PURIFICATION OF ANTIBODIES BY IMMUNOADSORBENTS - ROSE, E.A.

M.Sc. Microbiology and Immunology

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The use of immunoadsorbents for the purification of antibodies has been investigated.

An immunoadsorbent was prepared by coupling bovine serum albumin (BSA) to ehtylene maleic anhydride copolymer (EMA) at 0°C, and used to adsorb rabbit anti-BSA antibodies from an ammonium sulphate precipitated globulin fraction of the antiserum. The antibodies were eluted by a column procedure with phosphate buffered 8 M urea or stepwise with 0.35 M glycine-HCl buffer pH 3.0, 2.5 and 2.0.

Using urea to elute and a modification of the original procedure, recovery, based on precipitable nitrogen was 42%. Purity was 50% based on the proportion of total nitrogen recovered which was precipitable. The yield was 10% higher than that obtained with the original procedure.

When tested with normal rabbit gamma globulin, suggestive evidence was obtained for negligible amounts of non-specific adsorption.

A batchwise method of elution was investigated and found to be less efficient than the column procedure.

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by

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I. INTRODUCTION AND PURPOSE

Serum proteins have been separated on the basis of their charge in an electrical field into four distant fractions (Tiselius, 1937, Tiselius and Kabat, 1939). Since that time several other methods of separation utilizing other physical properties of the proteins have been developed.

Based on their differences in solubilities, they have been separated by fractional precipitation at varying concentrations of salt, by adjustment to their isoelectric points at low ionic strength and by organic solvents. A variety of cations and anions have also been used (Isliker, 1957).

Chromatographic separation of serum proteins on ion-exchange resins such as diethylaminoethyl (DEAE) cellulose and carboxymethyl (CM) cellulose (Sober et al, 1957) probably represents the first successful application of chromatography to proteins. With the use of DEAE-cellulose, it has been possible not only to separate serum into several components, but also to subdivide gamma globulins into a number of fractions with differing physicochemical and immunological properties (Fahey et al 1958, 1959, Fahey, 1960). The technique is based on the differences in binding strengths

of the proteins to the resins.

Ultracentrifugation and gel filtration separate proteins on the basis of the size of the molecules. Gel filtration is a chromatographic technique using cross-linked dextrans (Flodin et al, 1962, Gelotte et al, 1962, Killander et al, 1962, Baumstark et al, 1964) and other types of cross-linked polymers such as polyacrilamide gel. Gels of varying pore sizes are available. Molecules larger than the pores of a given gel do not penetrate the gel and appear first in the effluent. Smaller molecules which penetrate are eluted by displacement in order of decreasing size.

All these physicochemical methods have been useful to separate the antibody containing fractions of serum thus effecting concentration of the antibodies. However, no immunologically pure preparation of antibodies can be obtained by them.

Highly purified antibodies are being used in the investigation of the synthesis, structure and function of antibody molecules. Studies of antibody specificity utilizing the technique of equilibrium dialysis have been reported to be most useful when purified antibodies have been used (Karush, 1962) and it seems that in studies on the chemical

composition of the antibody molecule, the nature of the antigen combining site or the control of antibody production the use of purified antibodies would be most desirable.

In this Department studies on the control of antibody synthesis using an *in vitro* system are in progress. This project was, therefore, undertaken to investigate the possible use of an immunoadsorbent to produce antibodies of high purity for use in this system.

II. LITERATURE REVIEW

Antibodies are serum proteins which have the unique property of reacting specifically with antigens which induce their formation. They are globulins and are largely in the gamma region on electrophoresis. Most antibodies are similar in their physico-chemical properties. Because of these similarities, it has not been possible to obtain immunologically pure preparations of specific antibodies by physical techniques. In recent years, however, specific methods have been developed for their purification. Such methods take advantage of the affinity of antibody to its homologous antigen and have given the purest preparations.

A. Basic Principles of Specific Purification

Although methods may vary in experimental details, the basic principles of specific purification are the same. These are:

(1) Formation of insoluble antigen-antibody complexes

(2) Separation of the complexes from other serum proteins

(3) Dissociation of the complexes

(4) Separation of the antibodies from the antigens.

Steps 1 and 2 ordinarily present no problem. It is with steps 3 and 4 that most difficulties have been encountered, those associated with the latter being mainly responsible for the development of immunoadsorbents.

(1) Formation of Insoluble Antigen-Antibody Complexes

a. Nature of the Reaction

Insoluble antigen-antibody complexes are formed either by the union of antibody molecules with a specific soluble antigen followed by separation of the product from solution as a precipitate, or by the reaction of antiserum with antigen in some insoluble state. With the former, the visible insoluble product is a secondary manifestation of a primary invisible union between antigen and antibody, and occurs usually in the presence of an appropriate concentration of salt. With the latter, only the primary union may occur as in the case when a soluble antigen is made insoluble by attachment to an insoluble support.

As early as 1906, Ehrlich suggested that the union of antigen and antibody was the result of a chemical reaction in which both reactants combined in constant proportions. Later, it was established that the reac-

tion could indeed be explained on a chemical basis, but the concept that antigen and antibody reacted in constant proportions could not be accepted since it had been shown that a given quantity of antitoxin would neutralize varying quantities of toxin (Danysz, 1902).

Attempts to explain the combination of antigen and antibody in multiple proportions resulted in a number of suggestions, some of which now form the basis for understanding the antigen-antibody reaction.

Bordet (1939) likened it to a colloidal reaction in which antibody is adsorbed by antigen, the amount of antibody adsorbed being dependent on the surface area of the antigen and the concentration of the antibody. He considered the visible stage of precipitation or agglutination to be nonspecific representing a "salting out" phenomenon in the presence of an electrolyte.

That adsorption was not likely to be the mechanism of reaction was suggested when it was demonstrated that the amount of antibody removed from a solution did not increase indefinitely with its concentration as would be expected with an adsorption phenomenon. Credit for this

observation goes mainly to Heidelberger (1934) who, by the use of quantitative chemical procedures showed that when increasing amounts of antigen are added to a constant amount of antiserum the amount of antibody precipitated increases to a maximum then declines until in extreme antigen excess no precipitate is formed.

This was explained in terms of a "lattice theory" originally proposed by Marrack (1938) and modified by Heidelberger (1934) and Pauling (1940). It suggests that the reactions leading to precipitation are a series of successive bimolecular reactions the rates of which are proportional to the concentrations of the reactants (Heidelberger and Kendall, 1935). Thus:

$$A + G \underset{K_2}{\overset{K_1}{\rightleftharpoons}} AG$$

where A is antibody and G is antigen.

In a reaction similar to polymerization the product would be

$$AG + AG \longrightarrow AG.AG$$

or in the presence of excess antibody

$$AG + A \longrightarrow AG.A$$

The products combine until large aggregates are formed and precipitate. Lattice formation would be possible if the antibody molecule were at least bivalent and antigen were multivalent (Marrack, 1938). Strong evidence for the bivalency of antibody has been obtained by a number of workers. By ultracentrifugal analysis and electrophoretic studies of products formed in antigen excess, antibody excess and at equivalence, and subsequent calculation of the molecular ratios of antigen and antibody, it was concluded that the maximum valency of antibody was greater than one, and at most two (Pappenheimer et al 1940, Marrack et al, 1951, Singer and Campbell, 1952).

The extent to which precipitation occurs depends primarily on the ratio of antigen to antibody (Dean and Webb, 1926). With optimal proportions large aggregates are formed and maximum precipitation occurs. If either antigen or antibody is in excess the aggregates formed are too small to precipitate and remain in solution as "soluble complexes". Thus, the composition of the precipitate varies according to the proportion of anti-

gen and antibody in the mixture rather than the concentration of the antibody.

b. Specificity of the Reaction

Perhaps the most remarkable characteristic of the antigen-antibody reaction is its specificity. Antibody produced to a given antigen will react only with that antigen or with other chemical determinants having a closely similar structure.

The first inference to the relation of antibody specificity to the chemical structure of the antigen came from the work of Wells and Osborne (1913). They studied the antigenic specificity and cross reactivity of a variety of plant proteins, and suggested that specificity of antigen was dependent on the chemical structure of portions of the antigen molecule.

More decisive evidence came from the work of Landsteiner (1945) who prepared conjugated azoproteins and showed:

a. that antibodies could be produced to small haptenic groups of known chemical structures,

b. that such antibodies would combine with the haptens, and

c. the haptens could specifically inhibit precipitation of the conjugated azoprotein.

This discovery has made it possible to examine experimentally the molecular structure of the combining region of the antibody molecule. By systematic alteration of the structure of a haptenic group, it is possible to observe the effects such changes have on the combining capacity of an antibody produced to the unaltered hapten, and thus relate structure to specificity.

It is assumed that the antigen combining site of the antibody molecule has a structure complementary to that of the antigen, but it is not known how this structure is introduced into the molecule to make it different from other globulin molecules. There have been suggestions that the different specificities may derive from variations in the folding of the completely synthesized molecule as it is released (Pauling 1940) or to differences in the positions of disulphide bonds linking the chains of the molecule (Karush, 1962). Based on more recent studies of proteins in general, it is now generally accepted that the final configuration of the molecule is dependent on its amino acid sequence. This suggests that

in at least portions of the antibody molecule there are amino acid differences. Much work is being done to determine the structure of the antibody molecule. Sequence studies have revealed a constant region and a variable region where there are amino acid differences. (Cohen and Milstein, 1967, Lennox and Cohn, 1967) The antigen combining site is in the variable region, and it is conceivable that this variability makes possible a large number of combining sites with different specificities.

c. The Nature of the Bonding

Because specificity is determined ultimately by chemical structure one might expect that a definite bond is required between both molecules. Presumably, if the physical conformation of the reactive sites of antigen and antibody corresponded closely, the formation of chemical bonds would be facilitated (Pauling, 1940). Van der Waals attraction, ionic attraction and hydrogen bond formation have been suggested to be involved. These forces are short-ranged and in themselves non-specific, but specificity could arise as a result of the spatial configuration of the molecules. Pauling

(1962) maintains that complementariness of structure is the only possible explanation of specificity compatible with our present knowledge of intramolecular forces. If two molecules possessed mutual complementary configurations so that their surfaces conformed closely to each other, strong interatomic van der Waal attraction would exist between them. If they contained oppositely charged groups these would then be brought closely together, and assuming maximum hydrogen bonding, the total energy of interaction would be great and the molecules would be attracted to each other strongly.

The presence of any of these forces has not been successfully demonstrated. Most of the work in this direction has been confined to a study of the role of ionic groups. Experiments involving alteration of the ionic groups on both antigen and antibody molecules by acetylation, deamination or guanidation (Marrack and Orlans, 1954, Nisonoff and Pressman, 1959, Habeeb, Stelos and Singer, 1959) and by methylation (Ram and Maurer, 1959) have resulted in reduction or abolition of the precipitin reaction, but since such treatments will not selec-

tively affect the combining sites but all other groups as well, the results cannot be taken as conclusive evidence for the role of ionic groups in antigen-antibody reactions (Hughes-Jones, 1963).

(2) Isolation of Complexes from other Serum Proteins

Insoluble complexes formed between antigen and antibody mixed in suitable proportions will precipitate in the presence of an appropriate concentration of salt leaving nonspecific and uncombined proteins in solution. Such complexes are easily isolated by centrifugation, and can be washed with saline essentially free of all uncombined serum proteins (Heidelberger et al, 1935a). With small volumes of serum two washes with chilled saline are adequate. Loss of antibodies due to solubilization of the precipitate is negligible, the error calculated to be just slightly greater than three micrograms per millilitre of saline.

If antigen is made insoluble by attachment to an insoluble support suitable for columnar operation, removal of uncombined serum components may be effected by passing saline or an appropriate buffer through a column to which the antiserum has been applied. Adequate

washing can be determined by spectrophotometric analysis of the effluent preferably by a continuous flow spectrophotometer, or by colorimetric determination for proteins. In either case a negative reading is taken as an indication of the absence of uncombined material.

(3) Dissociation of Complexes

The combination of antigen and antibody is firm but in general reversible (Humphrey and White, 1963). Evidence for this has been obtained by the technique of equilibrium dialysis. Antibody and hapten were dialyzed against hapten and it was shown that a definite equilibrium could be reached (Eisen and Karush, 1949).

If one accepts the theory that antigen-antibody reactions follow the law of mass action, it is not difficult to conceive that any disturbance of the equilibrium could reverse the reaction. Indeed, it has been observed that in an excess of antigen no precipitate forms while specific precipitate may be dissolved with an excess of antigen.

Dissociation of antigen-antibody complexes as a means of obtaining pure preparation of antibodies has found wide use over the years. Use of the method is based upon the assumption that no profound chemical changes take place

in the molecules when antigen and antibody are combined, and if any such changes did occur, it was assumed that they were reversible (Chow and Wu, 1937). Antibodies produced by dissociation of complexes should therefore, be functionally active.

The degree and ease with which dissociation occurs is determined partly by the nature of the antibodies and partly by the type of dissociating agent. Heterogeneity among antibody molecules has been recognized as early as 1945 by Landsteiner, who stated that antibodies formed to one determinant group though related, are not entirely identical but vary around a main pattern. A single preparation of antibody consists of a population of molecules having varying degrees of affinity for the corresponding antigen (Saha et al, 1966, Kitagawa et al, 1967). Those of low affinity would be expected to dissociate more readily than those which bind more strongly. In addition, there are reports that there is increasing firmness of union between antigen and antibody with passage of time (Boyd, 1940, Mougal et al, 1963, Bennett et al, 1963). This creates a problem of achieving a satisfactory degree of dissociation. A good yield, in most cases, requires the use of

drastic conditions which may cause partial denaturation of the antibodies. A number of methods have been developed for dissociating antigen-antibody complexes. Each has been designed with the hope of procuring a combination of good yield and high purity. Some are applicable to a variety of systems, others are more restricted in their use. Nonspecific methods as changes in temperature and salt concentration, carbon dioxide, alkali, acid, urea and more specific ones as enzymatic degradation of antigen and displacement of antigen by simple haptens have given varying degrees of success.

Dissociation by Changes in Temperature

The reaction leading to complex formation between antigen and antibody is exothermic. One would, therefore, expect that with increased temperatures the tendency would be towards dissociation (Boyd, 1966). Thus, cold agglutinins adsorbed to washed erythrocyte stroma at $0^{o}C$ have been eluted at $37^{o}C$ with saline to give an antibody preparation of high purity (Gordon, 1953, Fundenberg and Kunkel, 1957). Antibodies to blood group antigens have been isolated by heating agglutinated erythrocytes to $56^{o}C$ (Landsteiner and Miller, 1925). In general, the method is of restricted use,

since with most antigen-antibody systems the extent of dissociation is limited, high temperatures must be used and the degree of purity is considerably reduced.

Dissociation by High Salt Concentration

Dissociation of antibodies from specific precipitates and agglutinates by a high concentration of salt has had most success with the pneumococcus polysaccharide anti-polysaccharide system, and is probably applicable only to carbohydrate systems.

Precipitates of pneumococcus polysaccharide and antipolysaccharide (Heidelberger and Kendall, 1936), as well as agglutinates of killed pneumococcus (Heidelberger and Kabat, 1938) have been dissociated with 15% NaCl to give a preparation which was 80-100% precipitable in most cases, and in a yield of up to 40%.

It has been suggested that salt at such a concentration causes a shift in the equilibrium so that free antibody is released (Kabat and Mayer, 1961, Heidelberger and Kendall, 1936).

Dissociation by Carbon Dioxide

Specific precipitates have been dissolved with aqueous carbon dioxide in the absence of salt at near neutral

pH (Tozer et al, 1962). Precipitates were mixed with aqueous carbon dioxide and carbon dioxide gas passed through the mixture for one to two hours. The dissolved preparation was chromatographed on carboxymethylcellulose columns equilibrated with saturated aqueous carbon dioxide. Antibodies were eluted with sodium hydrogen carbonate solution. Yield was satisfactory but precipitability was low due to the presence of antigen and subsequent formation of soluble complexes. Approximately 97% of the total antibodies could be accounted for suggesting that there was little denaturation.

Dissociation by Alkali

Chickering (1915) and Chow and Wu (1936) were among the first to demonstrate that antibodies could be dissociated from a precipitate of pneumococcus polysaccharide and its specific antiserum by treatment with dilute alkali. The alkali-treated preparation was allowed to stand overnight then neutralized with acid. Antibody was recovered from the solution by dialysis against 0.9% NaCl, and was 90% precipitable by the nomologous antigen.

A modification of the method was used for purifying antibodies to egg albumin (Liu and Wu, 1938). Yield

was 40-50% and it was 96% precipitable.

Under alkaline conditions dissolution of a precipitate occurs at about pH 11.0 but dissociation does not begin under pH 11.7 (Kleinschmidt and Boyer, 1952, Turner and Boyer, 1952). At pH 12.0 and above, alterations in the physical properties of the globulin are noticeable, and its reactivity with antigen is greatly reduced (Kabat, 1939). It seems then, that alkalinity sufficient to give complete dissociation of antigen-antibody complexes causes alteration of the antibody globulin.

Dissociation by Acid

Acid has been used extensively for the dissociation of antigen-antibody complexes since the procedure was introduced in 1923 by Ramon who used it to purify diphtheria antitoxin.

Since antigen and antibody molecules are probably charged (Singer, 1957) one would expect them to be affected by changes in pH. This was substantiated when an antigen-antibody complex was exposed to varying pH values and it was noticed that as pH fell from neutrality increasing amounts of antibody and decreasing amounts of complexes could be detected. Dissociation is probably due to a shift in equilibrium giving free antibodies and soluble complexes. Since under these conditions complexes will react with dissociated

antibodies, optimum pH conditions must be established for complete dissociation. In most cases a pH value of from 3.0 to 2.3 is adequate; below this some breakdown of the molecules seems to occur (Kabat, 1939).

Not all acids are equally effective in their ability to dissociate antigen-antibody complexes, some have been found to give incomplete dissociation (Bennett and Haber, 1963) and even at very low pH they fail to dissociate antibodies of certain systems (Weliky et al, 1964). Other disadvantages include a considerable amount of denaturation if antibodies are allowed to remain in contact with acid for extended periods of time. Kabat (1939) found that horse pneumococcal antibodies treated at a pH lower than its isoelectric point aggregates nonspecifically. A similar observation was made by Turner and Boyer, (1952) for diphtheria antitoxin. This tends to reduce their efficiency since the net result is a decrease in yield.

It seems also, that the activity of the recovered antibodies at least in some systems, is affected. Eagle (1938) reports changes in biological qualities of horse diphtheria antitoxin and horse pneumococcal antibodies treated with formol and there is some suggestion that the

complement fixing activity of acid-treated rabbit pneumococcal antibodies might be destroyed (Weil, et al, 1939). Antibodies dissociated below pH 3.8 showed partial or complete loss of complement fixing ability as well as decrease in mouse protective ability; there was, however, no difference in the amount of precipitable nitrogen, a result which is not surprising since the antigen combining and complement fixing sites are now known to be on separate portions of the antibody molecule. More recently, Keller and Sorkin (1965) reported that aggregated globulin which is known to fix complement (Christian, 1960, Marcus, 1960) loses this property if aggregated by acid treatment. Despite these drawbacks, a survey of the literature indicates that acid is the most extensively used non-specific dissociating agent.

