie brilliant blue R.

Fig. 5.1 (b)
Myeloma sera 'blue' (top) and S.T. (bottom) with a sample from a human serum pool run in between. Electrophoresis on cellulose acetate followed by staining with Ponceau S.

Fig. 5.1 (c)
'Gradipore' electrophoresis of (from left to right) myeloma serum 'blue', myeloma serum 'red', human serum G.C., myeloma serum S.T., myeloma serum M.V. and human serum pool.
Fig. 5.2 (a)

Immunoelectrophoresis of pooled human serum (wells 1-3) and pooled myeloma sera (IgD) (wells 4-6) against (a) sheep anti-human serum, (b) (c) (d) (e) rabbit antisera raised to a myeloma (IgD) pool and (f) rabbit anti-IgG.

Fig. 5.2 (b)

Immunoelectrophoresis of pooled myeloma sera (IgD) (wells) against six rabbit antisera (troughs) raised to a pool of purified IgD paraproteins and adsorbed with glutaraldehyde-polymerised IgD.
The antibody was of very low titre and was demonstrated in the antisera from five of the six rabbits.

Injections were resumed after three months using purified IgD from a myeloma pool. Each rabbit received 100 μg of protein in 1 ml of saline/Freund's complete adjuvant. A booster injection of the same dose was given four weeks later and the rabbits were bled after a further ten days. All animals produced antisera with a high titre of antibodies to IgD - 1:16/1:32 by Ouchterlony gel diffusion test. These were rendered specific by adsorptions with a human serum pool polymer.

Two further groups of rabbits were injected with 50 and 100 μg doses of purified myeloma IgD (S.T.). Two booster injections were given at four week intervals, and the animals were bled ten days after each of the three injections. Twenty ml of blood was collected on the first two occasions. The resulting antisera were adsorbed with polymerised IgG, prepared from a human serum pool, to remove any anti-light chain activity (Fig. 5.2).

All injections were given intramuscularly, primary injections into the four limbs and boosters into the hind limbs only.

(ix) Purification of Myeloma IgD by Physicochemical Techniques

The procedure adopted was essentially that of Griffiths and Gleich (175) with minor modifications.

(1) Five ml of myeloma serum was dialysed for 18 hr. at 4°C against 1 litre of 0.01 M phosphate buffer pH 5.4 containing EDTA and ε-amino caproic acid at concentrations of 0.01 M and 0.005 M respectively. (KH₂PO₄ 1.36 g, EDTA 3.72 g and ε-amino caproic acid 0.656 g made up to 1 litre with distilled water.

(2) Centrifuged 15 min. at 12,000 g. This resulted in pelleting of the pH 5.4 precipitate and flotation of a lipid
fraction. The latter proved to be quite considerable in the two myeloma sera studied.

(3) The clear serum fraction was stirred continuously at 4°C during the dropwise addition of an equal volume of saturated ammonium sulphate over 15 min. The serum was left overnight at 4°C.

(4) The precipitate resulting from the previous step was collected by centrifugation at 2,500 g for 15 min. at 4°C. and washed twice by resuspension in fresh 50% saturated ammonium sulphate. The precipitate was dissolved in the minimum volume of 0.15 M saline and chromatographed on a 75 x 1 cm column of Sephadex G-25 (fine) equilibrated with 0.01 M sodium phosphate - 0.005 M ε-amino caproic acid pH 7.66. (NaH$_2$PO$_4$2H$_2$O 1.56 g, Na$_2$HPO$_4$ 12.8 g and ε-amino caproic acid 6.56 g to 10 litres with distilled water).

(5) The protein was loaded onto a column of DE 32 cellulose 20 x 1.5 cm equilibrated with the same phosphate buffer. Ten 6 ml fractions were collected and protein was eluted from the column by phosphate buffer (300 ml gradient from 0.01 M to 0.1 M) (Fig. 5.3). Samples were concentrated by ultrafiltration using a diaflo PM 30 membrane and examined by immunoelectrophoresis. Stepwise elution with phosphate buffer in 0.005 M increments of concentration was also performed.

(6) Fractions containing IgD were pooled, concentrated to approximately 3 ml and dialysed against 0.05 M phosphate buffer pH 8.0, 0.15 M with respect to sodium chloride and 0.005 M with respect to ε-amino caproic acid at 4°C for 16 hr.

(7) The IgD fraction was loaded onto a Sephadex G-200 column (120 x 2.5 cm) equilibrated in the same phosphate buffer. Fractions from the column were analysed by immunoelectrophoresis and polyacrylamide disc gel electrophoresis.
When necessary trace amounts of IgG contaminating the preparation were removed by immunoadsorption. Sheep antibodies specific for the Fc region of human IgG were coupled to diazotised N-(3-aminobenzyl) cellulose, and the resulting immunoadsorbent was used in a 'batch' procedure. Aliquots of the immunoadsorbent were stirred with IgD preparation at room temperature for 1 hr, and then recovered by centrifugal filtration.

Samples of IgD were stored deep frozen at -40°C.
Rabbit anti-human IgD (6)

Aliquots (10 ml) of rabbit antiserum raised to human myeloma IgD were centrifuged at 12,000 g for 20 min. at 4°C to separate any precipitated material by pelleting and fat by flotation. An equal volume of saturated ammonium sulphate was added dropwise to the clear serum fraction with continuous stirring, thus rendering the solution 50% saturated with respect to ammonium sulphate. The mixture was kept at 4°C for 18 hr. before collecting the precipitate by centrifugation at 2,500 g for 15 min. at 4°C and washing it twice with 50% saturated ammonium sulphate. The precipitate was dissolved in 5 ml PBS and dialysed against five changes of 1 litre of PBS over 2 days. The antiserum fraction was rendered \( \delta \)-chain specific by immunoadsorption with glutaraldehyde polymerised pooled human serum. The success of the immunoadsorption was judged initially by immunoelectrophoresis and immunodiffusion, and then by radioimmunoassay. The antiserum was tested for residual anti-IgG and anti-light chain activity by determining whether or not IgG inhibited the binding of \( ^{125}\text{I}-\text{IgG} \) and \( ^{125}\text{I}-\text{IgD} \) respectively.

Purification of Myeloma IgD by Immunoadsorption

Method 3 (Chapter 4) was used to purify myeloma IgD (S.T.). The immunoglobulin fraction of rabbit anti-human IgD (6) |BR2| was coupled to either diazotised N-(3-aminobenzylxoxymethyl) cellulose or glutaraldehyde-activated aminodipropylamine cellulose. The immunoadsorbent was packed on top of a column of G25 Sephadex. Elution was effected with either 50 mM glycine-HCl or 1 M propionic acid.

Purification was also achieved by the batch procedure (Method 5, Chapter 4).

Purification of "Normal" IgD by Immunoadsorption

(a) (Method 6, Chapter 4). A 200 ml sample of fresh serum (spiked
with $^{125}$I-IgD (S.T.) was mixed with c. 10 ml packed volume of rabbit anti-IgD (δ) BR2 immunoadsorbent in a 500 ml screw-top bottle. The bottle was rotated about its long axis on a rocking-roller mixer (Luckhams) for 2 hr. at room temperature. The mixture was centrifuged at 1500 g for 2 min., and the immunoadsorbent was resuspended in PBS and mixed with an equal volume of Sephadex G-25. The mixture was packed into a column on top of a column of Sephadex G-25 (35 x 1.5 cm). Elution was effected with either 50 mM glycine-HCl or 1 M propionic acid. The IgD was located by monitoring the radioactivity emerging from the column, and by measuring the ability of fractions to inhibit the binding of $^{125}$I-IgD in a solid-phase radioimmunoassay (Chapter 3).

(b) (Method 3, Chapter 4). Serum was fractionated by 50% ammonium sulphate precipitation and IgD was purified from the immunoglobulin fraction.

In both cases, column fractions were concentrated in Amicon B15 cells.

(xii) **Purification of Rabbit Anti-Human IgD (δ) Antibodies**

Immunoadsorbents were prepared by coupling pure myeloma IgD (S.T.) to cellulose and used according to Method 3 (Chapter 4) to purify anti-human IgD (δ) antibodies from the immunoglobulin fractions of rabbit antisera.

(xiv) **Radioimmunoassay of IgD**

This was performed according to the protocol for IgD solid-phase radioimmunoassay (Chapter 3).
Neuraminidase Treatment of Purified Myeloma IgD

IgD (S.T.) (2 mg in 0.5 ml PBS) was dialysed against two changes of 0.1M acetate buffer pH 5.0. A 100 μl aliquot of a solution of neuraminidase,* at a concentration of 2 Units/ml acetate buffer, was added to the dialysis sac. The sac was put into a universal bottle containing 10 ml 0.1 M acetate buffer at 37°C. The temperature was maintained for 1 hr before transferring the dialysis sac into fresh PBS at 4°C. Dialysis was against two changes of PBS overnight. Samples were examined by electrophoresis and isoelectric focusing in polyacrylamide gels.

*Neuraminidase from Clostridium perfringens - Type VI (Sigma), Lot 92C-8090, containing 0.78 Units of neuraminidase activity per mg of protein - bovine submaxillary mucin as substrate.)
Results and Discussion

Characterisation of IgD Myeloma Sera

A preliminary study involving acetate and 'Gradipore' electrophoreses has been described in Methods, section (vii). Further electrophoretic studies were conducted with sera S.T. and M.V. Immunoelectrophoretic analysis of the whole myeloma sera with light chain specific antisera (Mercia) revealed free \( \lambda \)-type light chains in serum S.T. as well as identifying \( \lambda \)-type light chains in the myeloma protein. Serum M.V. failed to react with either \( \lambda \) or \( \kappa \) light chain specific antisera, and light chains were identified as being of the \( \lambda \)-type only after D.T.T. reduction of the purified immunoglobulin.

When examined immunoelectrophoretically with \( \delta \)-chain specific antisera raised to the myeloma pool both myeloma proteins were found to migrate to the \( \beta_1 \) region. As the plates were developed transient secondary arcs were observed which could have been due to myeloma protein polymers. This possibility was also raised by the results of the 'Gradipore' electrophoresis which indicated a molecular weight of the order of 360,000 daltons in keeping with a dimeric form of IgD.