Dissociation by Urea

Urea has a solvent action on protein bringing them into solution without entering into chemical combination (Burk and Greenberg, 1930, McMeckin et al 1949). It is also capable of breaking hydrogen bonds, and in view of the possible implication of hydrogen bonding in antigen-antibody reactions it was natural that the usefulness of urea as a dissociating agent should have been investigated.

Early studies with egg albumin anti-egg albumin and diphtheria anti-diphtheria systems (Kleinschmidt and Boyer, 1952, Turner and Boyer, 1952) revealed that precipitates could be dissolved by a 2 molar urea solution at pH 9.8. Dissociation, however, could be initiated only at pH 10.75. To obtain any significant amount of dissociation higher pH values were necessary and this was accompanied by denaturation. A more recent study (Bata et al, 1964) showed that urea not only inhibits precipitation but at high concentrations will dissociate antigen-antibody complexes at near neutral pH. Treatment of a specific precipitate with 6 molar urea gave about 20% dissociation. The degree of dissociation seemed to be influenced by the composition of the precipitate, being greatest in the zones of antigen and antibody excess. It was suggested that this might be an indication that urea acts more effectively on loose aggregates.

Interpretations of the mechanism of urea dissociation are conflicting. Karush (1958) and Nisonoff (1959) reported that in the presence of urea, rabbit antibodies to various haptens lost their binding capacity, but this was restored when the urea was removed. Karush attributes

this action to a reversible disruption of the configuration of the antigen combining sites, while Nisonoff explains it as a direct competition of urea for these sites. Another suggestion is that urea does not break bonds to release free antibody and antigen but rather breaks up the precipitate into soluble complexes which can be separated by ion-exchange. This suggestion was based on results from experiments in which specific precipitates formed with fluorescein labelled antigen and dissolved in 8 molar urea were chromatographed on diethylaminoethyl (DEAE) cellulose and found to contain about 0.6% antigen. Similar findings have been reported for other systems when fluorescein labelled antigen was used (Bennett and Haber, 1963). The fact that complete dissolution of precipitates can occur under conditions which are not adequate for dissociation, suggests that the interactions between aggregates leading to precipitate formation may be weaker than the primary interactions (Kleinschmidt and Boyer, 1952), and it is possible that the formation of soluble complexes may, in general, be the first step in dissociation of antigen and antibody.

Yields of antibody obtained by urea dissociation are usually lower than those obtained by acid elution, but

urea has the advantage that it can be removed by dialysis without causing denaturation to the antibodies.

Dissociation by Enzymic Degradation of Antigen

In 1946 Feiner and co-workers showed that if an aggregate of Micrococcus lysodeikticus and its specific antibodies was treated with lysozyme, free antibodies could be demonstrated in the supernatant. The objective of their experiment was not purification of antibodies, but it revealed the possibility of adopting the technique to this purpose.

Dissociation of antigen-antibody complexes by this method has not had wide application, and its use is restricted to systems where there are specific degrading enzymes for the antigen or hapten. However, a preparation reported to be 80-90% precipitable was obtained by treating a precipitate of dextran-antidextran with a small amount of dextranase (Kabat, 1954). The precipitate dissolved as the dextran was digested to produce oligosaccharides which were removed by dialysis. The method was also applied to the preparation of pneumococcus anti-polysaccharide antibodies which had been precipitated with dextran (Swineford et al, 1959).

Dissociation by Haptens

The usefulness of haptens for dissociating immune complexes is based upon their ability to combine with antibodies but not precipitate them. The principle is one of competition between hapten and the determinant of antigen for antibodies. Antibodies which have a stronger affinity for the hapten than for the antigen are displaced to form soluble hapten-antibody complexes. Haptens of high affinity, used in high concentrations will dissociate antibodies from antigen completely or nearly so, but their subsequent removal from the antibodies is likely to be difficult (Karush and Marks, 1957, Farah et al, 1960). A partial solution to the problem is the displacement of strongly bound haptens by less strongly bound ones (Saha et al, 1966) which can in turn be removed by dialysis (Kaplan and Kabat, 1966), gel filtration on Sephadex (Basset et al, 1961), or a combination of gel filtration and ion-exchange chromatography on DEAE-Sephadex (Saha et al, 1966) or Dowex (Farah et al, 1960).

Haptens of low affinity will dissociate only a fraction of the antibodies precipitated, and this represents a selected population of molecules which have a higher affinity for the hapten but in general they seem to offer a satisfactory compromise.

Yields and purity are generally high, but in most instances antigen is not completely removed (Karush and Marks, 1957, Farah et al, 1960) and subsequent removal of hapten results in precipitation of some antibody.

4. Separation of Antibodies from Antigen

Antibodies which have been dissociated from antigen can be isolated by centrifugation if the antigen is insoluble, or by selective precipitation if antigen and antibody have different physical properties as differences in molecular sizes, solubilities in the presence of certain compounds, or at certain electrolyte concentrations. Viruses have been separated from their antibodies by centrifugation (Isliker and Strauss, 1959) anti-hapten antibodies precipitated with a high concentration of salt leaving the hapten in solution (Karush and Marks, 1957) and pneumococcus polysaccharide precipitated as an insoluble complex of calcium (Heidelberger and Kabat, 1939).

In general, when antigens are soluble, separation from their corresponding antigens after dissociation of an antibody-antigen complex is more difficult. Separation is facilitated when the antigens have been modified so that they become insoluble during dissociation, or when they have been made insoluble by coupling them to insoluble materials.

An attempt to solve the problem of separation was made by Sternberger and Pressman (1950) who modified the antigen by diazotization prior to reacting it with antibody. Such azoproteins will precipitate antibodies to native protein, and their acidic groups will combine with aluminum at alkaline pH to form insoluble salts. Antigen-antibody precipitate was therefore dissociated at pH 12.0 and antigen was precipitated by adding a calcium aluminate suspension. Haurowitz and co-workers (1947) took advantage of the fact that azoproteins precipitate nonspecifically at pH below 4.0 to prepare antibodies in high yield.

A somewhat similar, but more general technique was developed by Singer, Fothergill and Shainoff (1959). Antigen was first reacted with a sulphur compound to produce a thiolated antigen. When a precipitate formed with this modified antigen and its antibody was dissociated at acid pH and a bifunctional organic mercurial - 3,6 bis (acetoxymercurimethyl) dioxane (MDD)- was added, a cross linkage formed between the sulphydryl and mercuric groups. The product is insoluble and precipitates leaving the antibodies in solution. Thiolation did not alter the structure of the protein appreciably, and the antibodies recovered were 90% of the

maximum precipitate obtained with the native protein. Purity of antibodies prepared in this way may be as high as 98%.

A novel approach to the problem was used by Farah et al (1960) who used streptomycin to remove dinitrophenyl-BGG (DNP-BGG) from the antibody preparation. Streptomycin forms insoluble salts with proteins that are polyanionic and it has been suggested that the use of streptomycin might be extended to other antigen-antibody systems if the antigen can be made more anionic by appropriate substitutions, without altering its antigenic determinants.

Synthetic antigens have been separated by ion-exchange chromatography on DEAE-cellulose (Slobin and Sela, 1965) DEAE-Sephadex and Carboxymethyl-Sephadex (Freedman et al, 1966) or by alteration in pH resulting in precipitation (Basset et al, 1961). With ion-exchange chromatography the principle was based on the fact that under the conditions of the experiment, at neutral pH the antigen had a strong affinity for the resin and remained firmly fixed to the uppermost part of the column while antibody was readily eluted. It was suggested that the method might be applicable to anti-protein antibodies where the antigen has a sufficiently different charge from the antibody or where the

charge on the protein can be altered without drastically affecting its antigenic properties.

If antigen is attached to insoluble supports which are suitable for chromatographic operations, dissociation and separation are accomplished simultaneously. Antibodies are eluted as they are dissociated and only removal of the dissociating agent from the preparation is required for completion of the procedure. Haptens are usually removed by ion-exchange chromatography or dialysis, and urea by dialysis. Acids and alkali are promptly neutralized followed by dialysis against an appropriate buffer solution to adjust pH and ionic concentration.

B. Development of Specific Purification Methods

1. Dissociation of Precipitates and Agglutinates

a. Dissociation of Precipitates

Attempts at isolation of antibodies from immune sera probably began with the work of Gay and Chickering (1915). Their purpose was twofold. First they wanted to investigate the nature of the "protective bodies" and second, they thought that for therapeutic purposes a preparation low in protein content would reduce the

incidence of serume sickness which often accompanied the administration of antiserum prepared in horse. They discovered that a precipitate formed between an extract of pneumococcus and its specific antiserum had a low protein content. This was an indication that the "Protective bodies" comprised only a small fraction of immune serum. Shortly after Chickering (1915) found that the supernatant fluid from a precipitate suspended in a normal salt solution and heated for one hour at 42°C gave protection, but not if it were allowed to stand at room temperature for twenty four hours. Furthermore, if sodium carbonate were added before heating, the suspended particles flocculated and later settled leaving a clear supernatant fluid which had enhanced protective capacity and contained precipitins and agglutinins. However, the preparation contained a substance which produced active immunity, suggesting the presence of antigen.

The method was later reinvestigated by Felton (1932) who found that the treatment increased the solubility of the precipitate but that no real dissociation occurred. He also investigated the use of strontium hydroxide for dissociation and strontium and calcium phosphates for

precipitating the specific soluble substance, and was able to get up to 80% dissociation. Thus, these workers showed that it was possible to dissociate specific precipitates of antibodies and antigen and thereafter separate one from the other.

This realization prompted a flurry of work, most notably that of Heidelberger and his co-workers (1932, 1935a, 1936, 1940) who made extensive studies on the precipitation reaction, and introduced its use for the quantitation of antibodies in immune sera.

As developed by previous workers, dissociation of precipitates was applicable only to a polysaccharide-protein system, but it was later extended to the protein-protein system. This probably marked the first notable advance in specific antibody purification.

b. Dissociation of Antibody from Cellular Antigens

Some antigens are naturally present as constituents of insoluble particles and have been used for isolating antibodies without the problem of separation encountered with soluble antigens. Intact bacterial cells have been used to adsorb specific antibodies from antisera and the antibodies

eluted by an agent which will not disrupt the bacteria (Huntoon, 1921, Heidelberger and Kabat, 1938, Lee and Wu, 1940, Robbins et al, 1965). Because of the complexity of bacteria, antibodies are produced to a variety of antigenic determinants. Adsorption with whole organisms however, will remove only antibodies to surface antigens, and so this method is useful only when this type of antibody is required.

Isoagglutinins too have been adsorbed to erythrocytes and eluted by heating at 56°C (Landsteiner and Miller, 1925), but because of the fragility of erythrocytes it is difficult to get a preparation of high purity unless the cells have been modified. Boiled red cells, for example, retain their ability to adsorb specific agglutinins and give better results (Isliker, 1957). A more effective procedure is the use of erythrocyte stroma (Isliker, 1953). Stroma treated with fomaldehyde was combined with an anion exchange resin for columnar operations. The stroma was bound to the resin presumably by electrostatic attraction and at an ionic strength of 0.15u stroma-resin complex was insoluble. With slight modification the method was used to isolate complete and incomplete Rh antibodies.

Tissue localizing antibodies have been prepared by adsorption to and elution from specific tissues. Antibodies to rat lung, kidney and spleen tissues were prepared in rabbits by injection of tissue extracts. They were radioiodinated and purified by adsorption to the corresponding rat tissues, and subsequent elution by heating at 60°c (Korngold and Pressman, 1953) or by acid at pH 2.4 (Tsuzuku et al, 1967). Acid elution resulted in dissolving fewer tissue components than did heat elution. In both cases antibodies, when reinjected, localized preferentially in the organs against which they were produced.

2. Physical Entrapment of Antigen into Gels and Adsorption to Insoluble Supports

Bernfeld and Wan (1963) prepared cross-linked polyacrylamide gels with antigens entrapped within the lattice. However, small amounts of proteins leaked out from the gel even after exhaustive washing, limiting the use of the method.

A more useful method was the adsorption of soluble antigens to the surfaces of insoluble substances making them artificially insoluble. Artificially prepared com-

plexes of soluble antigens and erythrocytes have been widely used in serological reactions to investigate the presence of small amounts of antibodies in sera (Neter, 1956). Erythrocytes have not been generally used as an insoluble support for antigens except for the purification of antibodies to those viruses which interact naturally with erythrocyte membrane. On the basis of this property, antibodies to Influenza PR 8 virus have been isolated by reacting the virus with erythrocyte stroma at 4°C to produce a stroma-virus complex which was allowed to adsorb antibodies. At 37°C the virus-antibody complex elutes spontaneously from the stroma. The complex was dissociated either at pH 7.5 and antibody precipitated by a polyelectrolyte, or at acid pH and the virus removed by high speed centrifugation (Isliker and Strauss, 1959, Isliker, 1954). A disadvantage of the method was the loose union between virus and stroma which was reflected in a partial dissociation when in contact with antibody, but recovery of antibody was about 55% and purity from 70-90%.

Other forms of insoluble supports have long been used for soluble antigens. Porter and Pappenheimer (1939) studied the antibody-antigen reaction on built up stearate

films. The reaction was specific since antibody would not adsorb to the films in the absence of antigen. Vekerdi (1955) studied the reaction by reacting antigen-coated charcoal and glass powder with antibody while Bruijn (1956) used Chamberland filters to adsorb non-precipitating antibodies from syphilitic serum. Bentonite, a type of aluminum silicate clay which has cation exchange properties has been widely used for the adsorption of antigenic material in certain serological reactions such as diagnosis of trichinosis (Bozicevich et al, 1951), detection of rheumatoid factor (Bozicevich et al, 1958), detection and measurement of antibodies to DNA in lupus erythematosus (Bozicevich et al, 1960). Latex particles have been used in a similar manner and were first employed by Singer and Plotz (1956) for the detection of rheumatoid factors.

Glass has been treated so that antigen adheres irreversibly and can be used to adsorb antibodies (Sutherland and Campbell, 1958). Although there is some non-specific adsorption and capacity is low it was calculated that the method could yield sufficient amounts of antibody for analytical data especially when there is a low concentration of antibodies or limited amount of antiserum.

The use of antigens adsorbed to insoluble supports had the advantage that antibodies could be separated with greater ease from antigens after dissociation. However, it also had severe limitations. Antigens are adsorbed non-specifically and the strength of the linkage between antigen and the carrier is questionable. It is possible therefore, that while under a given set of conditions no active protein is eluted, a change in ionic strength, pH or temperature might bring about desorption of antigen to contaminate the antibody preparation. Moreover, only small amounts of antigen can be adsorbed, so that the capacity to remove antibodies from solution is limited.

3. Immunoadsorbents

In the search for a method of purification which would allow clean, easy separation of antibodies from soluble protein antigens after their dissociation, it was suggested (Campbell et al, 1951) that attachment of antigen by chemical linkage to an insoluble carrier might provide a solution. The basic idea was that a specific reacting group anchored covalently to an inert, insoluble material could react with its complementary material and remove it from solution without contaminating the preparation provided

the covalent linkages formed are such as not to be broken under the conditions of elution. Thereafter, a number of methods for the preparation of immunoadsorbents - as these preparations came to be known - were developed; many of them for specific purposes, or as attempts to overcome difficulties encountered in the application of existing methods (Weliky and Weetall, 1965).

a. Properties

The use of immunoadsorbents for purifying antibodies from protein mixtures has the advantage that it requires only the primary interaction of antibody and antigen thus eliminating the problem of soluble complex formation. In addition, it is particularly useful when the concentration of antibodies is too small, or their properties such that they cannot be isolated by the conventional precipitin techniques. Non-precipitating antibodies represent such a class (Carter and Harris, 1967).

Other properties of immunoadsorbents are probably best discussed by enumerating some of the criteria which should be met by a good adsorbent:

1. It should be insoluble, and soluble antigens should be fixed to it by covalent bonding.
2. The capacity, avidity and specificity of antigen for antibody should not be impaired by its linkage to the carrier substance.
3. The insoluble support by itself should not adsorb plasma proteins, but if small amounts are adsorbed, they should be easily removable by washing.
4. The adsorbent should have a high capacity for antibodies, and should release quantitatively the adsorbed antibodies without significant change in their activity.
5. It should possess mechanical properties which allow its ready removal from a reaction mixture, and preferably permit its use in columnar operations.
6. It should be stable with time retaining biological activity during use and storage.

Since the antigenic determinants of natural antigens are unknown, it is conceivable that the coupling reactions may on occasions involve the determinants themselves, or closely situated groups, resulting in reduction or complete loss of antibody binding capacity (Sehon, 1967). The usefulness of a particular method of modifying the antigen is,

therefore, governed by the type of antigen and has to be determined individually.

b. Preparation

1. Covalent Linking Reactions

Binding of biologically active proteins to insoluble carriers by covalent bonds is carried out through functional groups on the proteins which are non-essential for their biological activities and should be performed under conditions which will not cause denaturation.

The functional groups of proteins suitable for covalent binding under mild conditions include alpha and epsilon amino groups, alpha, beta and gamma carboxyl groups, sulphydryl and hydroxyl groups of cysteine and serine respectively, the imidazole group of histidine and the phenol ring of tyrosine. Amino groups react readily with acylating and alkylating agents, aldehydes, isocyanates and diazonium salts; hydroxyl groups with acylating agents, sulphydryl groups with organomercurial compounds and alkylating agents and imidazole and phenol groups with diazonium salts (Silman & Katchalski, 1966).

Chemical binding of proteins is very frequently done through acylated and arylated groups by diazonium

linkage. Binding is effected by using carriers containing suitably acylated groups such as acyl halides, acid anhydrides, activated carboxylic groups. In addition, amino groups of proteins can be acylated or arylated so that new functional groups can be inserted and used for binding to the carrier. Binding through polydiazonium salts has the advantage that it can be performed rapidly even at low temperatures and in neutral aqueous solutions.

2. Supporting Media

Polymers used for preparation of immunoadsorbents are those which have groups that can be coupled covalently to proteins under conditions that are known to induce such chemical reactions. In making a choice of a given carrier to be used, it must be realized that a functional group which is highly reactive in low molecular weight compounds may be less reactive when incorporated into a polymer. Moreover, the carrier may alter the environment so that the strength of interaction between insoluble antigen and antibody is affected, and consequently the degree of elution of adsorbed antibodies may also be affected.

Physical properties of the carrier have also to be considered. Solubility, mechanical stability, swelling

characteristics, surface area, electrical charge, hydrophobic or hydrophilic nature all play a role in determining the maximal amount of protein which can be covalently bound and the stability and biological activity of the insoluble product. Highly cross-linked polymers have high mechanical stability, but a more loosely linked polymer network is to be desired since it allows more ready access of antibody to antigen.

The chemical nature of the carrier will affect its affinity for a given protein and subsequently its ability to bind that protein. Of particular importance is the ability of the carrier to adsorb proteins or other compounds by non-covalent bonds. This may cause physical adsorption of the biologically active protein during the coupling reaction, and nonspecific adsorption of various other materials.

Among the insoluble carriers frequently used are: cellulose and its derivatives, polystyrene, red blood cell stroma, and synthetic polypeptides.

a. Cellulose Adsorbents

Cellulose is a plant fibre composed of linear polymers of β - D-glucose. It is hydrolysed when boiled in strong acids, but its insolubility is not appreciably

affected by dilute acids.