Isoelectric focusing of the myeloma serum (S.T.) and pooled 'normal' human serum revealed a complex pattern of lines perculiar to the former and presumably attributable to the myeloma protein (Fig. 5.3). This was confirmed by P.M. Johnson (214 and personal communication) who compared the isoelectric focusing pattern with those obtained for several IgG myeloma. On the basis of circular dichroism studies, which revealed that \( \kappa \)-chains absorbed far more strongly at 294 nm than did \( \lambda \)-chains, Johnson et al. (209) suggested that the isoelectric focusing patterns of IgG myeloma proteins are influenced by molecular constraints dependent upon the light chain type. A more complex isoelectric
Fig. 5.3

Isoelectric focusing patterns from IgD myeloma serum S.T. (2 and 4) and a normal human serum pool (1 and 3) over the pH ranges 4-9 (1 and 2) and 5-8 (3 and 4).
focusing pattern could therefore be attributed to a κ-type light chain. Such an explanation cannot be proposed in the case of IgD (S.T.) because this protein has a λ-type light chain. The complexity of the IgD pattern is comparable to those obtained for IgA myeloma proteins. The high carbohydrate content which the two immunoglobulins have in common may be implicated, and in particular the high sialic acid contents of the two proteins which contribute to their low isoelectric points.

The possibility that the complexity of the IgD isoelectric focusing pattern was due to partial loss of sialic acid residues was considered. Preparations of IgD (S.T.) were treated with neuraminidase to remove exposed sialic acid residues. Unfortunately the enzyme treatment appeared to have no effect on the protein.

Purification of Monoclonal IgD

Dialysis against buffer at pH 5.4 resulted in the precipitation of a euglobulin fraction which contained at least one protein which otherwise contaminated subsequent fractions of the preparation. Immuno-electrophoresis patterns obtained for the sera after removal of copious amounts of lipid by centrifugation and flotation no longer showed diffuse anodal tailing from the origin (Fig. 5.5).

DEAE-cellulose chromatography failed to resolve IgG and IgD. The protein trace from a column eluted with a 0.01 to 0.10 phosphate buffer gradient is shown in Fig.5.4(a). Fractions were examined by immunoelectrophoresis, and IgG was found in fractions 18 to 28 with peak concentrations in fractions 19 and 23. IgD was first detected in fraction 21 and its concentration reached a peak in fractions 25 and 26. The more anodal component of the IgD fraction (Fig. 5.5) was eluted from the column in fractions 49 et seq. Stepwise elution of protein
Fig 5.4 (a)
Elution profile for myeloma serum S.T. from a DE-32 cellulose column. Protein was eluted in phosphate buffer, pH 7.66, in a gradient from 0.01 to 0.1M.

Fig 5.4 (b)
Gel filtration of an IgG containing fraction on Sephadex G-200. 'Fraction' contained fractions 23 to 29 from DE-32 column (Fig 5.3).
Fig. 5.5 (a)

Fractions 16-33 from DEAE-cellulose column (20 x 1.5 cm). IgG is present in fractions 16-26. The first and last wells contained whole myeloma serum and sheep anti whole human serum was placed in all the troughs.

Fig. 5.5 (b)

Fractions 18-33 from DEAE-cellulose column (20 x 1.5 cm). IgD is present in fractions 21-33 with a peak concentration in fractions 25 and 26 - demonstrated by single radial diffusion technique. The first and last wells contained whole IgD myeloma serum and rabbit anti human IgD (Fc) was placed in all the troughs.
from the ion-exchange column was also investigated, but again fractions containing IgD were heavily contaminated with IgG. Similar elution profiles were obtained for both myeloma sera (S.T. and M.V.).

A 120 x 2.5 cm column of Sephadex G-200 was found to be adequate for the resolution of IgD and IgG (Fig. 5.4 (b)). The purity of the IgD fraction was ascertained by polyacrylamide disc gel electrophoresis and solid-phase radioimmunoassays for IgG and IgD. IgG contamination in later fractions of the IgD peak were removed by batch immunoadsorption with anti-IgG antibodies coupled to bromoacetyl cellulose.

Monoclonal IgD (S.T.) was successfully purified from the immunoglobulin fraction (50% ammonium sulphate) of the serum according to the procedure described in Methods, section (xi). The Sephadex effectively retarded the acid front so that the bulk of the protein was eluted from the column before the pH dropped (Fig. 5.6). The first peak represented IgD as did the fourth (Table 5.1). The second and third peaks of radioactivity contained relatively little protein, and these were shown to represent iodinated IgD fragments which resulted from degradation of the purified iodinated protein on storage. The fractions were adsorbed with anti-IgG antibodies coupled to bromoacetyl cellulose to remove trace amounts of IgG (Table 5.1), and then examined by disc gel electrophoresis, immunoelectrophoresis and isoelectric focusing (Fig. 5.7), and ultracentrifugation (Fig. 5.8). The preparation was judged to be homogenous by the electrophoretic techniques, but the ultracentrifugation analysis revealed a high molecular weight component. This may well have been an IgD aggregate, the existence of which has been postulated earlier on the strength of observations in immunoelectrophoresis and 'Gradipore' electrophoresis.
The amount of IgD recovered by elution when the column was used for a second time was less, but so also was the amount adsorbed as indicated by the amount recovered in the wash peak. Losses due to irreversible adsorption and the prolonged washing prior to elution were less on the second run, presumably because of the blocking of high avidity antibody.

The combination of batch immunoadsorption and column washing and elution gave a good yield of IgD (Table 5.1) with only a marginal increase in the amount of IgG contamination. The immunoadsorbent was not mixed with Sephadex G-25 prior to packing in the column and consequently IgD dissociated by the eluting agent was carried with the acid front until it passed into the Sephadex portion of the column where the two were resolved. Readsorption of IgD was thus prevented, and a single protein peak emerged from the column ahead of the acid front (Fig. 5.9). Elution of readsorbed IgD may have accounted for the late protein peak observed in Fig. 5.6.

The batch procedure using Amicon filtration tubes was combined with stepwise elution of the protein in an attempt to reduce the amount of IgG contamination. The overall yield of IgD was slightly less than that obtained by combining batch immunoadsorption with column elution, but the larger part of the contaminating IgG was removed in the pH 4.0 and 3.0 fractions (Table 5.1 and Fig. 5.10). These results may be explained in one of two ways. Firstly, the IgG contamination may arise as the result of non-specific physical adsorption to the carrier which is reversible at pH 4.0. Secondly, the possibility still remains that the immunoadsorbent employed to render the anti-IgD serum specific failed to remove some low avidity antibody directed against IgG.
A direct quantitative comparison of the column and batch techniques is difficult because of the need to either dilute or use larger quantities of the IgD containing immunoglobulin fraction in the batch procedures in order to achieve suspension of the immunoadsorbent.

Fig. 5.6
Elution profiles from G25 Sephadex/G25 Sephadex - anti IgD (6) immunoadsorbent column. (Methods, section (xi)). Elution was achieved with 50 mM glycine-HCl pH 2.5 and protein concentration, radioactivity and pH were monitored. (-- -- - radioactivity $[^{125}\text{I}-\text{IgD}$, ................ - optical density 280 nm, and - - - - - pH).
Table 5.1

Purification of myeloma IgD (S.T.) by immunoadsorption

<table>
<thead>
<tr>
<th></th>
<th>IgD added</th>
<th>IgD recovered* in wash peak</th>
<th>IgD recovered* by elution</th>
<th>IgG contamination mg</th>
<th>% Recovery IgD Added</th>
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<tr>
<td></td>
<td>U</td>
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<td>4545</td>
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*Measured by solid-phase radioimmunoassay. Units (U) refer to MRC/WHO standard 67/37. Immunoadsorbents based on 1 - diazotised N-3-aminobenzethylocellulose; 2 & 3 - butyraldehyde activated aminoditolamine cellulose.
Fig. 5.7
Myeloma IgD (S.T.) purified by immunoadsorption -
(a) from left to right: - isoelectric focusing pattern in polyacrylamide gel, pH range 3-10; isoelectric focusing pattern in polyacrylamide gel, pH range 4 - 9; acrylamide gel electrophoresis pattern.
(b) Immunelectrophoresis of IgD (S.T.) against rabbit anti IgD (\(\delta\)). Upper well, IgD purified by immunoadsorption. Lower well, IgD purified by immunoadsorption and Sephadex G200 gel filtration.
Fig. 5.8

Sedimentation patterns of myeloma IgD (S.T.) purified by immunoadsorption (Methods (xi)). Sedimentation from right to left. Photographs taken at 8 min. intervals after reaching 44,000 r.p.m. at 20°C. Protein dissolved in 0.05 M phosphate buffered saline, pH 7.66.
Fig 5.9
Elution of IgD (S.T.) from anti IgD immunoadsorbent. Eluting agent - glycine-HCl pH 2.0. Antibody coupled to glutaraldehyde-activated aminodipropylamine cellulose. (Method 6, Chapter 4).

Fig 5.10
Elution of IgD (S.T.) from anti IgD immunoadsorbent. Eluting agents - glycine-HCl buffers. Antibody coupled to glutaraldehyde-activated aminodipropylamine cellulose. (Method 5, Chapter 4).
Stability of IgD on Storage and on Heating

Immunoelectrophoresis of heated (56°C) and unheated myeloma serum (Fig. 5.11) revealed little or no change in the IgD pattern. Heating at 56°C for 30 min caused precipitation of the lipid fraction of the serum which characteristically gave anodal tailing from the well. The protein was not stable when stored in solution, at a concentration of 3 mg/ml, at 4°C for 2 weeks. The immunoelectrophoresis pattern was considerably modified by the extension of the IgD line in the anodal direction. When examined by ultracentrifugation the solution was found to contain a major component of approximately 3.5 S and a minor 7 S component. The nature of the former, a degradation product of the 7 S IgD, is uncertain.

Sedimentation Velocity

The sedimentation constant \( S_{20,w}^{0} \) of myeloma IgD (S.T.) was 6.84, determined by extrapolating values to infinite dilution. The correlation coefficient, \( r \), was 0.96, and a value of 0.77 was assumed for the partial specific volume (197) (Fig. 5.12). The protein was purified by ammonium sulphate precipitation followed by immunoadsorption and Sephadex G200 chromatography.