Except for end groups, there are three reactive hydroxyl groups per glucose unit to which coupling of a particular substance may be done directly, or derivatives made by addition of other groups which may undergo further reactions.

The reactivity of the cellulose will depend on the extent to which a solvent penetrates its fibres and the past history of the resin, such as its exposure to solvents and high temperature and the method of regeneration. Its physical properties will be determined by the changes its hydroxyl groups have undergone. Hydroxyl groups undergo the usual reactions of alcohols forming such derivations as esters, ethers, halides, amines and urethanes some of which are frequently used as intermediate products of synthesis (Weliky and Weetall, 1965).

As early as 1932 Landsteiner and Scheer demonstrated that synthetic dipeptides could be coupled to proteins through diazonium bonds without loss of antigenicity. They coupled haptens to erythrocyte stroma and used the conjugates to adsorb homologous and heterologous antibodies (Landsteiner and Scheer, 1936). A similar procedure was

used by Campbell and his co-workers (1951) to develop the first well characterized cellulose immunoadsorbent. Cellulose was treated with p-nitrobenzyl chloride followed by reduction, diazotization, and coupling to bovine serum albumin (BSA). The procedure was tedious and complicated, but yielded a product which gave an antibody preparation of high purity and precipitability after elution with hydrochloric acid at pH 3.0.

A modification of the method in which unreacted free diazonium sites were blocked with β-naphthol was later used to prepare immunoadsorbents of BSA (Weetall and Weliky, 1964, Malley and Campbell, 1963), human gamma globulin (Weetal and Weliky, 1964, Talmage et al, 1954, Webb and LaPresle, 1964), ragweed pollen extract and Timothy pollen extract (Malley and Campbell, 1963). All preparations were at least 80% precipitable by their antigens, but the modification resulted in increased nonspecific binding at low pH.

The method was further modified by Gurvitch (1961) and used by Mougal and Porter (1963). Cellulose was treated with N (M-nitrobenzyloxy)-methyl pyridinium chloride at 125^{o}C followed by reduction of the nitro groups to produce "amino cellulose". The latter was dissolved in a solution

of cupric ammonium hydroxide from which it was precipitated by acid as a suspension. Such a treatment greatly increased the surface area so that the amount of antigen fixed was increased, and an immunoadsorbent of extremely high capacity was produced. The method was used to produce adsorbents of gamma globulins, albumins of various animal sources, and liver and brain extracts (Gurvitch, 1964).

Proteins and haptens containing basic amino groups have been coupled directly to carboxymethylcellulose (CMC) in the presence of a tetrahydrofuran solution of N, N-dicyclohexylcarbodiimide (DCC) (Weliky et al, 1964, Weetall and Weliky, 1964). The reaction was believed to involve acid activation and was found to go to a greater extent at pH 3.5 to 5.0. The coupling is probably due to the formation of an amide bond between the amino group of the protein and the carboxyl group of CMC. BSA, human gamma globulin, keyhole limpet hemocyanin, ϵ - DNP-lysine and p (p'amino-phenylazo) phenylarsonic acid have been coupled by this method. Recovery of antibody from such adsorbents ranged from 20% to 60% and purity was greater than 90%. An advantage of the method is that no blocking of groups is required.

The method has been modified (Weetall and Weliky, 1964), by first coupling benzidine to CMC in the presence of a tetrahydrofuran (THF) solution of DCC. The resulting arylaminocellulose obtained was coupled to BSA and unreacted diazonium groups were blocked with β - naphthol. Nonspecific adsorption was higher than when CMC was coupled directly, and seemed to be dependent on the number of β -naphthyl groups present. DCC has also been used in the preparation of a benzenearsonic acid cellulose derivative (Vannier et al, 1965). Tyramine was first coupled to CMC in the presence of DCC, and the product coupled to diazotized p-arsanilic acid. The immunoadsorbent had a high capacity and yields were good.

A somewhat different procedure was developed for the preparation of immunoadsorbents for isolating anti-hapten antibodies (Lerman, 1953, Kreiter and Pressman, 1964). Powdered cellulose in benzene was brominated by refluxing over phosphorous tribromide, followed by etherification with resorcinol and coupling to diazotized p-benzenearsonic acid. The adsorbent had a capacity of 2 milligram antibody protein per millilitre of adsorbent. Recovery was effected by a gradient or stepwise elution with increasing concentrations of a specific hapten solution.

An efficient adsorbent was recently prepared from an arylamino cellulose derivative with the use of an "active" ester of N-hydroxysuccininimide (Behrens et al, 1967). The starting material was commercial aminoethyl cellulose which was treated with sodium hydroxide and N,N-dimethyl formamide to increase its reactivity. Unreacted amino groups were blocked by acetylation and nitro groups were reduced. The amino aryl derivative was then diazotized and coupled to BSA. Any unreacted groups present were blocked with glycine. Antibody binding capacity was high and purity was between 80 and 90%. Using I^{131} BSA it was shown that about 0.2% of the antigen was eluted. Nonspecific adsorption was 1 to 2%.

Aminoethylcellulose has also been coupled to 6-trichloromethyl purine in a tetrahydrofuran-water mixture at pH 10-10.5 to produce an adsorbent for the isolation of purine specific antibodies. (Weetall and Weliky, 1965). Antibodies were eluted with hydrochloric acid pH 2.3, the yield was 82% and the preparation was 89% precipitable. The procedure is applicable to the isolation of antibodies to any purine or pyrimidine capable of being converted to a derivative which can be coupled to an amino group.

An immunoadsorbent prepared from bromacetylcellulose

was developed by Jagendorf and co-workers (1963) and used by Robbins et al (1967) for isolating antibodies to proteins, synthetic polypeptides and haptens, and by Haimovich (1967) for isolating anti-human IgG antibodies. Powdered cellulose was treated with bromacetic acid in dioxane, and bromacetyl bromide. Antigen was reacted with bromacetyl cellulose in a phosphate-citrate buffer, at which stage antigen was physically adsorbed. Antigen adsorption was pH dependent and varied with the particular antigen. The resulting product was adjusted to pH 8.9 when chemical bonds were formed. Unreacted bromine was blocked by treatment with ethanolamine, and any antigen bound noncovalently was removed by treatment with 8M urea. Antibodies were eluted with 0.1M acetic acid pH 2.3 and by specific hapten (Robbins et al, 1967). Yields varied from 40% to 90% and antibodies were about 90% precipitable. Nonspecific adsorption was low but the preparation contained 3 to 5 milligrams of antigen per gram of adsorbent.

In general, the versatility of cellulose and the variety of reactions it can undergo make possible the coupling of a vast number of proteins. Adsorbents prepared from cellulose have high capacities and low nonspecific adsorption, and it might be that hydrophilic polymers are more

efficient supporting materials than are hydrophobic materials such as polyaminostyrene which has a capacity about thirty times lower than that of cellulose (Sehon, 1967) and non-specific adsorption as high as 28%. One disadvantage reported with cellulose adsorbents is the tendency to retain 'tightly bound' antibodies (Lerman, 1953, Bennett et al, 1963), but this might be overcome if antibody is allowed to remain complexed with antigen for a minimum period of time (Weliky et al, 1964).

b. Ion-exchange Resins

The investigation of ion-exchange resins as supporting media in the production of immunoadsorbents has been reported (Isliker and Strauss, 1954, Isliker, 1953, 1957). Human serum albumin (HSA) and PR 8 Influenza virus were coupled to the acid chlorides of ion-exchange resins and to diazotized sulfonated resins, a treatment which wholly or partially deprived the resins of their ion-exchange properties. Antibodies were recovered by acid elution in high yields, but the results varied with the batch of conjugate used. Apart from the work of these authors, there seems to be no other report of the use of these resins in the preparation of immunoadsorbents.

c. Polyaminostyrene

Polystyrene is a nonpolar compound devoid of ion-exchange properties. For this reason its use as a supporting medium for soluble antigens was investigated in an attempt to reduce non-specific adsorption associated with cellulose and other ion-exchange resins.

Polystyrene was nitrated and reduced to produce polyaminostyrene. The latter was diazotized and coupled to antigen and free unreacted diazo groups were blocked with glycine. Antibodies to normal human serum (NHS), human serum algumin (HSA), bovine serum albumin (BSA) and water soluble extract of ragweed pollen (WSR) (Gyenes et al 1958, Gyenes and Sehon, 1960, Webb and LaPresle, 1961) Rous Sarcoma virus (Chubb, 1967), human gamma globulin (HGG) (Oreskes, 1966) were purified using acid elution at pH 3.0. Recovery varied with the batch of polyaminostyrene, and frequently the adsorbents displayed a high degree of nonspecific binding of serum proteins. However, with a commercial preparation in which the number of amino groups were unknown, recovery was 35% and purity 82%. These findings correlated with those of Yagi and Pressman (1959).

The capacity of polyaminostyrene resins to adsorb

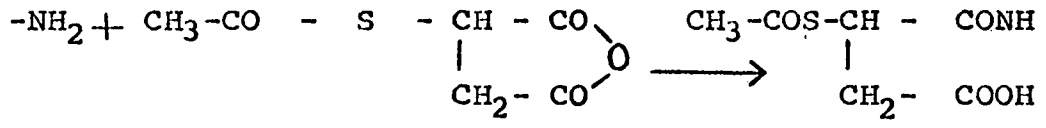
antibody seems to depend on the number of amino groups introduced, highly substituted preparations were less efficient. This might be due to the participation of active antigenic sites in linkage (Yagi et al, 1960). However, preparations with reduced amino groups were hard to wet and therefore, inconvenient to use.

The high nonspecific adsorption of these resins might be explained by the observation that preparations in which diazo groups were blocked with β -naphthol could still tightly bind a considerable amount of antigen which was found to be not chemically bound. Physically adsorbed antigen had an antibody adsorbing capacity similar to chemically bound antigen (Yagi et al, 1960, Richter et al, 1962, Delorme et al, 1962). This finding casts uncertainty about the nature of the effective antigen on chemically coupled antigen resins. It is possible that the activity of the resin may be due to a great extent to physically adsorbed antigen existing with chemically coupled antigen, a situation which could make these adsorbents unfavourable for antibody purification.

d. Insoluble Protein Polymers

Insolubilization of biologically active proteins can be accomplished by the use of bi or multifunctional cross-linking reagents to produce immunoadsorbents of high capacity.

The reaction may occur by two methods, namely: formation of a three dimensional network as a result of intermolecular cross-linking of the protein and binding of the protein to an insoluble carrier. Cross-linking reagents are of two classes: "homo" bifunctional reagents possessing two identical functional groups as bisdiazobenzidine (BDB) and "hetero" bifunctional reagents possessing two different functional groups. Because of the different chemical reactivities of the two functional groups the latter are particularly useful in binding proteins to suitable native and synthetic carriers. Thus, an insoluble protein polymer was prepared (Onoue et al, 1965) by the introduction of sulphydryl groups into rabbit serum albumin by reaction with S-acetyl-mercaptosuccinic anhydride at pH 8.0 followed by alkaline hydrolysis of the S-acetyl group. The reaction sequence is envisaged as:



(Protein) (Mercaptosuccinic Anhydride)

The resulting modified protein was insoluble at below pH 4.5 - 4.0. The acetyl group on the mercaptosuccinyl residue was

removed by adjusting the pH to 11.5 and the product was cross-linked with the trifunctional reagent tris (1(2-methyl) aziridinyl phospho oxide) (MAPO) to give a product which became insoluble during cross-linking over a pH range of 2.0 to 9.0. p-Arsanilic acid, BSA and insulin were diazotized and coupled to the insoluble RSA polymer and used to remove specific antibodies from immune sera. The adsorbent had a capacity of up to one gram of antibodies per gram of adsorbent in some systems. Recovery was 80% and purity upward of 90%.

Serum proteins have also been insolubilized for use as adsorbents by polymerization with ethyl chloroformate (Avrameas and Ternynck, 1967). Polymers or copolymers of IgG, serum algumin and normal serum proteins could be prepared simply by mixing the protein with ethyl chloroformate at room temperature at a pH value near its isoelectric point. The resulting materials were either precipitates or colloids, which were washed extensively and used in a batchwise procedure. Most of the polymers were stable in a number of detergents and disaggregating agents, and the authors suggested that stability may be dependent on the number of ionic groups present. Acetylation abolished insolubility in 8M urea indicating that free amino groups are involved in linkage during

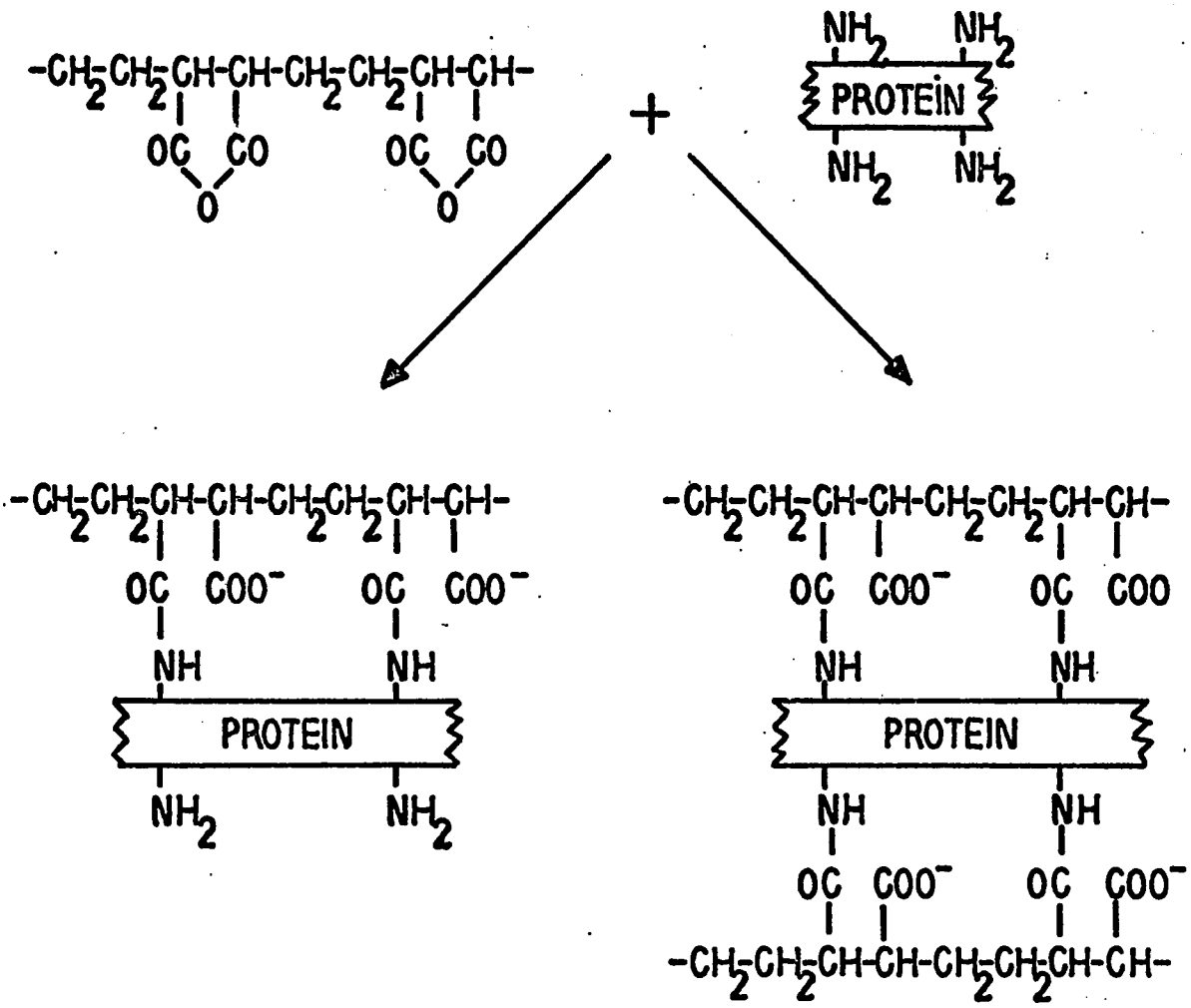
polymerization. Such groups may form covalent bonds with carboxyl groups. Adsorbents prepared in this way had a capacity of up to 0.6 gram of antibodies per gram of antigen, and the eluted material was 90%-97% precipitable.

Soluble antigens have also been cross-linked with formaldehyde to produce adsorbents of high antibody capacity (Metcalfe et al, 1966). A p-aminophenylether derivative of choline (CPE) was diazotized and coupled to BSA. The resulting product was made insoluble by addition of formaldehyde at pH 3.0 and 25°C. Hapten dissociation with choline gave an antibody preparation of high yield which was 84% precipitable.

Bovine serum albumin has been made insoluble without loss of antigenic determinants by heating at pH 3.7 (Hirata and Campbell, 1965). The insoluble product had a high antibody capacity, but had the disadvantage that on prolonged storage it became soluble. Also, it is applicable only to those proteins which retain their antigenic determinants under conditions of denaturation needed to make them insoluble.

e. Synthetic Copolymers

A method originally developed for coupling enzymes to insoluble polymers (Levin et al, 1964) was recently adapted as a general method for the production of immunoadsor-


PROTEIN
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bents (Centeno and Sehon, 1966, Sehon, 1967). Soluble protein antigens were coupled to a commercial copolymer of maleic anhydride and ethylene (Ethylene-maleic anhydride - EMA) at 0°C with constant stirring. The resulting conjugate was collected by centrifugation, washed extensively and used either in batchwise procedure or mixed with Sephadex G-25 or cellulose powder and used in columnar operations. Antibodies were recovered by stepwise acid elution with glycine-HCl buffer at pH 3.0, 2.5 and 2.0, or with phosphate buffered 8M urea pH 7.4. Yields were not reported but purity was given as 91% to 97%.

The reaction is believed to occur as shown on page 53a.