Molecular Weights of IgD (S.T.) Heavy and Light Chains

The conditions of dithiothreitol (DTT) reduction chosen were such as to result in only partial reduction of the IgG and consequently reduction intermediates were present in the final mixture. IgD (S.T.) proved to be more susceptible to reduction and, except for a small amount of a heavy-light chain (HL) intermediate, was degraded completely to heavy (H) and light (L) chains. The results of SDS electrophoresis of reduced IgG and IgD are shown in Fig. 5.13. The identification of HHL, HH and HL
Fig. 5.11

Stability of IgD.

All troughs contained rabbit anti-IgD (§).

Wells contained (top to bottom):

(i) Freshly thawed myeloma serum (S.T)
(ii) Myeloma serum stored at 4°C for 3 days (0.1% sodium azide added).
(iii) Myeloma serum heated at 37°C 30 min.
(iv) Myeloma serum heated at 56°C 30 min.
(v) Freshly prepared IgD (S.T.)
(vi) Purified IgD stored at 4°C in presence of 0.1% sodium azide for 2 weeks.
Fig. 5.12

Sedimentation patterns of myeloma IgD (S.T.) purified by ammonium sulphate precipitation, immunoabsorption and Sephade X G.200 gel filtration. Sedimentation from right to left. Photographs taken at 8 min. intervals after reaching 59,780 r.p.m. at 20°C. Protein dissolved in 0.05 M phosphate buffered saline, pH 7.66.
Fig. 5.13
SDS electrophoresis of reduced and alkylated IgG and IgD (S.T.) in 5% polyacrylamide gels. (Left - IgG and right IgD).

Standard curve constructed from the mobilities of IgG reduction products assuming molecular weights of 22,500 and 53,000 for light and heavy chains. (Confirmed by reference to external standards - see text).
IgG reduction intermediates and H and L chains was made by reference to external standards. (Chymotrypsinogen A, 25,700; egg albumin, 46,000 and human serum albumin, 66,000). A semilog plot of molecular weight and mobility revealed a linear relationship (Fig. 5.13). By reference to this standard curve the molecular weights of IgD (S.T.) H and L chains were found to be 70,500 and 26,000 daltons respectively. Assuming two H and two L chains per molecule of IgD, the molecular weight of the whole molecule would be 193,000 daltons.

The IgG used in this study was purified from pooled human serum which presumably contained both κ and λ light chains. The poorly defined band immediately behind the band with fastest mobility (Fig. 5.13) represents the λ light chain.

The value of 70,500 daltons for the H chain is in good agreement with values obtained by electrophoresis and gel filtration by Perry and Milstein (179) and Leslie et al. (176) within the accuracies of the methods. The value of 26,000 daltons for the L chain reported here is higher than that of other IgD myeloma proteins at 22,500 daltons (176). This value of 22,500 daltons has been widely reported as the molecular weight of immunoglobulin light chains. It is possible that the difference is due to an artefact attributable to the SDS technique. Higher values have been obtained using this technique as reported by Ahmad-Zadeh et al. (210) and Virella and Coelho (211). In the first case values of 25,170, 26,407, 25,589 and 26,351 daltons were estimated for human IgM, IgA and IgG light chains and Bence-Jones protein respectively. These values are in good agreement with that found for IgD (S.T.) light chains in the present study, the only difference being that 5% gels were used as opposed to 10% gels. Virella and Coelho (211) reported a difference between the
mobilities of λ and κ light chains as determined by SDS electrophoresis. In 5% gels the molecular weights of κ and λ chains were estimated as 22,845 and 28,395 daltons respectively. IgD (S.T.) light chains are of the λ type.

Carbohydrate has been shown to reduce the mobilities of proteins in SDS gel electrophoresis (212), a factor attributable to increased molecular weight and possible hydration of the carbohydrate moiety. Carbohydrate would account for the reduced mobility of IgD (S.T.) light chain.

Assuming a mean residue weight of 109, the molecular weight of IgD (H), as determined by SDS electrophoresis, is consistent with an immunoglobulin chain with five domains as is the case with both μ and ε chains. Taking the values of 70,500 and 52,500 daltons for the molecular weights of IgD (H) and IgG (H) and carbohydrate contents of 12% and 3% respectively, the chains would contain 468 and 567 amino acid residues. These figures compare with values of 570 (μ), 550 (ε) and 45 (γ).

Amino Acid Analysis

Amino acid analyses were performed on the purified IgD paraproteins from sera S.T. and M.V. (1 and 2 respectively, Table 5.2). The results represent the means of two separate analyses. Purification was achieved by ammonium sulphate precipitation and immunoabsorption. IgG, purified from serum S.T. was analysed for comparison. Levels of lysine, aspartate and tyrosine were lower in IgD than IgG while levels of threonine, serine, glutamate and alanine were higher. The relatively low levels of proline in IgD paraproteins noted by Saha et al. (178) were not found in the proteins studied here.
Table 5.2

Amino Acid Analysis of Immunoglobulins

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<th>IgD (1)</th>
<th>IgD (2)</th>
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<tr>
<td>Lysine</td>
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<td>66</td>
<td>71</td>
</tr>
<tr>
<td>Histidine</td>
<td>26</td>
<td>36</td>
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<tr>
<td>Arginine</td>
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</tr>
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<tr>
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moles of amino acid per mole of immunoglobulin *

Norleucine was included as an internal standard.

*Assuming molecular weights of 193,000 and 153,000 and carbohydrate contents of 12 and 3 percent for IgD and IgG respectively.
Immunoglobulin D (IgD) Levels in Myeloma Sera and 'Normal' Sera

IgD levels were measured by solid-phase radioimmunoassay as described in Chapter 3, and expressed in terms of either International Units (MRC/WHO standard 67/37) or mass. The immunoadsorbent was prepared from immunoadsorbent purified rabbit anti-human IgD (6) antibodies raised to an IgD paraprotein pool. Competitive-binding assays were set up with labelled IgD (purified from paraprotein pool) and dilutions of standard 67/37, and the resulting standard curves were used to determine the concentrations of IgD in dilutions of sera S.T. and M.V. and in a fraction of purified paraprotein S.T. (Table 5.3). The concentrations of IgD in the two myeloma sera studied in detail were 4,226 ± 152 I.U. (S.T.) and 1,373 ± 74 I.U. (M.V.).

Assuming a molar extinction coefficient at 280 nm \(\varepsilon_{280}^{1\%} = 14.5\), a S.T. preparation contained 1.027 mg/ml, one I.U. was equivalent to 1.67 ± 0.03 µg of paraprotein S.T. The assays were repeated with purified paraprotein S.T. as a standard and then 1 I.U. was equivalent to 1.55 ± 0.03 µg.

The mean IgD concentration in a group of 30 subjects was found to be 26.8 ± 17.2 I.U./ml. (arithmetic mean) within the range 5.4 to 75.4 I.U./ml. (MRC/WHO standard 67/37). Values were determined using 20, 50 and 200 fold dilutions of 'normal' serum. Assuming a value of 1.67 µg IgD per I.U., the mean IgD concentration was 44.8 µg/ml.

Purification of 'Normal' IgD

The batch/column method described in Methods (xii) was adopted for the purification of IgD from normal serum B.C., which contained 4550 I.U. or 7.6 mg of IgD per 100 ml.
Table 5.3  Solid-phase radioimmunoassay of human immunoglobulin D (IgD).

(a) Standard Curve: MRC/WHO standard 67/37

<table>
<thead>
<tr>
<th>Purified Myeloma IgD (S.T.)</th>
<th>IgD Myeloma (S.T.)</th>
<th>IgD Myeloma (M.V.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
<td>µg/ml</td>
<td>I.U./1.027 mg</td>
</tr>
<tr>
<td>1/1000</td>
<td>1.027</td>
<td>614</td>
</tr>
<tr>
<td>1/2000</td>
<td>0.513</td>
<td>600</td>
</tr>
<tr>
<td>1/4000</td>
<td>0.256</td>
<td>640</td>
</tr>
<tr>
<td>1/8000</td>
<td>0.128</td>
<td>600</td>
</tr>
</tbody>
</table>

**Mean 613.5 ± 16 I.U.**

**Mean 4226 ± 152 I.U./ml**

**Mean 1373 ± 74 I.U./ml**

**1 I.U. = 1.67 ± 0.03 µg**

**7.06 ± 0.25 mg/ml**

**2.29 ± 0.12 mg/ml**

(b) Standard Curve: Purified Myeloma IgD (S.T.)

<table>
<thead>
<tr>
<th>MRC/WHO 67/37</th>
<th>IgD Myeloma (S.T.)</th>
<th>IgD Myeloma (M.V.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.U./ml</td>
<td>µg/ml</td>
<td>Dilution</td>
</tr>
<tr>
<td>5.00</td>
<td>7.80</td>
<td>1/20,000</td>
</tr>
<tr>
<td>2.50</td>
<td>4.00</td>
<td>1/40,000</td>
</tr>
<tr>
<td>1.25</td>
<td>1.90</td>
<td>1/80,000</td>
</tr>
<tr>
<td>0.63</td>
<td>0.95</td>
<td></td>
</tr>
</tbody>
</table>

**Mean 7000 ± 163**

**Mean 115 ± 113**

**1 I.U. = 1.55 ± 0.03 µg**

**7.00 ± 0.16 mg/ml**

**2.11 ± 0.11 mg/ml**
The immunoglobulin fraction of antiserum BR2 raised to an IgD pool was coupled to glutaraldehyde-activated aminodipropylamine cellulose. Two grams of the immunoadsorbent contained of the order of 28 mg of antibody. A total of 1250 I.U. of IgD was recovered from the immunoadsorbent by elution with 50 mM glycine-HCl pH 2.0, representing 14% of the total IgD. Assay of the serum after adsorption revealed 2100 I.U. remaining unadsorbed. Therefore, 51% (1250 ex 2450 I.U.) of the adsorbed IgD was recovered by elution, the remainder either having been lost in the washings after physical adsorption or retained by the immunoadsorbent. The eluate was contaminated with IgG (c.60%) and some albumin (not quantitated).