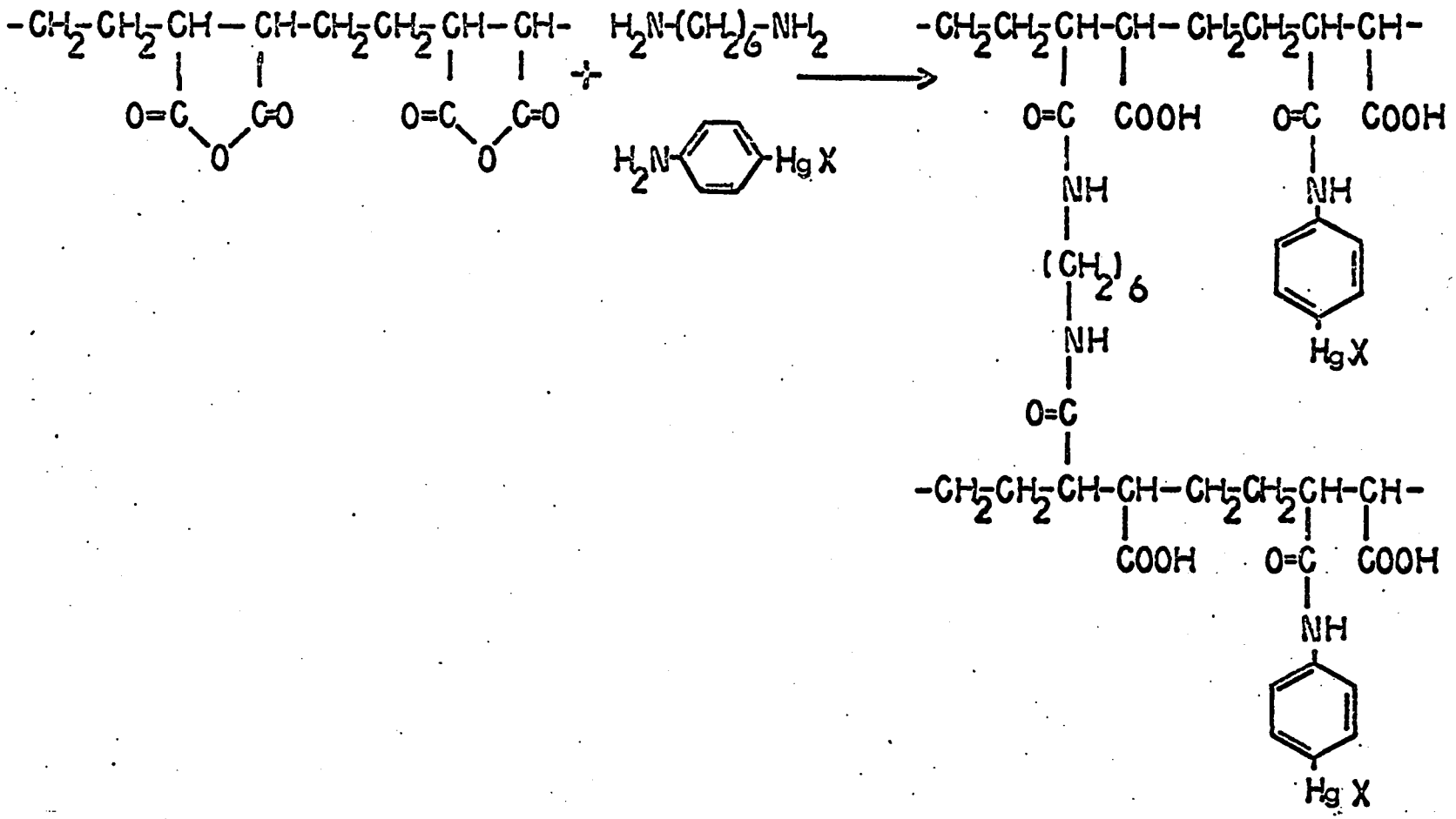
Linkage occurs through amino groups of the protein and the anhydride groups of the carrier to form amide links and subsequent production of free carboxyl groups. The authors stated that with small antigen molecules, at low antigen concentration or with antigens having a small number of free amino groups the conjugate might not be extensively cross-linked, a situation which might result in a tendency to become solubilized.

A modification of the method referred to as "graft" polymerization was used to isolate rabbit antibodies to ragweed. A tightly cross-linked conjugate was first prepared

by reacting EMA with a large molecular weight heterologous protein eg. BSA without blocking all the amino groups. The low molecular weight ragweed soluble extract was then grafted to this matrix by addition of the antigen to the former conjugate and subsequent addition of more EMA.

An adsorbent produced from modified EMA copolymer has recently been reported (Liener, 1967). EMA was reacted with p-mercurianiline in the presence of hexamethylenediamine as a cross-linking agent to produce a water insoluble resin capable of combining reversibly with thiols. The reaction sequence occurs as shown in the diagram on page 55a.

Proteins with one or more sulphydryl groups were bound by the resin and eluted with cysteine at pH 7.2 with 90% recovery. Although the resin was not used for the isolation of antibodies the possibility of employing it for this purpose might be investigated. Nonspecific adsorption of basic non-sulphydryl proteins has been noted but since these are not eluted by cysteine this should present no problem. The adsorbent has two disadvantages: its physical properties are such that it cannot be used in columnar operations, and it cannot be regenerated for re-use.



f. Polysaccharide

Sephadex G-75, an insoluble cross-linked dextran preparation has been used to adsorb antidextran antibodies (Schlossman and Kabat, 1962, Gelzer and Kabat, 1964). Antibodies were recovered by hapten elution with haptens of varying sizes.

Similarly Concanavalin A, a plant protein with globulin-like properties suggesting a close analogy with an antibody-antigen system has been isolated from Canavalia ensiformis (jack bean) by adsorption to Sephadex G-50 and elution with glucose. The purity of the preparation was above 97% (Agrawal, B and Goldstein, I, 1967).

Peptides and proteins have also been coupled to Sephadex, cellulos and starch by reacting with cyanogen halides. The method involves the formation of an active intermediate by treatment with an aqueous cyanogen halide solution under alkaline conditions, and the coupling of the intermediate with the protein (Axén et al, 1967). Carbohydrate derivatives containing primary amino groups presumably form cyanamide intermediates capable of reacting with proteins with subsequent formation of guanidino derivatives. The charge of the

protein is essentially unchanged, a fact which migh contribute to higher retention of biological activity. Various allergens were coupled to Sephadex by this method, and the adsorbents used to detect the corresponding antibodies (Wide et al, 1967).

g. Miscellaneous

A recent communication reported the use of hemocyanin for the isolation of small quantities of antibodies (Rutishauser et al, 1967). The principle is based on the properties of hemocyanin at different pH values. Hemocyanin in its native form at pH 6.5 to 6.9 has a molecular weight of 7.5×10^6. At pH 8.5 it dissociates to units of molecular weight 814,000. When precipitates of dissociated hemocyanin and antibodies to the native form are adjusted to the pH favouring association, free antibodies can be detected in the supernatant fluid after removal of the precipitate. Advantage was taken of this property to isolate antibodies to pneumococcal polysaccharide from a hemocyanin-glucuronide conjugate (Corneil and Wofsy, 1967).

There has been one report of the isolation of bacterial antibodies by an immunoadsorbent prepared by poly-

merizing bacteria with benzidine and mixing them with non-ionic cellulose for use in columns (Weetall, 1967). While the degree of purity of the eluted antibodies could not be determined, no serum proteins other than gamma globulin could be detected by immunoelectrophoresis. Only one type of bacteria was used, but the method should be applicable to various other types provided that the polymerization process does not result in loss of antigenic determinants.

To date, immunoadsorbents have provided the purest antibody preparations, but none has been completely satisfactory. Many of the methods are tedious and complicated, and overall yields are not generally high. One disadvantage which should not be overlooked is that often the antibody population isolated differs in its range of specificities from that of the whole immune serum. Antibodies having a high affinity for antigen are very often so tightly bound that they are not eluted.

C. Other Applications of Immunoadsorbents

The preceding discussion served to illustrate that immunoadsorbents have been usefully employed to obtain antibodies of high purity. The usefulness of immunoadsorbents is, however, not restricted to this purpose. They have been successfully employed in such areas as the detection and

quantitative assay of antibodies, demonstration and analysis of heterogeneity of antibodies to a given antigen, isolation of specific antigens from a mixed population and ezyme studies.

Quantitative Assay of Antibodies

Prior to the development of immunoadsorbents, quantitative determinations of the antibody content of immune sera could be made only by the quantitative precipitation technique introduced by Heidelberger et al (1935) (Heidelberger, 1939). Because of the inhibiting effect of excess antigen or antibody, the optimum amount of antigen needed to give maximum precipitation had to be determined, and this was best done by preparing a precipitin curve using a constant amount of antibody and varying amounts of antigen. Such a curve is applicable only to a given antiserum since it has been shown (Humphrey and Porter, 1956) that the character of the precipitin curve varies with the phase of immunization even within a single animal. Moreover, only precipitating antibodies could be measured. Obviously then, there were disadvantages in this method, and it was not until 1954 that Talmage et al demonstrated that the use of immunoadsorbents was a more efficient way of making absolute quantitative assays of antibodies in immune sera. Thereafter, Gurvitch et al (1962)

used adsorbents of albumin conjugated to "aminocellulose" to adsorb antibodies, and estimated the quantity either by measuring the increased uptake of a dye after correction had been made for nonspecific adsorption or by Lowry's method of protein determination.

A very sensitive method was used by Yagi et al (1960) to assay quantities of antibody too small to be measured by the conventional quantitative methods. As little as 0.2 micrograms of antibody per milligram globulin could be detected using radioiodinated globulin and measuring the amount of radioactivity specifically adsorbed. Bovine serum albumin made insoluble by heating was also used to estimate the antibody content of sera and it was found that the quantity detected was greater than that estimated by quantitative precipitation. The difference was presumed to represent non-precipitating antibodies (Hirata and Campbell, 1965).

A somewhat unique situation is the detection of antipenicilloyl antibodies by penicilloylated bacteriophage (Haimovich et al, 1967). Coupling of haptens to bacteriophage had previously been reported (Mäkelä, 1966), and the ability of such conjugates to react with specific antibodies demonstrated. Modification of phage by treatment with penicillin

G inactivated some of the virus, but the survivors retained their ability to react with and be inactivated by antibody (Haimovich and Sela, 1966). The presence of penicilloyl antibodies in the sera of individuals with a history of allergy to penicillin was demonstrated by phage inactivation, and it was found that the method was much more sensitive than the hemagglutination assay.

Immunoadsorbents have only recently been adapted to the determination of the absolute antibody content of immune sera, but already it has been demonstrated that they are extremely useful for this purpose.

Heterogeneity of Antibodies

Antibodies to a given protein antigen may consist of a mixed population containing antibodies directed against different antigen determinants, as well as antibodies with different avidities for the same antigen determinant. This type of heterogeneity has frequently been demonstrated and studied by the use of immunoadsorbents. Gradual elution of such a heterogeneous population of antibodies from an immunoadsorbent-antibody complex leads to fractionation in which the antibodies with low affinity are the first to be eluted.

Using a polyaminostyrene-HSA adsorbent and eluting with two different acidic solutions Webb and Lapresle (1961)

showed that antibodies having different specificities required different pH for maximum elution. Under the conditions of their experiments, antibodies were eluted in three fractions each containing antibodies directed against different fragments of HSA. Similar findings were obtained by Gurvitch (1962) Centeno and Sehon (1966).

Arquilla and Finn (1963) studied the uptake of insulin antibodies from guinea pig and rabbits by insulin-sheep stroma-cellulose conjugates and found that when such immunoadsorbents were completely saturated with antibodies from the immune serum of one animal they could still adsorb additional antibodies from the immune serum of another animal.

By eluting human antidextran antibodies from a sephadex adsorbent with oligosaccharides of varying sizes, Schlossman and Kabat (1962), Gelzer and Kabat (1964), obtained populations of molecules differing in their affinities and were able to suggest an upper limit for the size of the antigen combining site. Heterogeneity in binding properties has also been shown by Saha et al (1966), Cheng and Talmage (1966), Kitagawa et al (1967).

Inhibition of precipitation by haptens has been used to study the affinity of antibodies for known chemical structures and to obtain information on the structure of the

antigen combining site. Frequently, however, specific binding of haptens by serum albumin and other proteins cause complications, which, it seems, might be avoided by the use of immunoadsorbents (Farah et al, 1960).

Enzyme Studies

A number of enzymes have been made insoluble by chemical attachment of insoluble supporting media, and used as specific catalysts in suspension or in column form to produce specific chemical changes in large amounts of substrate without contamination. In addition, water insoluble enzymes make possible the reproduction of conditions surrounding enzymes in their native state while embedded in membranes and therefore serve as models for the study of the effect of altered environment on their mode of actions (Silman and Katchalski, 1966).

In some instances coupled enzymes lost their activity on storage, but many remained stable even after lyophilization. Activity is dependent upon the nature of the carrier, some might increase while others decrease enzyme stability.

One application of a water insoluble enxyme of special interest is the use of insoluble papain to partially degrade rabbit gamma globulin into fragments similar to those described by Porter (Cebra et al, 1961, 1962, Cebra 1964).

III. MATERIALS AND METHODS

A. Preparation of Antiserum

New Zealand white rabbits were immunized with a 1% (w/v) solution of bovine serum albumin (BSA) (Nutritional Biochemicals Corp.) in 0.85% sodium chloride. They were inoculated intravenously in the ear vein with 1 ml of the antigen. Injections were given three times per week for three weeks, a total of nine injections. The animals were test bled three days after the last injection, and were bled out by cardiac puncture the following day if they showed good antibody response evidenced by strongly positive ring tests. The blood was collected in 250 ml glass centrifuge bottles and placed at 37°C for three hours until clot formation occurred. The clots were rimmed, and the bottles placed at 4°C for 4 hours, after which they were centrifuged in an International centrifuge Model UV at 1430 x g for 30 minutes. The serum was collected, centrifuged again, pooled, dispensed in vials in 20 ml volumes and frozen. The constant antibody optimal ratio (AOR) was determined on a sample of the pooled serum by reacting 1 ml of the anti-serum diluted 1:10 with 1 ml volumes of twofold serial dilutions of a 1% BSA solution.

B. Methods of Characterization

1. Agar Electrophoresis and Immunoelectrophoresis

Electrophoresis in agar was done on glass microscope slides using a LKB electrophoresis equipment and a discontinuous barbital buffer system as described by Hirschfeld (1960) (See Appendix). Electrophoretic patterns were developed with goat anti-rabbit serum (Hyland Laboratories, Los Angeles, Calif.) diluted 1:2.

2. Double Diffusion in Agar Gel

Immunodiffusion in agar gel was done on 25 x 75mm microscope slides using the same agar preparation as for electrophoresis.

Slides were precoated with agar and 3.5 ml of a 1% solution of No 2 Ionagar pipetted on to each slide. When the agar had gelled the slides were put in a humid chamber at $4^{o}C$ for at least 2 hours. Wells were punched and the agar removed by suction. Antigen and antibody wells were filled two to three times with the test samples, and twofold serial dilutions of antigen, and precipitation allowed to proceed for eighteen hours. Following adequate precipitation, the slides were washed with two changes of a 1% sodium chloride solution for six and sixteen hours respectively,

and distilled water for one hour. They were dried with strips of filter paper over them, and stained with 0.1% thiazine red in 1% acetic acid. The agar was de-colourized in a solution of 1% acetic acid in 70% ethanol.

3. Disc Electrophoresis

Disc electrophoresis was performed in 7% polyacrylamide gel and Tris-glycine buffer pH 8.3 using the method of Davis (1964) (See Appendix).

4. Ultracentrifugation

Ultracentrifugation was done on fractions obtained by elution from Ethylene Maleic Anhydride-BSA immunoadsorbent at pH 3.0 and 2.5. The samples contained approximately 5 mg protein per ml. Analysis was carried out in borate-buffered saline pH 7.2 in a Beckman Model E centrifuge with rotor AN-D and Kel-F cell. The speed was 56,000 r.p.m. and temperature was maintained at 20°C. Photographs were taken at 16 minute intervals for up to 96 minutes for fraction I and 80 minutes for fraction II.

Calculation of sedimentation coefficients was done on the basis of a 1% solution.

Analysis was performed through the kindness of Dr. Anil Saha of the Division of Immunochemistry and Allergy Research, Royal Victoria Hospital.

C. Determination of Antibody Content

Antibody content was estimated by quantitative precipitation and subsequent determination of the nitrogen content of the precipitate. The difference between antigen nitrogen, previously determined, and total nitrogen at equivalence was taken as antibody nitrogen. Nitrogen determination was done according to the method of Kjeldahl as described by Mayer (1961).

Standardization of Antigen

a. Digestion of Protein

An antigen preparation containing 3 mg protein (BSA) per ml was arbitrarily chosen for standardization. Nitrogen determination was made on 1 ml volumes dispensed with volumetric pipettes into five 100 ml Kjeldahl digestion flasks. To this was added 2 ml concentrated sulphuric acid, a small amount of potassium sulphate-selenium catalyst, and one Hengar granule. A sixth flask in which saline was substituted for antigen served as a control.

The flasks were placed on an electrical digestion rack, and digestion allowed to proceed for six hours. The heaters were turned to medium temperature until most of the

water had evaporated before they were turned to maximum temperature. As digestion proceeded SO_3 fumes appeared, the preparation charred and finally cleared to become colourless.

At the end of the digestion period, the flasks were allowed to come to room temperature, then placed in an ice bath, and 10 ml of glass distilled water added to each.

b. Distillation of Ammonia

A Kemmerer-Hallet Type nitrogen distillation apparatus (Fisher Sci.Co.) was used for distillation. Before use, it was steamed out and the contents collected in a 125 ml flask containing 5 ml of a boric acid-methyl red indicator mixture (See Appendix) diluted with 5 ml water. For this procedure a Kjeldahl digestion flask with 10 ml distilled water and 9 ml saturated sodium hydroxide was attached to the apparatus which was supplied with steam from a jacketed flask containing distilled water and electrically heated. Distillation was done with the tip of the condenser below the level of the indicator mixture for 7 minutes, following which the flask was lowered, the tip of the condenser rinsed with a small amount of water and distillation allowed to

continue for an additional two minutes.

If the indicator remained pink, distillation of the saline control, followed by the sample was done. A change from pink to yellow indicates the presence of nitrogenous material. If this occurred during the steaming out process, the procedure was repeated until a suitable blank was obtained.

Distillation of the sample was done as in the above procedure except that the sodium hydroxide was let into the sample from a well built into the apparatus. The alkali was introduced before distillation was started. This was done slowly to prevent nitrogen from being released too rapidly, and to avoid excessive spattering.

c. Titration

The nitrogen distilled into the boric acid-methyl red mixture was titrated with N/70 hydrochloric acid delivered from a 5 ml buret which delivers 0.01 ml per drop. The endpoint was taken as the colour of the saline control. At no time was there any detectable nitrogen in the controls.

Nitrogen content was calculated by multiplying the volume of acid used to titrate by 0.2 and the amount of

protein was estimated by multiplying the amount of nitrogen by the factor 6.25.

Samples were analysed in sets of five and repeated until the values obtained for all five within a set corresponded to within 10 micrograms. The mean value was obtained and the standard error calculated.

2. Quantitative Precipitation

A preliminary qualitative precipitation test was done using twofold serial dilutions of a 1% BSA solution and antiserum diluted 1:10 to determine the range of dilutions to be used in the quantitative test. From this, the amount of antigen nitrogen which gave optimum precipitation was determined. Since undiluted serum was to be used in the quantitative test, calculations were made for ten times as much antigen.

The standardized antigen solution was diluted to give values in the range of those calculated from the qualitative test. Dilutions were made using volumetric flasks where possible and volumetric pipettes, with saline as the diluent.

Ten dilutions were set up in duplicates using conical centrifuge tubes with pouring spouts. One ml of

antigen appropriately diluted was mixed with 1 ml of undiluted serum (or antibody preparation). Tubes in which saline was substituted for antigen served as antiserum control. The tubes were capped with parafilm, incubated in a water bath at 37°C for 1 hour and kept at 4°C for 7 days. They were mixed once daily by shaking the racks gently.

At the end of the time allotted for precipitation, the tubes were centrifuged at 4°C for 1 hour in an International centrifuge PR-2 model at approximately 1860 x g. The supernatant fluid was collected and saved to be tested for residual antigen and antibody. The tubes were drained on absorbent paper, the precipitates resuspended with the aid of a vortex mixer in 3 ml chilled saline, and centrifuged again. This was repeated and the washing discarded. After thorough draining the precipitates were dissolved with one or two drops of M/2 sodium hydroxide. The solution was transferred quantitatively to Kjeldahl digestion flasks. The tubes were washed five times with small volumes of distilled water and the washings added to the flasks. Digestion and determination of total nitrogen content were performed as described previously.

3. Supernatant Tests

The first supernatant fluid from the reaction mixture was tested for residual antigen and antibody. The fluid was pulled into small capillary tubes to a height of about 15 mm. A similar volume of antiserum was let into half the tubes and antigen (standardized solution) into the other half. The tubes were inserted into plasticine with an air space between the lower level of the liquid and the plasticine. They were incubated at 37°C for 1 hour and placed at 4°C for 48 hours.

D. Fractionation of Antiserum by Ammonium Sulphate Precipitation

Rabbit normal serum and antibovine serum were fractionated by precipitation with ammonium sulphate according to the procedure of Campbell et al (1963). Ammonium sulphate was saturated at room temperature and 75 ml added dropwise to 150 ml antiserum to give 33% concentration ammonium sulphate. The preparation was stirred constantly on a magnetic stirrer, and after all the ammonium sulphate had been added the pH was adjusted to 7.8 with 4N sodium hydroxide and stirring allowed to continue at room temperature for an additional two hours.

The mixture was centrifuged at room temperature in an International centrifuge at approximately 1400 x g for 30 minutes, and the precipitate resuspended in saline to the original volume. The procedure was repeated twice and the precipitate from the third centrifugation suspended in borate-buffered saline pH 8.4 to a final volume of one half the original volume.

The preparation was dialysed at 4°C in an Oxford multiple dialyser against several changes of 5 litre volumes borate-buffered saline. The dialysate was tested for the presence of ammonium ion using a 2% barium chloride solution. The dialysate which gave a negative test was checked by Kjeldahl procedure for the presence of nitrogen. If negative, the globulin preparation was made up to the original volume and centrifuged at 4°C for 1 hour at approximately 48,000 x g to remove a small amount of precipitate which formed during dialysis.

Activity was determined by a qualitative precipitation test using a twofold serial dilution of a 1% BSA solution and globulin diluted 1:10. It was also subjected to electrophoresis and the pattern developed with goat anti-rabbit serum. The remainder of the preparation was dispensed in 10 ml volumes, lyophilized, and stored at 4°C.

E. Fractionation of Globulin by Diethylaminoethyl Cellulose Chromatography

Globulins precipitated by ammonium sulphate were further fractionated by column chromatography on DEAE-cellulose according to the procedure of Peterson and Sober (1956) as modified by Fahey et al (1958).

Whatman DEAE-cellulose was washed repeatedly with large volumes of 0.1N sodium hydroxide, followed by distilled water, 0.1N hydrochloric acid and again 0.1N sodium hydroxide. This was followed by washing with distilled water to near neutrality. Washing were removed by suction and as much as possible of the "fines" removed.

A slurry of medium thickness was prepared from 16 grams (dry weight) resin, and a column 36.5 x 2.5 cm (outside diameter) was packed under pressure of ten pounds per square inch. It was washed with 0.01M phosphate buffer pH 8.0 until the effluent had a pH of 8.0. One hundred ml lyophilized globulin was reconstituted in 20 ml distilled water and dialysed overnight at $4^{o}C$ against 0.01M phosphate buffer pH 8.0. The sample was pipetted on to the column and rinsed with several small volumes of the buffer. The column was attached to a peristaltic pump (Harvard Apparatus Co. Inc.)

operated to give a flow rate of 96 ml per hour, an LKB UVicord continuous flow spectrophotometer fitted with a 3 mm optical cell, and an LKB automatic fraction collector. Elution was effected by pH and molarity gradients starting with 0.01M phosphate buffer pH 8.0 and ending with 0.3M phosphate buffer pH 4.5. This was achieved by a continuous flow of buffer from a one-litre Florence flask)with magnetic bar) acting as a mixing chamber and attached by a syphon to a 500 ml flask which served as a reservoir. One litre starting buffer (0.01M, pH 8.0) was placed in the mixing chamber and 500 ml of the final buffer (0.3 M, pH 4.5) in the reservoir. The effluent was collected in 10 ml volumes and operating time was approximately 16 hours.

With the absorbence pattern as a guide, the effluent was pooled into three fractions, shell-frozen in an alcohol-dry ice mixture, and lyophilized in a Virtis mechanically refrigerated "Freez-mobile". They were then reconstituted with distilled water to the volume of the sample applied to the column, and dialysed against borate-buffered saline pH 8.4. A small amount of precipitate formed during dialysis and was removed by centrifugation.

For comparison with the original serum, the

preparations were diluted 1:50, and activity determined by precipitation with twofold dilutions of a 1% BSA solution. Electrophoresis was done and the pattern developed with goat anti-rabbit serum. The remainder of the fractions were dispensed separately in 5 ml volumes and frozen.

Purity of the preparation was determined, and calculated as the percentage of precipitable nitrogen present per millilitre.

F. Isolation of Antibodies by BSA-Cellulose Immunoadsorbent

Preparation of an immunoadsorbent of BSA coupled to carboxymethyl cellulose (CM-cellulose) according to the procedure of Weliky et al (1964) was attempted.

Whatman CM-cellulose powder CM 70 was converted to the acid form by stirring with 4N hydrochloric acid, followed by washing with distilled water and acetone, and drying on a Buchner funnel.

A solution of 500 mg BSA in 25 ml distilled water was prepared and CM-cellulose (approximately 2 grams) added with stirring until the mixture had thickned. A preparation of 1.0 gram N,N'-dicyclohexyl-carbodiimide (DCC) in 2.5 ml tetrahydrofuran (THF) and 5 ml distilled water (two phases) was added, followed by more CM-cellulose to a total of 3.0 grams.

The preparation was allowed to stand at room temperature for two days, following which a 10 ml volume of water was added, and the cellulose dispersed and filtered on a Buchner funnel. The resin was transferred to a beaker and washed extensively with large volumes of 0.005M sodium carbonate, 0.01M hydrochloric acid and water.

A column 30 x 1.0 cm (inside diameter) was packed under a pressure of ten pounds per square inch. The column was washed with 1% sodium chloride acidified with hydrochloric acid to pH 2.3 until the effluent gave no absorbance at 220 mμ. Absorbance was measured by a Zeiss spectrophotometer. It was further washed with a 1% sodium chloride solution pH 7.0 until there was no absorbance and the pH of the effluent was near neutrality. A sample of 2 ml anti-BSA was applied to the column and washed through with small volumes of 1% sodium chloride pH 7.0 until protein could be detected in the effluent. The effluent was collected and passed through the column several times. After the last pass the effluent was collected to be tested for residual antibody activity. The column was then washed with 1% sodium chloride pH 7.0 until there was no absorbance at 220 mμ, when it was attached to a recorder, fraction collector and pump.

Elution was carried out with 1% sodium chloride pH 2.3 flowing from an open reservoir to which additional fluid could be added when necessary.

The eluted material was neutralized with 0.2M sodium hydroxide and along with the effluent from the last pass of the antiserum through the column, was concentrated by ultrafiltration and tested for antibody activity by double diffusion in agar gel.

G. Isolation of Antibodies by an Ethylene Maleic Anhydride-BSA Immunoadsorbent

1. Preparation of the Conjugate

An ethylene maleic anhydride (EMA) - BSA conjugate was prepared by reacting a solution of BSA with a 0.6% solution of EMA in acetone (or dioxane) at $0^{o}C$ according to the procedure of Centeno and Sehon (1966). EMA is a commercial copolymer available from Monsanto (Canada) Ltd., and was a gift from Dr. Centeno of the Division of Biophysical and Immuno-Chemistry, Department of Chemistry, McGill University.

Preliminary experiments were done to establish the amount of EMA which would give optimum coupling and a product in which essentially all the protein in the solution would be bound. On the basis of the results conjugates were prepared

using a proportion of 17 ml EMA to 100 mg BSA. EMA which had been maintained at 0°C in an ice bath, was added dropwise with stirring (on a magnetic stirrer) to the solution of BSA, which was prepared in distilled water the pH of which had been adjusted to 5.95. The protein solution was also maintained at 0°C. The conjugate separated out in flakes and was collected by centrifugation in a Servall centrifuge at 4°C for 10 minutes using a speed of approximately 125 x g. The sediment was resuspended in 0.15M sodium chloride containing 3 ml of a 1.0 M sodium hydrogen phosphate solution per 100 ml sodium chloride, and washed three times, centrifuging at approximately 625 x g for 20 minutes. It was finally suspended in a similar solution to which approximately 50 mg of BSA had been added and the pH adjusted to 7.8 with 1.0 M sodium hydrogen phosphate, and stirred overnight at 4°C. At the end of this time the immunoadsorbent was centrifuged and washed with three changes of 0.15 M sodium chloride, three changes of 0.35 M glycine-hydrochloric acid buffer pH 3.0, and again with three changes of sodium chloride. At this stage it was necessary to use a higher speed to sediment the conjugate and it was usually centrifuged at 12,000 x g for 20 minutes.

When antibodies were to be eluted with phosphate buffered 8 M urea, the conjugate was washed three times with 0.15 M sodium chloride followed by three washes with phosphate buffer pH 7.4. Further washing with urea was done after the column had been packed (Materials and Methods, Section G, Part 4).

2. Determination of Activity of the Immunoadsorbent

The EMA-BSA conjugate is in the form of a fine precipitate and can be used in batchwise or columnar operation. For the latter it has to be mixed with large amounts of Sephadex G-25 (coarse) (Pharmacia, Uppsala, Sweden) or neutral cellulose to keep it dispersed and to achieve a high flow rate. Columnar operation was the method of choice for the experiments to be described. The first experiments were done to determine whether the conditions of preparation were adequate to produce an adsorbent which would remove specific antibodies from immune sera.

Series I : Isolation of Antibodies from Anti-BSA and Concentration by Lyophilization

A small column 30 x 1.0 cm was packed from a preparation of 100 mg adsorbent mixed with 5 grams (dry weight) Sephadex. The mixture was let into the column through a funnel in which an electrical stirrer kept the Sephadex dispersed. Packing was done rapidly by opening the stopcock attached to the outlet shortly after packing began. The column could not hold all the sephadex and it appeared that only a small portion of the adsorbent actually got into it. Nevertheless, its ability to bind antibodies could be determined. For this a 2 ml volume of antiserum was pipetted on to the column, which was then washed with 0.15M sodium chloride from a reservoir attached to the column by polyethylene tubing.

When the wash was free from protein as determined with Biuret reagent (Appendix), the column was attached to a continuous flow spectrophotometer and fraction collector. Antibodies were eluted with glycine-hydrochloric acid buffer at pH 3.0, 2.5 and 2.0. The fractions were collected in 2 ml volumes, pooled according to the pH at which they were eluted, shell-frozen, and lyophilized. Each lyophilized fraction

fraction was reconsituted to 2 ml and dialysed against veronal buffer pH 8.6. Antibody activity was determined by gel diffusion, and characterization was by immuno-electrophoresis (Materials and Methods, Section B, Part 2).

Series II : Isolation of Antibodies from Ammonium Sulphate Precipitated Globulin and Concentration by Sucrose

The procedure outlined in Series I was repeated using a larger column which could accommodate 100 mg adsorbent, and substituting a globulin preparation for whole antiserum. The eluted material was concentrated by solid sucrose instead of lyophilization.

The effluent was collected as soon as it came off the column, dialysed against borate-buffered saline pH 8.4 for about 3 hours and the dialysing bags placed in solid sucrose. As soon as the sucrose had liquified, the bags were transferred to a new batch of sugar.

The preparation was tested for activity in gel diffusion against a twofold serial dilution of 1% BSA solution.

Series III : Concentration by Polyethylene Glycol

Polyethylene Glycol, M.W. 20,000 (Fisher Scientific Co.) was substituted for sucrose for concentrating

antibody preparations. The method used was that described by KOhn (1959). A small amount of the polymer was crushed and poured into dialysis tubing one end of which was knotted. It was moistened with a small amount of water and tne tubing was immersed in a graduated cylinder containing the protein solution. The preparations were put at $4^{o}C$. Some precipitate developed during concentration and was removed by centrifugation.

The preparations were tested for activity by gel diffusion and immunoelectrophoresis.

Series IV : Concentration by Ultrafiltration - Method I

Antibody preparations eluted from columns were dialysed against two 5-litre volumes of borate-buffered saline pH 8.4 and concentrated by ultrafiltration units fitted with collodion membranes (Membranfilter, Gottingen). Negative pressure dialysis was carried out against buffered saline at 750 mm Hg. The units were kept in an ice bath throughout the procedure.

Method II

An LKB ultrafiltration unit was used. It consisted of a nylon filter frame over which 1 inch (flat width) dialysis tubing was pulled. The distal end of the tubing

was tied, and the open end of the frame fitted with a rubber stopper with glass tubing through the centre. The unit was attached to negative pressure and immersed in the protein solution. Ultrafiltration was performed at $0^{o}C$.

3. Determination of the Capacity of the Immunoadsorbent

Experiments to determine the capacity of the immunoadsorbent were based upon the volume of globulin which would saturate the antigenic sites of a given immunoadsorbent preparation.

A conjugate prepared with 100 mg protein was mixed with 13 grams (dry weight) Sephadex and a column 42.0 x 1.5 cm (I.D.) packed. A lyophilized sample of globulin equivalent to 10 ml was reconstituted in 2 ml water, applied to the column and rinsed with saline. The effluent was monitored and showed an absorbance higher than was anticipated for non-specific protein. It was assumed that the effluent contained unbound antibodies. This was confirmed by a strong precipitation line given in gel diffusion, and was taken as evidence that the amount of adsorbent used could not accommodate the amount of antibodies in 10 ml of globulin. The experiment was repeated with 5 ml globulin and the same amount of adsorbent. The eluted material gave a degree of

absorbance, similar to the 10 ml sample, and no antibodies were detected in the effluent when tested in gel diffusion. This volume was therefore taken as the maximum amount of this globulin preparation which should be applied to a column containing 100 mg of adsorbent.

4. Determination of Stability of Immunoadsorbent for Re-use

A column was prepared with 200 mg adsorbent and a 10 ml globulin sample applied. Antibodies were eluted with phosphate buffered 8M urea pH 7.4, and the absorbance recorded. The column was washed with large quantities of saline, followed by phosphate buffer pH 7.4 and another 10 ml globulin sample applied and eluted. A reproducible absorbance pattern at 257 mμ was taken as an indication that the adsorbent could be re-used, at least for a second time.

5. Preparative Chromatography

a. Use of Neutral Cellulose as Dispersing Media for Immunoadsorbent

Neutral cellulose powder (W & R Balston Ltd., England) was washed repeatedly with distilled water followed by saline. A BSA-EMA conjugate prepared from 500 mg BSA was mixed with the washed cellulose powder and a column 3.5 cm

(O.D.) packed to a volume of 400 ml. Packing was done under pressure of 3 pounds per square inch. Lyophilized globulin equivalent to 25 ml was reconstituted in 5 ml volume and applied to the column. The column was washed and antibodies eluted with glycine-hydrochloric acid buffer at pH 3.0, 2.5 and 2.0. The flow rate was regulated with a peristaltic pump to give 6.6 ml per minute. Fractions were collected in 5 ml volumes, pooled according to pH and dialysed against two 5 litre volumes of borate-buffered saline pH 7.2. They were concentrated by ultrafiltration to one half the volume of the sample applied to the column. Total nitrogen content was determined on each fraction by the Kjeldahl procedure. Since these fractions were to be examined by ultracentrifugation and a minimum protein concentration of 5 mg per ml was desired, fractions I and II were further concentrated to 3.5 and 2.5 ml respectively. Ultracentrifugation was done as described in Section B, Part 5 of Materials and Methods.

b. Use of Sephadex as Dispersing Media for Immunoadsorbent

1. Elution with Glycine-HCl Buffer

The immunoadsorbent was prepared from 200 mg BSA

and mixed with 40 grams (dry weight) Sephadex G-25 coarse. A column 42 x 2.5 cm (I.D.) was packed in the manner described in Section G, part 2. A sample equivalent to 10 ml globulin was reconstituted from the lyophilized state in 2 ml distilled water and applied. The column was washed and antibodies eluted with glycine-hydrochloric acid buffer at pH 3.0, 2.5 and 2.0. The buffer was changed from one pH value to the other when the elution pattern showed a return to baseline. This varied with the concentration of the sample applied and the rate at which the antibodies were eluting, but usually from 200 - 300 ml buffer at pH 3.0, and 100 ml each at pH 2.5 and 2.0 were found to be sufficient.

2. Elution with Urea

If antibodies were to be eluted with urea a preparation containing only 180 mg BSA was mixed with 40 grams sephadex. The proportions of adsorbent to Sephadex were altered because at alkaline pH the adsorbent was swollen and the flow rate of the column reduced. A volume of approximately 200 ml phosphate-buffered 8M urea pH 7.4 (Appendix) was passed through the column followed by exhaustive washing with phosphate buffer to remove the urea. After the sample was applied, the column was washed with phosphate buffer and eluted with approximately 200 ml phosphate-buffered 8M urea.

Modification of Procedure

In some experiments the phosphate buffer wash was followed by a wash with 0.15M sodium chloride acidified with hydrochloric acid to pH 3.0. This converted the adsorbent to its acid form as used in the glycine-hydrochloric acid system. The column was then washed with large volumes of 0.15M sodium chloride pH 7.0 until the pH was approximately 5.0, at which time the sample was applied. Uncombined proteins were removed by washing with 0.15M sodium chloride, and antibodies were eluted with phosphate-buffered 8M urea.

The effluent was collected in 5 ml volumes, pooled and dialysed against two 5-litre volumes of borate-buffered saline or 0.3M sodium chloride buffered with phosphate at pH 7.4.

The preparation was concentrated by ultrafiltration and centrifuged at $4^{o}C$ to remove any precipitate which formed during concentration. Activity was determined by gel diffusion. Quantitative precipitation was set up and the precipitates analysed for their nitrogen content. The difference between antigen nitrogen added, and total nitrogen precipitated at equivalence was taken as the nitrogen content of

the antibodies. Yields were calculated as the percentage of nitrogen recovered from the sample, while purity was estimated as the percentage of the recovered nitrogen which was precipitable by antigen.

c. Batchwise Elution of Antibodies

Preliminary experiments were done to determine the length of the reaction time required for complete absorption of antibodies by the immunoadsorbent. This was done by reacting 100 mg adsorbent with 2 ml globulin for 25, 60, 90 and 120 minutes in polyethylene centrifuge tubes (with magnetic bars) suspended over a magnetic stirrer. At the end of each time interval, the tubes were centrifuged at 1450 x g for 30 minutes in a Servall refrigerated centrifuge. The supernatant fluid was collected and concentrated by ultrafiltration to 0.5 ml and tested in a ring test for residual antibody activity. All preparations showed complete absorption of antibodies, and 25 minutes was chosen as the reaction time in subsequent experiments using a batchwise elution.

A 5 ml globulin sample was absorbed with 100 mg adsorbent for 25 minutes, centrifuged and the adsorbent washed with chilled saline until there was no detectable absorbance at 280 mμ with a Beckman DU spectrophotometer.