The method left some room for improvement in terms of the efficiency of the immunoadsorption. This could have been improved by the use of more avid antibodies with the accompanying risk of losing antigen in undissociable antibody-antigen complexes. In the absence of such antibodies, it was sought to improve the immunoadsorption by reducing the reaction volume, excluding certain serum proteins from the system and using partially purified anti-IgD antibodies. The antibodies were purified by adsorption on to and subsequent elution from IgD (S.T. and M.V. together) coupled to glutaraldehyde activated aminodipropylamine cellulose, and themselves immobilised on the same carrier. The immunoglobulin fraction of serum B.C. was precipitated by 45% saturation with ammonium sulphate. The fraction was exhaustively dialysed against five changes of PBS over 2 days and made up to 25 ml of solution. The total IgD concentration was 4100 I.U., and 3700 I.U. were adsorbed. After washing at pH 7.66 and pH 5.0, 2900 I.U. were recovered by elution with 50 mM glycine - HCl pH 2.0. Disc gel electrophoresis of the concentrated eluate revealed that the preparation was
homogenous (Fig. 5.14). However, IgG contamination was detectable by radioimmunoassay and accounted for approximately 2% of the total protein. Immunoelectrophoresis gave no evidence of IgD degradation products. Ultracentrifugation analysis revealed a single peak of approximately 7S (Fig. 5.15). Insufficient data was available to enable an accurate determination of the sedimentation coefficient to be made.
Fig. 5.14
Polyacrylamide disc gel electrophoresis.
From left to right: 'normal' IgD (B.C.) purified by ammonium sulphate precipitation and immunoadsorption; myeloma IgD (S.T.) and whole serum (S.T.).
Sedimentation patterns of two preparations of normal IgD (B.C.) purified by immunoadsorption. Sedimentation from right to left. Photographs taken at 8 min. intervals after reaching 50,740 r.p.m. at 20°C. Note that the meniscuses in the two cells do not correspond.
Conclusion

The physical and immunochemical data amassed here confirm that the protein purified from normal serum was in fact IgD as judged by its similarity to the IgD paraproteins S.T. and M.V. These in turn had been shown to identify with IgD paraproteins described in the literature.

Immunoelectrophoresis showed both the myeloma and normal IgDs' as having comparable β mobilities. A degradation product with greater mobility was detected in the myeloma serum and in the pure IgD fractions upon storage at 4°C. This was identified with the 'Fc-like' piece reported elsewhere (174, 175).

A value of 6.84 $S_{20, w}^0$ for the sedimentation coefficient of paraprotein S.T. compared favourably with values of 6.92, 7.0 and 7.2 $S_{20, w}^0$ reported for other paraproteins (1973, 177, 180). Ultracentrifugation also revealed a high molecular weight component in some preparations which, in the absence of any other positive identification, was assumed to be an IgD dimer. This conclusion was supported by the result of 'Gradipore' gradient polyacrylamide gel electrophoresis, which showed the IgD in whole myeloma serum as having a molecular weight of 360,000 daltons. The value of 193,000 daltons determined by summing the molecular weights of heavy and light chains, as determined by SDS gel electrophoresis, may be on the high side. This is because of the high value attributed to λ chains with this technique. If a value of 22,500 daltons is assumed for light-chains a molecular weight of 186,000 is obtained. The molecular weight of H chains, 70,500 daltons, is consistent with the existence of a fifth domain, in common with ε and μ chains.

The isoelectric focusing pattern of the myeloma protein S.T. differed from that reported by Johnson et al. (214) in that the purified IgD appeared to be considerably less heterogeneous. The difference may have
its origin in the method of purification employed. The possibility that the immunoadsorbents showed a specificity for a particular IgD (S.T.) sub-population has to be considered.

The concentration of IgD in the myeloma sera S.T. and M.V. were 4226 and 1373 I.U./ml respectively, while the mean concentration in a group of normal subjects was 26.8 I.U./ml. The determinations of these values by solid-phase radioimmunoassay, using antisera raised to an IgD paraprotein pool and either paraprotein or IgD reference serum as standard, highlighted the pitfalls likely to be encountered when measuring levels of normal protein in terms of paraprotein standards and vice versa. The problem hinges around the antibody population of the antiserum. By raising antisera to a single IgD protein one is ignoring the possibility of isotypic sub-class variants and allotypic variants. The resulting antiserum will contain antibodies specific for a restricted population of IgD variants. The antisera used here for radioimmunoassay and purification of 'normal' IgD were raised against an IgD pool to minimise the chances of this occurrence.

Very encouraging results were obtained with the immunoadsorption purification procedures. The relatively high levels of IgD in the myeloma sera facilitated purification, but IgD fractions were always contaminated with IgG. Further adsorption of anti-IgD sera prior to immobilisation failed to reduce the eventual level of IgG contamination confirming that the contamination arose from non-specific adsorption to the immunoadsorbent. Similar contamination was encountered when purifying IgD from normal serum, but in this case the level was significantly reduced by using immunoadsorbent purified antibodies which allowed the attainment of higher specific antibody:carrier ratios. The higher degree of purification achieved was attributed to the use of smaller amounts of
carrier and the presence of very little non-antibody protein. Ninety percent of the IgD in an immunoglobulin fraction from 'normal' serum was adsorbed onto the solid-phase, and 78% was recovered on elution, the level of IgG contamination being 1.4%. IgG was most easily removed from the fractions by immunoadsorption with immobilised anti-IgG.