The adsorbent was eluted with 10 ml volumes of glycine-hydrochloric acid buffer once at pH 3.0, twice at pH 2.5 and three times at pH 2.0. Elution was done at room temperature using an International centrifuge at approximately 200 x g for 10 minutes.

The fractions were neutralized immediately with an appropriate amount of 1.0M sodium bicarbonate and dialysed at 4°C against borate-buffered saline pH 8.4. They were then concentrated by ultrafiltration to 5ml each, and their protein content determined colorimetrically according to the method of Lowry as described by Kabat and Mayer, and by their O.D. 280/260 ratios.

Protein Determination

A standard curve was prepared using a BSA solution which had been standardized by Kjeldahl procedure of nitrogen determination and estimated to contain 420 micrograms nitrogen per ml. The solution was diluted to contain from 6 to 52 micrograms nitrogen since for the colorimetric procedure it has been reported that best results are obtained in a range of 5 to 35 micrograms nitrogen (Kabat and Mayer 1961).

To 0.2 ml of each dilution was added 1 ml copper-tartrate sodium carbonate-sodium hydroxide (Appendix) solution ,

and the mixture allowed to stand at room temperature for 10 minutes. Next, 0.1 ml of Folin reagent (British Drug Houses, (Canada) Ltd.) previously titrated and diluted to 1.0 N, was added with vigourous mixing, and the mixture again allowed to stand for a minimum of 30 minutes when a blue colour developed. Colour intensity was determined by measuring the optical density at 750 mμ in a Beckman DU spectrophotometer fitted with a special sample holder and a 1 cm cell with a capacity of 60 microlitres.

Optical density readings were plotted against protein concentration.

Determination of Protein Content of Preparation Obtained by Batchwise Elution

Optical density readings were made at 280 and 260 mμ in a Zeiss spectrophotometer on the three fractions obtained by batchwise elution. With the aid of a nomograph (Warburg and Christian, 1942) the amount of protein per ml was determined.

Using the values obtained by spectrophotometry as a guide, the fractions were appropriately diluted for colorimetric determination and treated in the same way as the standard curve.

Protein content of an antibody preparation obtained by columnar operation was also determined by these methods, and the efficiency of the column and batch-wise procedures compared.

5. Determination of Non-specific Adsorption

To determine how much non-specific material was being retained by the immunoadsorbent and subsequently eluted, 5 ml normal rabbit globulin obtained by ammonium sulphate precipitation, was applied to a column containing 100 mg of conjugate, and eluted with approximately 300 ml phosphate-buffered 8M urea or glycine-hydrochloric acid buffer. The effluent was dialysed, concentrated, and its nitrogen content determined by the Kjeldahl procedure.

IV. EXPERIMENTAL RESULTS

A. Characterization of Antiserum and Globulin Preparations

Rabbits were immunized with a 1% solution of BSA for a period of three weeks, and the pooled serum submitted to immunoelectrophoresis in agar gel. The electrophoretic pattern was developed with goat anti-rabbit serum (Hyland Laboratories, Los Angeles, Calif.), and served as a reference for comparison of preparations obtained at each step in purification.

Globulin obtained by ammonium sulphate precipitation was similarly submitted to immunoelectrophoresis. A comparison of this fraction with whole antiserum is illustrated in Fig. I. There were small amounts of globulins in the beta region, but the preparation was predominantly gamma globulin. The optimum antibody ratio was determined by reaction with twofold serial dilutions of a 1% BSA solution and found to be 1:128.

Globulins precipitated by ammonium sulphate were fractionated by DEAE-cellulose chromatography. Fig. II shows the elution pattern at 257 mμ. The effluent was pooled into three fractions as indicated, concentrated by lyophilization, reconstituted, dialysed and submitted to immuno-

electrophoresis. The immunoelectrophoretic pattern (Fig.I) revealed gamma globulin in all fractions, but fractions II and III contained small amounts of alpha and beta globulins in addition to gamma globulin. The continuous elution of gamma globulin reveals the heterogeneity existing in this class of globulin.

A progressive increase in electrophoretic mobility was demonstrated in the three fractions, globulins with the slowest mobility being the first to be eluted.

B. Antibody Content of Antiserum and Globulin Fractions

a. Standardization of Antigen

A solution of BSA containing 3 mg protein per ml was chosen for standardization. Nitrogen content was determined on 1 ml volumes by the Kjeldahl procedure as outlined in Materials and Methods, Section C, Part 1. Analysis was done in sets of five samples each. The mean of the samples of the set in which the values corresponded to within 10 to 12 µg nitrogen was determined and the standard error calculated. On this basis the preparation was estimated to contain 420 ug nitrogen per ml $\pm$ 3.

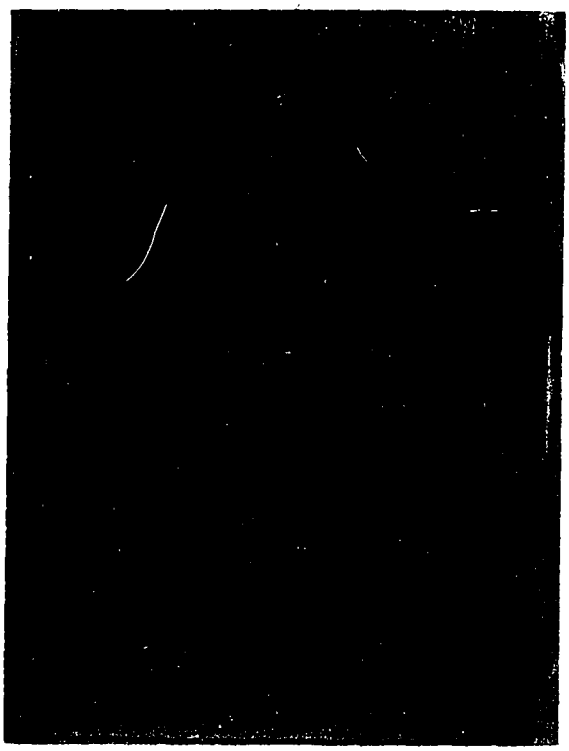


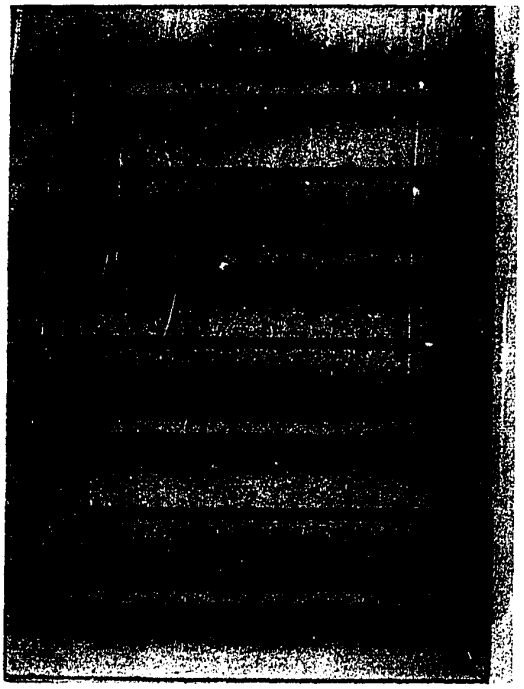
Fig. I. Immunoelectrophoresis of whole anti-BSA, $(NH_4)_2SO_4$ ppt'd globulin fraction, and globulins fractionated by DEAE-cellulose chromatography. The electrophoretic pattern was developed with goat anti-rabbit serum.

Slide 1: Upper well - Whole anti-BSA
Lower well - $(NH_4)_2SO_4$ ppt'd globulin fraction

Slide 2: Upper well - Whole anti-BSA
Lower well - DEAE-cellulose fraction I

Slide 3: Upper well - Whole anti-BSA
Lower well - DEAE-cellulose fraction II

Slide 4: Upper well - Whole anti-BSA
Lower well - DEAE-cellulose fraction III



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Slide 1: Upper well - Whole anti-BSA
Lower well - $(NH_4)_2SO_4$ ppt'd globulin fraction

Slide 2: Upper well - Whole anti-BSA
Lower well - DEAE-cellulose fraction I

Slide 3: Upper well - Whole anti-BSA
Lower well - DEAE-cellulose fraction II

Slide 4: Upper well - Whole anti-BSA
Lower well - DEAE-cellulose fraction III

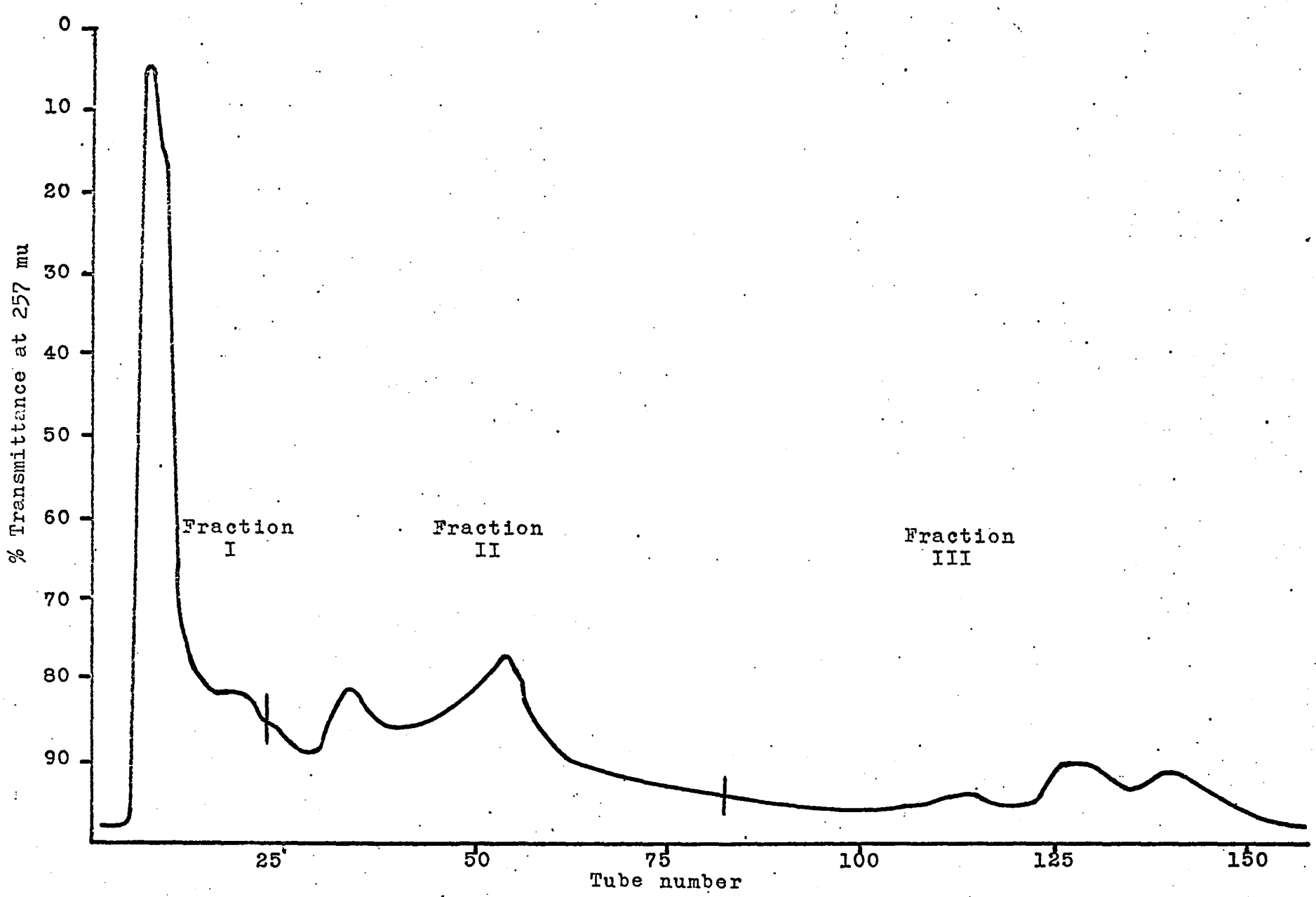


Fig.II Fractionation of $(NH_4)_2SO_4$ precipitated globulin fraction by DEAE-cellulose chromatography.

b. Quantitative Precipitation

The antibody contents of whole antiserum and globulins obtained by ammonium sulphate precipitation and DEAE-cellulose fractionation were determined by quantitative precipitation as described in Materials and Methods, Section C, Part 2.

The antigen was diluted to contain from 8 to 210 µg nitrogen per ml, and quantitative precipitation was done by reacting 1 ml of the antibody preparation with varying amounts of the antigen contained in 1 ml volumes. Total nitrogen was determined on the precipitates and antibody nitrogen was calculated as the difference between total nitrogen and antigen nitrogen precipitated at equivalence. The values obtained for the three preparations are shown in Table IA, IB and IC respectively, and are represented graphically as precipitin curves in Fig. III. Only fraction I of the DEAE-cellulose preparation was analysed. Also included in the tables are the results of tests for residual antibody or antigen in the supernatant fluid of the precipitation reactions.

As indicated in the first table whole antiserum

had approximately 0.94 mg nitrogen per ml which was specifically precipitable by antigen. After ammonium sulphate precipitation, this had declined to approximately 0.38 mg nitrogen per ml, representing a loss of 40%. This loss was due, probably, partly to the precipitation procedure and partly to lyophilization. Lyophilized globulin when reconstituted showed a substantial amount of denatured material which was usually removed by centrifugation prior to use of the globulin.

C. BSA-Cellulose Immunoadsorbent

Attempts were made to produce an immunoadsorbent according to the procedure of Weliky et al (1964) in which bovine serum albumin was coupled to CM-cellulose by means of a coupling agent, N,N-dicyclo-hexylcarbodiimide (DCC). Coupling was assumed to occur by formation of amide bonds between the amino groups of the protein and the carboxyl groups of DCC.

Cellulose, previously treated with 4N HCl, and DCC were added to a solution of 400 mg BSA in 25 ml distilled water and the mixture allowed to stand at room temperature for two days. At the end of this time the mixture was still moist. The preparation was washed as outlined in Materials

Table 1A. Determination of antibody nitrogen by addition of increasing amounts of BSA to a constant volume of rabbit anti-BSA - Whole Antiserum.

Total nitrogen - 10,680 µg/ml

Ag N added µg/ml	Total N ppt'd µg/ml	Ab N by difference µg/ml	Supernatant Tests
8.4	107	99	Excess Ab
35.0	367	332	Excess Ab
42.0	444	402	Excess Ab
46.6	509	462	Excess Ab
52.5	545	492	Excess Ab
60.0	627	567	Excess Ab
84.0	785	701	Trace excess Ab
105.0	918	813	No Ab or Ag
140.0	1080	940*	No Ab or Ag
210.0	1042	832	Excess Ag

* Antibody nitrogen at equivalence

Table 1B. Determination of antibody nitrogen by addition of increasing amounts of BSA to a constant volume of rabbit anti-BSA - Ammonium Sulpnate precipitated

Total nitrogen - 1520 µg/ml

Ag N added µg/ml	Total N ppt'd µg/ml	Ab N by difference µg/ml	Supernatant Tests
8.4	98	80	Excess Ab
42.0	335	293	No Ab or Ag
60.0	436	376*	Trace excess Ag
84.0	445	361	Excess Ag
105.0	409	304	Excess Ag
140.0	360	220	Excess Ag

* Antibody nitrogen at equivalence

Table 1C. Determination of antibody nitrogen by addition of increasing amounts of BSA to a constant volume of rabbit anti-BSA - DEAE-cellulose Fraction I**

Total nitrogen - 2236 µg/ml

Ag N added µg/ml	Total N ppt'd µg/ml	Ab N by difference µg/ml	Supernatant Tests
8.4	72	63.6	Excess Ab
21.0	164	143.0	Excess Ab
42.0	300	258.0	Excess Ab
60.0	528	468.0	No Ab or Ag
84.0	675	591.0*	No Ab or Ag
105.0	666	561.0	Trace excess Ag

* Antibody nitrogen at equivalence
** Five times concentrated

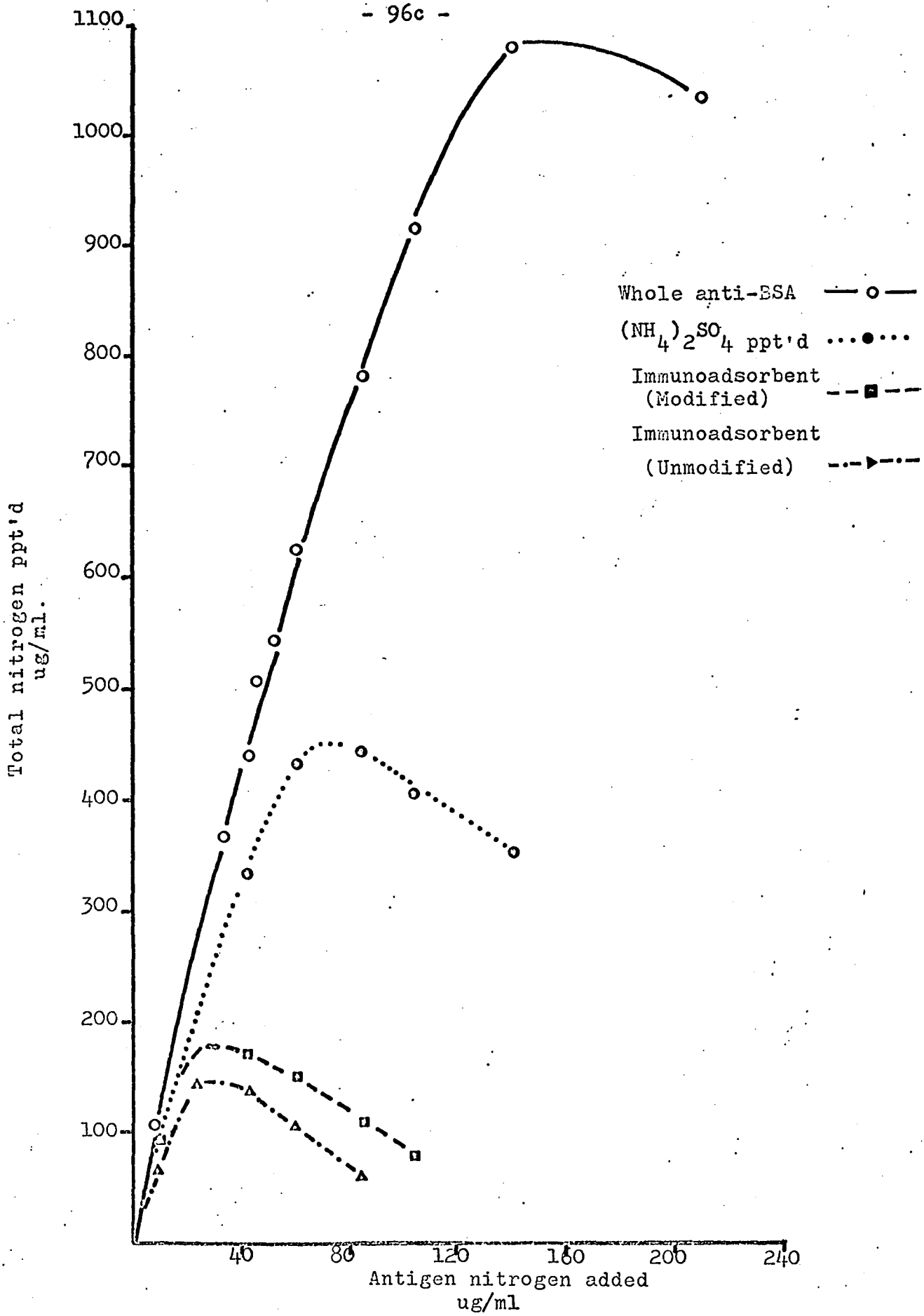


Fig. III. Precipitin curves of quantitative precipitation reaction of BSA with rabbit anti-BSA.

and Methods, Section F, and a column of the adsorbent prepared.