Using the whole immunoglobulin fraction of IgD anti-serum coupled to cellulose, the highest degree of purification of IgD from myeloma serum was achieved with the two tier immunoadsorbent: Sephadex G 25 column. Contamination with IgG was 4.1%, and this fell to 2.8% when the immunoadsorbent was reused.

Purified antibodies specific for IgD may find an application in the development of a solid-phase radioimmunoassay for specific IgD class antibodies along the lines of the radioallergosorbent technique (RAST).
CHAPTER SIX

In Conclusion
The most efficient immunoadsorbents are prepared by coupling antibody to diazotised N-(3-aminobenzylloxymethyl) cellulose, via tyrosine residues, and to glutaraldehyde-activated aminoalkylcellulose, via amino groups. Both activated carriers incorporate spacer arms, that in the case of glutaraldehyde activated aminodipropylaminecellulose being 14-C long. The lengths of the spacer-arms and the degree of activation of the carrier have both been shown to influence the activity of the immobilised antibody. The longer the spacer arm the greater the distance between the carrier and the antibody and the less the steric effects of the carrier on the antibody. The more efficient the immobilisation, in terms of the number of covalent bonds, the greater the overall constraint placed upon the antibody structure and the greater the chance of bonding occurring via amino acid residues in the antigen-binding site. Both will result in loss of overall binding capacity, and the former may influence the avidity of the antibody. The phenomenon of 'immunoadsorbent avidity', attributable to high antibody packing density on the carrier, has been discussed, and it is thought that this may influence the yields of antigen recoverable by elution.

Coupling to glutaraldehyde-activated aminoalkylcellulose is recommended. The coupling conditions are not hazardous, as for example with cyanogen bromide, and were found to be more reproducible than those involving diazotisations. Of the methods tested this gave retention of the highest percentage of original antibody activity. However, the amount of antibody protein immobilised using the amino-alkylcelluloses described here was considerably less than was possible with diazotised N-(3-aminobenzylloxymethyl)cellulose preparations, and
the use of this derivative is to be preferred if a high protein:carrier ratio is required. Alternatively, the use of cellulose derivatives with larger numbers of amino groups, or carriers with primary amino groups as inherent parts of the chemical structure, such as beaded polyacrylamide (Biogel), may be used.

Sepharose has come to the fore due to the commercial enterprise of Pharmacia Ltd., who now market a range of Sepharose derivatives to which protein and carbohydrate may be coupled. These include cyanogen bromide activated Sepharose, carboxyl and amino derivatives, and the oxirane derivative which is suitable for the immobilisation of carbohydrates. The biggest attractions of these derivatives are their good column flow characteristics and their gel filtration properties. Although cellulose cannot match these particular properties it does have the advantage of being considerably cheaper, lyophilizable and chemically more robust. Column characteristics may be improved by mixing the cellulose-based immunoabsorbents with gel filtration media. However, the resulting separation of the eluted protein from the eluting-agent-front may not be in the best interests of the method. The possible readsoption of eluted antigen once it has passed ahead of the eluting-agent-front may result in a reduced yield. A two-tier column system, with the immunoabsorbent packed on top of a column of gel filtration material, has been shown to give the best results.

Column procedures are far less demanding than batch procedures on the time of their users and are therefore more attractive from the routine production point of view. Furthermore, column techniques have been shown
to be superior to batch techniques in terms of the purity and recovery of antigen. In use under these conditions acid eluting agents were more efficient than high ionic strength electrolytes such as sodium thiocyanate.

Cellulose-based immunoabsorbents are suitable for lyophilisation provided suspensions are first made 5% with respect to sucrose. Immunoabsorbent preparations kept in this way provide a very suitable "off the shelf" reagent" for standardising radioimmunoassays, as well as being convenient to use*.

The solid-phase competitive binding assays have been found to be less sensitive than the double-antibody assays in the systems tested, but sensitivity was still more than adequate for routine applications. A big advantage of certain immunoabsorbent preparations, those with bromoacetyl cellulose and reprecipitated N-(3-aminobenzoxylmethyl) cellulose in particular, is their small particle size, and resulting slow rate of settling, which makes tumble-mixing of the assay tubes during the incubation unnecessary.

Immunoabsorption has been used successfully to purify IgG from "normal" serum and IgD from both myeloma and "normal" sera. All the IgD preparations were contaminated with IgG and this was removed by immunoabsorption. The level of contamination in preparations from "normal" serum was reduced by using immunoabsorbent purified immobilised antibodies.

*Insulin, IgG and IgD have been assayed routinely using such reagents.


160. Dandliker, W.B., Alonso, R., de Saussure, V.A., Kierszbann, F.,
        Levison, S.A. and Shapiro, H.C. (1967) Biochemistry, 6, 1460-1467.
167. Stenius, B., Wide, L., Seymour, W.M., Holford-Strevens, V. and
168. Court, G. and Hurn, B.A.L. (1971) in Radioimmunoassay Methods
        (Kirkham, K.E. and Hunter, W.M. eds.) pp. 283-289, Churchill-
        Livingstone, Edinburgh and London.
        Chapter 17.
        3, 477-490.
        6, 437-443.
        565-568.
        9, 2115-2122.
178. Saha, A., Chowdhuty, P., Sambury, S., Behelak, Y., Heiner, D.C. and