The amount of antigen coupled was not determined, but it was assumed that if coupling of the antigen had occurred, antibodies would be adsorbed when antiserum was passed through the column. A 2 ml. volume of antiserum containing 420 µg nitrogen per ml as determined by Kjeldahl procedure, was applied to the column and rinsed in with saline. The effluent was collected and passed through several times before the column was washed. Because the UV recorder to which the column was attached showed that a large amount of material absorbing at 257mµ had passed through, it was suspected that antibodies were not being bound. The effluent was, therefore, collected, concentrated and tested in double diffusion for antibody activity. Strong lines of precipitation developed with the highest concentration of antigen used (1:10), indicating that antibodies were recovered almost quantitatively in the effluent.

The column was washed with saline until no protein could be detected in the wash, then eluted with 1% sodium chloride pH 2.3. In all instances a component which gave a green colour when acid was applied to the column was eluted.

The material was concentrated by ultrafiltration to the original volume and tested for antibody activity in double diffusion with twofold serial dilutions of a 1% BSA solution. The preparation showed no antibody activity and it was assumed that the material eluted was some minor serum component which bound non-specifically to the cellulose.

In the details of the procedure, no optimun pH for the reaction was given. In a report published later (Weliky and Weetall, 1965) it was mentioned that best results were obtained when a pH of 3.5 to 5.0 was used, and when the water in the reaction mixture was kept to a minimum. Therefore, experiments were repeated in which the volume of water used for preparing the protein solution was varied, and the pH of the reaction mixture was adjusted to approximately 4.0. The results were similar to those obtained initially, and it was presumed that the antigen was not being coupled to the cellulose. After several unsuccessful attempts to reproduce the method, it was decided to discontinue the investigation in favour of another procedure.

D. Purification of Antibodies by EMA-BSA Immunoadsorbent

1. Characterization of Antibodies

a. Double Diffusion in Agar Gel

Antibodies were purified by applying a sample of globulin prepared by ammonium sulphate precipitation, to an EMA-BSA immuno-adsorbent and eluted with 0.35M glycine-HCl buffer at pH 3.0,2.5 and 2.0, or with phosphate buffered 8M urea pH 7.4. The effluent was dialysed against 0.3M sodium chloride buffered with phosphate at pH 7.4, concentrated to the original sample volume, and tested for antibody activity in double diffusion with twofold serial dilutions of a 1% BSA solution.

In the first series of experiments whole antiserum was used instead of a globulin fraction. A 2 ml volume was applied to a column containing 100 mg immunoadsorbent. Antibodies were eluted with glycine-HCl buffer, the effluent neutralized with 0.2 N sodium hydroxide, and concentrated by lyophilization. The lyophilized material was reconstituted with 2 ml distilled water, dialysed against borate-buffered saline and tested in double diffusion for antibody activity. There was little or no activity, despite the fact that their ultraviolet absorbance at 257 mμ during elution,

indicated a high concentration of protein. In some cases a small amount of activity could be demonstrated when the antibody wells were filled several times, but even them, the highest dilutions of antigen were in excess as indicated by the closeness of the precipitation lines to the antibody wells.

It was suggested that antibodies might have been inactivated by lyophilization and concentration by solid sucrose was tried. Protein eluted from the column was first dialysed for three hours, then placed in dialysing tubing. The dialysing bags were covered with sucrose. Concentration was rapid in the first few hours, but gradually the sugar diffused through the membrane as the water moved out, until the sugar reached such a high concentration within the bag that little or no water passed out. The bags were transferred as soon as the sugar became wet, but diffusion was still negligible. Short periods of dialysis were tried without significant improvement. The preparations could, therefore, not be reduced to their original volumes, and in no instance could antibody activity be demonstrated.

Polyethylene glycol (Carbowax), M.W. 20,000 was tried without success. Experiments were set up as outlined

in Materials and Methods Section G, Part 1. Concentration was rapid, but visible turbidity developed in the preparations, which were filtered before testing them for activity. No activity could be demonstrated in the filtrates. Polyethylene glycol is known to aggregate gamma globulin, and it has been reported (Kabat and Mayer, 1961), that some preparations contain small molecular weight polymers which can diffuse through dialysis tubing. It is possible that this might have happened in these experiments.

Preparations concentrated by ultrafiltration retained much of their activity as demonstrated in Fig. IV. Purified preparations obtained by urea elution gave strong lines of precipitation with varying concentrations of antigen in double diffusion. Optimum reaction occurred with antigen diluted 1:80 as evidenced by the position and sharpness of the line. Precipitin lines were also obtained with the three fractions eluted by glycine-HCl buffer, but the preparations had to be concentrated several folds to demonstrate activity. Whole antiserum and ammonium sulphate precipitated fraction were included for comparison. The protein material which passed through the column without binding to the antigen, and the subsequent wash were concentrated and tested also

for residual antibody activity. Strong lines of precipitation developed indicating that not all the antibodies applied to the column were bound, or that antibodies were released during the washing procedure.

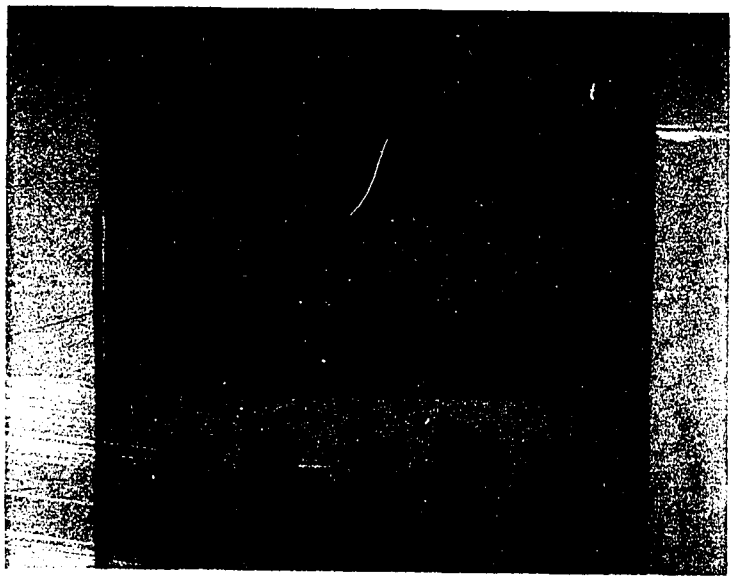


Fig. IV. Double diffusion in agar gel with twofold serial dilutions of Ag from 1:10 to 1:1280 reading L to R. Ag was put in the centre wells (unlabelled). Both Ag and Ab wells were filled three times. Purified Abs used in slides 10 & 11 were prepared by urea elution. Abs used in slide 12 were prepared by elution with glycine-HCl buffer pH 3.0, 2.5 and 2.0, and were concentrated several folds.

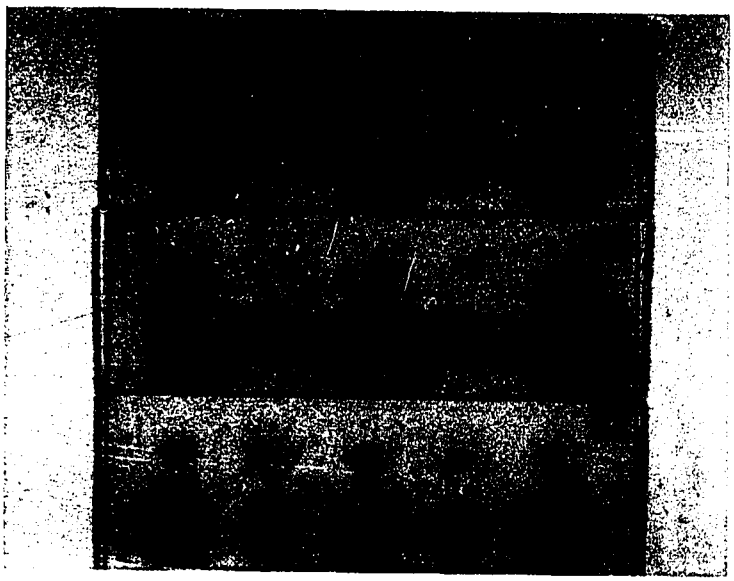
Slides 10 & 11 : a = Whole antiserum

b = $(NH_4)_2SO_4$ ppt'd globulin fraction

c = EMA purified Abs ; d = Wash

Slide 12 : a = Whole antiserum ; b = pH 3.0

c = pH 2.5 d = pH 2.0



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b = $(NH_4)_2SO_4$ ppt'd globulin fraction

c = EMA purified Abs ; d = Wash

Slide 12 : a = Whole antiserum ; b = pH 3.0

c = pH 2.5 d = pH 2.0

b. Immunoelectrophoresis

Immunoelectrophoresis in agar was used to characterize all preparations. Electrophoresis was performed in a 1% Ionagar gel for 90 minutes and developed with goat anti-rabbit serum diluted 1:2.

Since rabbit antibodies are found primarily among the gamma globulins, precipitation lines appearing in any other region and not showing continuity with the gamma line were interpreted as impurities.

Immunoelectrophoresis done on preparations obtained in the first series of experiments in which whole antiserum was applied to the immunoadsorbent and the effluent concentrated by lyophilization showed lines of precipitation not only in the gamma region but in the alpha, beta and albumin regions as well. The albumin line was particularly strong. It was assumed that the results were probably due to inadequate washing of the immunoadsorbent-antibody complex. A repeat of the experiment in which washing was done for a much longer time gave similar results. Other experiments were done to rule out technical errors which might have occurred in filling the antigen wells when preparing for electrophoresis. Again the results were similar.

Since molecules of the size of globulins are not expected to penetrate Sephadex G-25 (used to disperse the immunoadsorbent), it was unlikely that the contaminating proteins were released from the gel beads during elution. Moreover, the length of the washing time and the volume of wash fluid should have been adequate for a column of the size used (45 x 1.5 cm - I.D) to allow for removal of any proteins which might have penetrated the gel. It was, therefore, concluded that proteins were being bound non-specifically. This was confirmed by the authors (Centeno and Sehon) (Personal communication). Thereafter, all experiments were performed with a 33% ammonium sulphate precipitated fraction of serum. It should be mentioned that these impure preparations gave good lines of precipitation with antigen in double diffusion. It is known that non-antibody serum proteins help to increase precipitation.

When experiments were done using a globulin fraction and the effluent from the column was concentrated by sucrose or polyethylene glycol (PEG), no lines of precipitation were detected on immunoelectrophoresis. The sugar concentrated preparations were assumed to be too dilute to allow detection by this method of the amount of globulins present. With the

PEG preparations, globulins had probably been aggregated and removed when the preparations were filtered.

Immunoelectrophoresis patterns of purified preparations obtained by elution with urea and glycine-HCl buffer, and concentrated by ultrafiltration are shown in Fig. V. Whole antiserum or an ammonium sulphate precipitated fraction were included as references. Lines of precipitation appeared in the gamma region in all cases. Since the serum was obtained three weeks after primary stimulation and the IgM globulins have a short half-life none or only a very small amount of this class of globulins would be expected to be in these preparations. The lines, therefore, were most likely produced by antibodies of the IgG type. Results of ultracentrifugal analysis tend to support this view.

Urea eluted preparations showed an additional small line over the antigen well which was not seen in the acid eluted preparations. The line has not been identified.

Some amount of heterogeneity in electrophoretic mobility has been revealed in the acid eluted preparations. IgG (7S) globulins have electrophoretic mobilities extending from the slow gamma through the beta region and into the

alpha region. With the scheme of acid elution used, antibodies having the slowest mobility were eluted first (pH 3.0). The faster moving molecules could be eluted only at a lower pH (2.5 and 2.0). Since the pattern of elution is assumed to reflect the relative binding strengths of the antibodies, one could possibly make an association between charge and affinity to antigen. In a recent study Carter and Harris (1967) isolated rabbit non-precipitating antibodies and showed that they had a faster electrophoretic mobility than the precipitating antibodies resulting from a greater net negative charge. In their study antibodies of the non-precipitating type displayed properties similar to IgA globulins. They suggested that in view of reports (Onoue et al, 1964, 1966) that IgA globulins were associated with both a 7S and 9S component, that the non-precipitating antibodies of rabbits are IgA globulins. If these observations are correct, then perhaps the preparations obtained by acid elution do contain a high proportion of non-precipitating antibodies which might account for the relatively weak reactions of these fractions in double diffusion.

The diffuseness of the lines is due probably to the concentration of antibodies in the preparations or to

heterogeneity of the antibody molecules.

The pattern of urea eluted globulins is somewhat different, and resembles more closely that of the whole antiserum. Only one peak of material absorbing at 257 mμ was eluted by urea, and it is possible that urea elutes a different class of antibodies. Provided that the eluting agents did not alter the antibody molecules to cause changes in their electrophoretic mobilities, it is apparent that urea elutes much less of the faster migrating antibody molecules. The fact that better lines of precipitation were obtained in double diffusion with this preparation might suggest the absence of inhibition by non-precipitating antibodies.

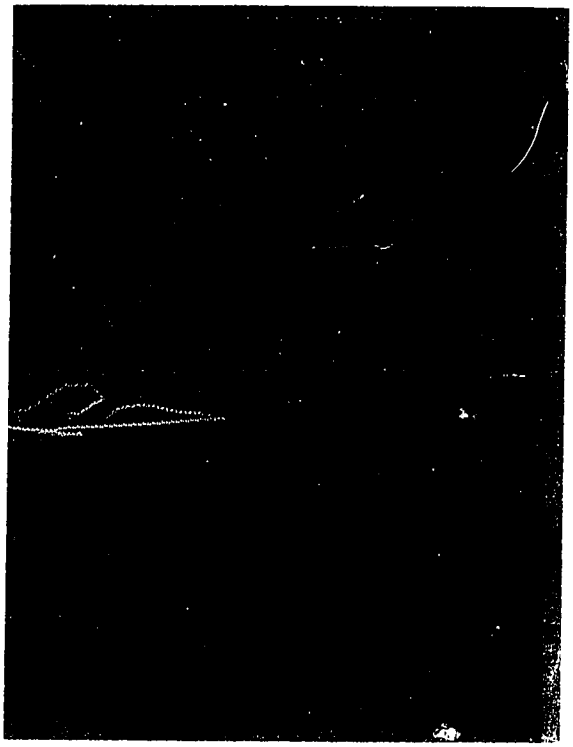
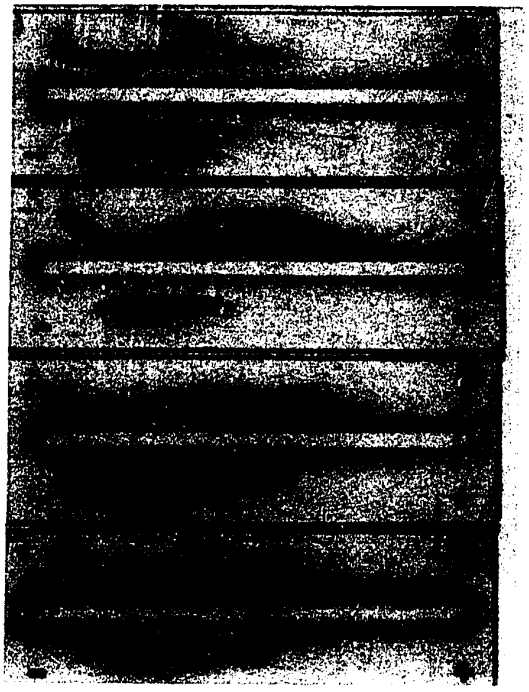


Fig. V. Immunoelectrophoresis of antibodies purified by EMA immunoadsorbent. Slide 5 : Abs eluted by phosphate-buffered 8 M urea pH 7.4. Upper well contained $(NH_4)_2SO_4$ ppt'd globulin fraction.
Slides 6, 7, 8: Abs eluted by glycine-HCl buffer pH 3.0, (6); 2.5 (7); and 2.0 (8). Upper wells contained whole anti-BSA. All troughs contained goat anti-rabbit serum.



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Slides 6, 7, 8: Abs eluted by glycine-HCl buffer pH 3.0, (6); 2.5 (7); and 2.0 (8). Upper wells contained whole anti-BSA. All troughs contained goat anti-rabbit serum.

c. Disc Electrophoresis

Since separation of molecules in disc electrophoresis is a function of "molecular filtration" and electrophoretic properties, it produces a degree of resolution that cannot be obtained by other electrophoretic procedures. For this reason it was chosen as an additional method to determine the purity of our preparations. Like the other methods, it cannot differentiate between antibody globulins and 'normal' globulins.

Disc electrophoresis was performed in polyacrilamide gel using Tris-glycine buffer pH 8.3. Whole antiserum and an ammonium sulphate precipitated globulin fraction were included for comparison. The results are shown in Fig. VI. Gamma globulin appeared as a diffuse band at the uppermost end of the separation gel. Except for one or possibly two narrow bands (not clearly shown in 3,4,5 of Fig. VI) which appeared in all cases at the juncture of the spacer and separation gels, only gamma globulin could be detected in the preparations purified by the immunoadsorbent. These bands might be either lipoproteins or glycoproteins, or perhaps small aggregates, although the latter should be retained

in the sample gel. These materials were not detected by immunoelectrophoresis possibly because of the small amounts present. Little differences in electrophoretic mobilities were detected by this method. The ammonium sulphate fraction showed an additional band migrating faster than the gamma globulin and was absent from the purified samples.

Since globulins of the 19S type would be retained at the lower end of the sample gel, most of the globulins in the diffuse band are probably of the 7S type.

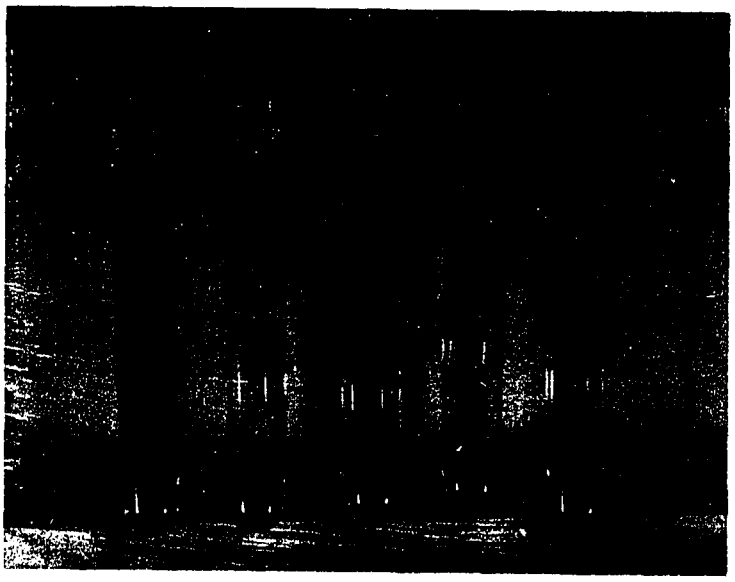
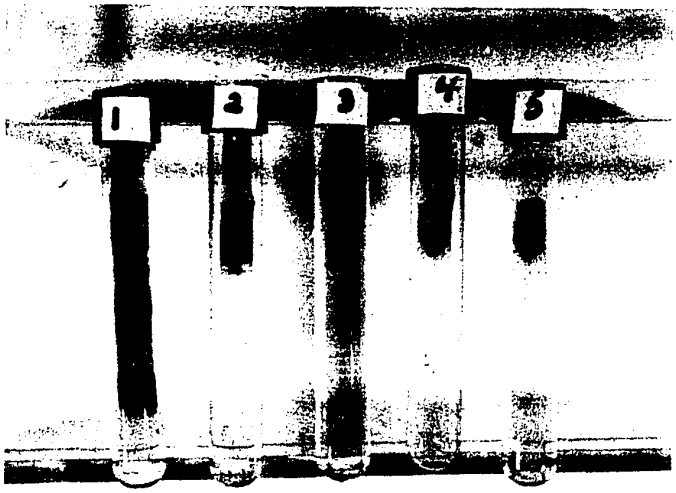


Fig. VI. Disc electrophoresis of whole anti-BSA (1), $(NH_4)_2SO_4$ precipitated globulin fraction (2), and antibodies purified by immunoadsorbent (3,4,5). Antibodies were eluted with glycine-HCl buffer pH 3.0 (3), pH 2.5 (4) and pH 2.0 (5). Electrophoresis was done in 7% polyacrilamide gel using tris-glycine buffer pH 8.3.



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d. Ultracentrifugation

Fractions of antibodies obtained by elution with glycine-HCl buffer at pH 3.0 and 2.5 were submitted to ultracentrifugation, and their sedimentation coefficients calculated. The sedimentation co-efficient for fraction I was estimated to be 7.328 S while that of fraction II was slightly higher being 7.416 S. The sedimentation pattern showed a single sharp peak for each fraction, and no significant differences were apparent from the photographs.

The values are somewhat higher than those reported for rabbit antibodies. Normal rabbit globulin has been calculated to have a sedimentation coefficient of 7.05S (Phelps and Putnam, 1960), and 6.5 S for antibodies to ovalbumin (Kabat and Mayer, 1961). Small amounts of aggregats could be expected to be present since the suspending buffer was of a low ionic strength, and we had observed the occurrence of aggregation previously, when the preparations were allowed to stand at $4^{o}C$. The fact that the photographs gave no indication of the presence of aggregates might have been due to the globulin concentration, the degree of sensitivity of the procedure, or both, and does not prove their absence. However, if these were aggregates, the sedimentation co-

efficients would probably be much higher.

Values reported for sedimentation coefficients are known to vary with the method of preparation of the globulin, and it is possible that this might account for the difference between the values obtained and those reported. The possibility of technical errors cannot be ruled out since calculations were made on the basis of a 1% solution and our preparations contained only 5 mg per ml protein.

2. Investigation of the Batchwise Elution Procedure and Comparison with Column Elution Procedure

Differences in weights of immunoadsorbent and Sephadex made it difficult to prepare large columns in which the adsorbent would be well dispersed. Slow packing resulted in a band of adsorbent on the top of the column, and this greatly reduced flow rates. As will be pointed out later high flow rates are needed to get good yields. With a batchwise procedure this difficulty would be avoided, and so it was investigated with a view to using it if results comparable to, or better than those of the column procedure could be obtained.

Optimal conditions for the procedure were determined by preliminary experiments (Materials and Methods,

Section G, Part 2c). In these experiments it was found that although the immunoadsorbent could be centrifuged at a high speed during the washing procedure following reaction with antibodies, a much lower speed had to be used during elution with acid (batchwise elution with urea was not done) in order to resuspend the immunoadsorbent-antibody complex. This might have resulted from conformational changes caused by the acid. A reaction time of 25 minutes was found to be adequate for complete adsorption of antibodies.

For experiments designed to investigate the possible use of the procedure, 5 ml globulin was reacted with 100 mg immunoadsorbent for 25 minutes, and antibodies eluted with glycine-HCl buffer. Protein determination was done colorimetrically according to the method of Lowry and calculated by comparison with a standard curve prepared with BSA. In order to determine if and how the protein fractions should be diluted to be within the sensitive range of the colorimetric test O.D. 280/260 ratios were taken and protein determined by the procedure of Warburg and Christian (1942). Protein content of antibodies obtained by column elution was determined in a similar manner. Table IV gives the amount of protein eluted by bathwise and column procedures. Values

obtained by the O.D. 280/260 ratios are included for comparison.

The figures show that the amount of protein eluted by the batchwise procedure was only about one-half that eluted using column elution. Mougal et al (1963) also obtained lower yields from batchwise elution. The procedure was therefore considered less efficient. Moreover, it was more time consuming, less convenient, and was accompanied with a greater risk of losing antibodies and of contaminating the antibody preparation with antigen. It, however, had the advantage over column elution that antibodies could be recovered in a more concentrated form.

3. Yield and Purity of Antibodies

In the experiments preceding those done for quantitative determinations, antibodies were eluted by glycine-HCl buffer and dialysed against borate-buffered saline pH 8.4. However, an excessive amount of precipitation developed during dialysis, due possibly to aggregation or to precipitation with contaminating antigen. Phosphate buffered 8 M urea was suggested as an alternative eluting agent (Sehon, 1967) and it was decided to try this. At that time no quantitative determinations had been done on antibodies

Table IV. Protein Determination by the Lowry Method and by the O.D. 280:260 ratio

Method of Elution	Lowry µg protein/ml	O.D. 280:260 µg protein/ml
Column:		
Fraction I	1600	1700
Fraction II	3487	3000*
Fraction III	13	150
Average :	1700	1600*
Batchwise:		
Fraction I	0	0
Fraction II	625	780
Fraction III	495	670
Average:	560	725

* Approximation

eluted by acid. Subsequently, experiments were done with this in mind, but due to inadequate binding of antibodies by the new preparations of immunoadsorbent, the concentrations of antibodies recovered were not sufficiently high to give reasonable amounts of precipitate for a quantitative precipitation test. Therefore, yield and purity are reported for preparations obtained by urea elution only. Typical patterns of elution by glycine-HCl buffer and by urea are shown in Fig.VII.

The capacity of the columns used in these experiments was not high enough to give sufficient antibodies in one run for a complete quantitative test, and it was decided to pool material from two runs on the same column if reproducible results could be obtained on reuse of columns. A preliminary experiment showed that the elution pattern obtained from a column being used for the second time was similar to that obtained from its first use under similar conditions. All quantitative work was, therefore, done on pools of antibodies, obtained from a preparation of immunoadsorbent used up to four times.

When antibodies were to be eluted by urea, the packed column was washed with about 200 ml 8 M urea in phos-

phate buffer pH 7.4 followed by extensive washing with phosphate buffer to remove all the urea. After such a treatment the immunoadsorbent has a gel-like consistency due to swelling. When a globulin sample was applied to such a column a significant amount of protein passed through unbound. A concentrate of this effluent, when tested in double diffusion gave strong lines of precipitation indicating the presence of much antibody. In previous experiments, when a similar sample from the same batch of globulin was applied to a column of immunoadsorbent in its acid form (Materials and Method, Section G, Part 1) only a small amount of protein passed through unbound. Since the only difference between the two preparations was the method of treatment, it was decided to see if the same immunoadsorbent, when converted to the acid form would give results similar to those of earlier experiments. When this was done, the U.V. recorder showed that only a small amount of protein was not bound. This was taken as evidence that the differences in activities were due to the treatment.

Antibodies were eluted with urea from immunoadsorbents in both forms, the effluents were dialysed, concentrated to their original volumes and analysed for their

nitrogen content. Table V shows the results obtained. Precipitin curves are included in Fig. IV.

Yield was calculated as the percentage of precipitable antibodies recovered from the sample applied to the immunoadsorbent. On this basis, the figure obtained for the unmodified method was 32% and for the modified method 42%. The amount of antibodies recovered by the modified method was therefore 10% higher than the original method, and the degree of purity was the same for both methods. Purity was 50% and was calculated as the proportion of the total nitrogen (in the recovered material) which was precipitable. It is possible that at alkaline pH when the immunoadsorbent is a gel, the conformation of the complex is such that some antigenic sites are concealed. Acid probably causes a change in conformation, antigenic sites become more accessible and consequently more antibody is bound.

Precipitable antibody nitrogen and total nitrogen were also determined on fraction I of the DEAE-cellulose preparation for comparison of the degree of purity obtained by the use of a non-specific method of antibody purification with that obtained from the immunoadsorbent. The results are shown in Table 1C. The purity of this preparation was 25%.

Table Va. Determination of antibody nitrogen by addition of increasing amounts of BSA to a constant volume of rabbit anti-BSA - Antibodies purified by immuno-adsorbent-unmodified method.

Total nitrogen - 244 µg / ml

Ag N added µg/ml	Total N ppt'd µg/ml	Ab N by difference µg/ml	Supernatant Tests
8.4	67	59	No excess Ab
21.0	143	122*	Trace Ag excess
42.0	140	98	Excess Ag
60.0	105	45	Excess Ag

* Antibody nitrogen at equivalence

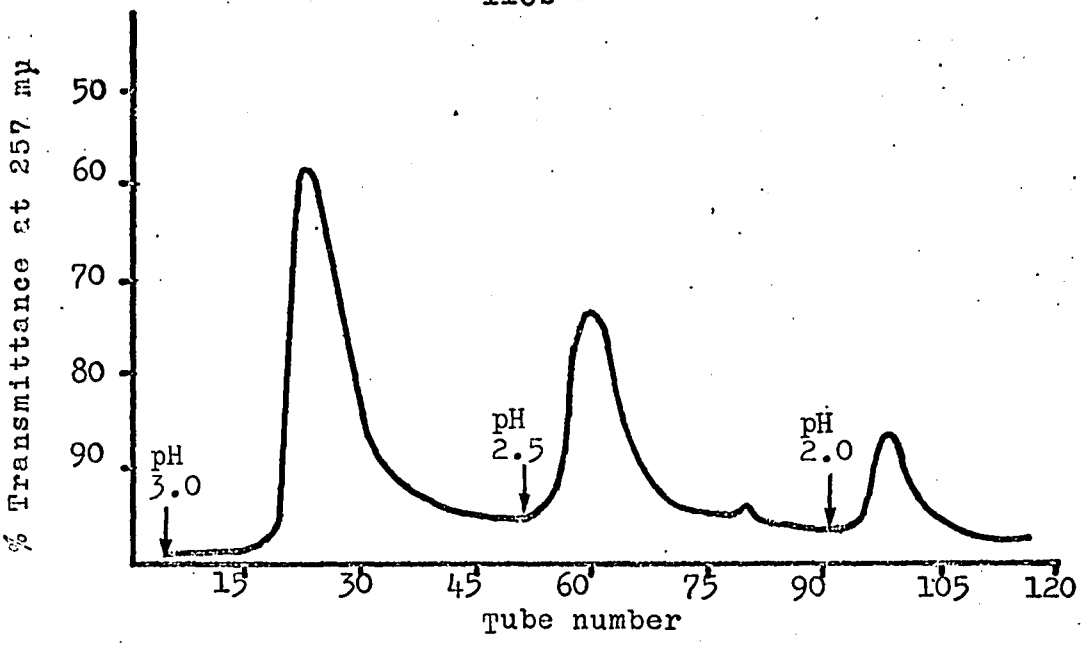
Table Vb. Determination of antibody nitrogen by addition of increasing amounts of BSA to a constant volume of rabbit anti-BSA - Antibodies purified by immuno-adsorbent-modified method.

Total nitrogen - 320 µg / ml

Ag N added µg/ml	Total N ppt'd µg/ml	Ab N by difference µg/Ml	Supernatant Tests
8.4	69	60	No excess Ab
21.0	181*	160*	-
42.0	170	128	Excess Ag
60.0	150	90	Excess Ag
84.0	112	28	Excess Ag

* Antibody nitrogen at equivalence calculated by extrapolation from precipitin curve Fig. IV.

A.



B.

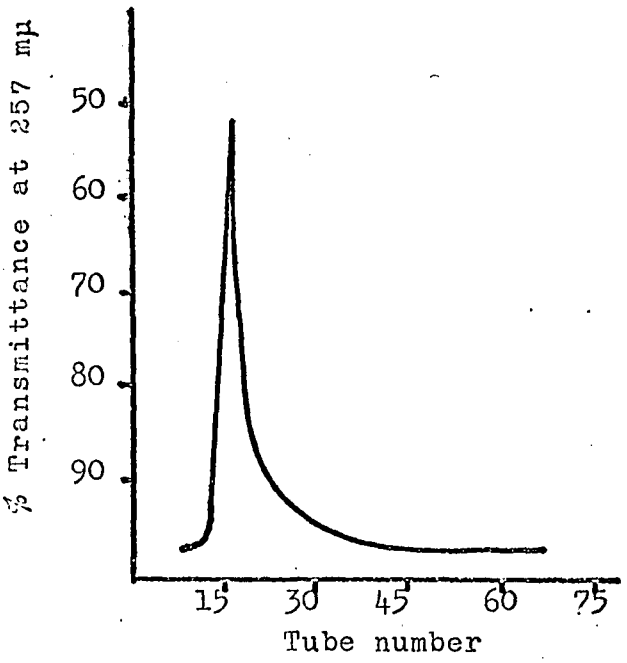


Fig. VII Elution pattern of antibodies purified by EMA immunoadsorbent. A : Elution with glycine-HCl buffer pH 3.0, 2.5 and 2.0. B : Elution with phosphate buffered 8 M urea pH 7.4.

4. Non-Specific Adsorption

Because of the relatively low degree of purity obtained compared to that reported by the authors (Centeno and Sehon,1966; Sehon, 1967) for antibodies purified with this immunoadsorbent, we decided to investigate whether it might have been due to non-specific adsorption. For this, normal rabbit globulin prepared by ammonium sulphate precipitation, was passed through a column of the adsorbent. The results of these experiments were suggestive but not conclusive.

In the first of these experiments, the absorbance pattern revealed that a substantial amount of protein was being retained. This was subsequently eluted by urea. The nitrogen content of the eluted material was approximately 660 ug per ml. It showed no antibody activity when tested in double diffusion.

The experiment was repeated with a globulin sample from a different rabbit, and the columns were eluted with both urea and glycine-HCl buffer. Only a trace amount of protein was eluted. The results of the latter experiments suggest that the high amount of adsorption observed in the first experiment was probably not due to a property of the

immunoadsorbent, but rather to the globulin preparation. It is possible that the rabbit from which the globulin was prepared, might, at some time, have been immunized either with BSA or some other material which cross reacts with it.

On the other hand, the fact that another immunoadsorbent prepared at the same time displayed poor binding capacity, it can be assumed that for the same reason (see Discussion) the adsorbent used in these latter experiments was also not binding. It is, therefore, not known whether a well functioning adsorbent would behave in a similar manner toward non-specific globulins. In this respect it is interesting to note that in earlier experiments when whole antiserum was used instead of a globulin fraction, proteins other than specific globulins were adsorbed and eluted with glycine-HCl buffer (Results, Section D, Part 1b). The question of non-specific adsorption by the immunoadsorbent, therefore, requires further investigation.

DISCUSSION

A method of purification was desired which would give large amounts of antibodies essentially free of other serum proteins and of globulins which were not specific for the antigen of choice. Several methods able to meet these requirements have been reported (Isliker, 1957, Weliky et al, 1964, Sehon, 1967). Immunoadsorbents prepared by chemically attaching soluble antigens to insoluble carriers seemed to offer the most advantages and therefore this procedure was chosen for investigation. A number of the methods developed, however, are tedious and complicated and we preferred to use one which was fairly simple, yet satisfactory. The two methods chosen seemed easy enough, and were reported to have given good results.

In the method of Weliky and his co-workers, BSA was attached to CM-cellulose by a coupling agent DCC. However, our attemps to produce a similar adsorbent were unsuccessful. A possible cause of failure might reside in the cellulose itself. The activity of cellulose is greatly influenced by the type of treatment it has undergone during processing. Thus, the authors found that with a new batch

of cellulose in which changes in processing were made, satisfactory coupling was obtained only if the cellulose had been converted to its acid form by treatment with 4N HCl.

The cellulose used in this study was obtained from a different manufacturer, and nothing is known of the manner in which it has been processed. Nevertheless, it was given the acid treatment as recommended. The length of time required for conversion was not known, but it was assumed that stirring for a few minutes in an excess of acid would be adequate. In a recent report (Campbell and Weliky, 1967) one-half to one hour was suggested by the authors. On one occasion the resin was left in acid overnight, so it seems that conversion should have occurred.

Assuming that the cellulose was in a reactive state, then failure of the adsorbent to remove antibodies from solution may have resulted from the fact that either antigen was not coupled, or that antigen was coupled but for some reason failed to bind antibodies. One possible explanation for the latter suggestion is the fact that in coupling reactions some antigenic determinants might be involved so that some or all the antigenic properties of

the molecule are lost.

Although one cannot completely rule out the latter possibility, we suggest that no antigen was coupled to the cellulose. The extent to which antigen was coupled, if at all, could have been determined by the use of isotopically labelled antigen, but this was not done, so that we have no experimental evidence to support our view.

In their recent report, the authors stated that the coupling reaction occurred to a greater extent in water at pH 3.0 to 5.0 than in tetrahydrofuran. In some cases water soluble diimides were used, and were added directly to the reaction mixture. If water insoluble DCC were used as in our studies, it was dissolved in a volume of THF amounting to 10-20% of the water used. In our experiments the ratio of THF to water was 1:2 as outlined in the procedure. From the report it seems that the amount of water in the reaction mixture is a critical factor. After standing at toom temperature for two days, the preparation should still flow if the flask were tilted. It was suggested that a dry preparation should be discarded. In the procedure used, the amount of water which should be used for preparing

the protein solution was not given, however, in most cases, our preparations were still moist after two days.

The extent to which any of these factors may have contributed to the results of the experiment is not known, and we cannot explain why even a small amount of antigen should not have been coupled since all the details of the procedure and the recommended modifications were followed closely.

There was some measure of success with the method of Centeno and Sehon, although the results were not as satisfactory as we would have liked them to be.

One property of this immunoadsorbent which makes it unsatisfactory for preparative work is its affinity for albumin. It was found that a significant amount of albumin binds to the adsorbent and so, of necessity, the starting material has to be a globulin fraction. From quantitative data it can be seen that when globulins are prepared by ammonium sulphate precipitation about 40% of the antibodies in the antiserum is lost, so that one starts with only 60%. This means that even with a high recovery from the immuno-adsorbent large quantities of antiserum would be required

to prepare any appreciable amount of purified antibodies.

The yield of antibodies from the EMA-BSA adsorbent was only 42% and this further reduced the efficiency of the overall procedure. Yields of this order have been reported for polyaminostyrene (Gynnes and Sehon, 1960), and cellulose (Weliky et al, 1964). With these immunoadsorbents, the starting material was whole antiserum, and these figures represent overall yield. In our system, overall yield would be about 14%.

One factor which contributed to the low yield was incomplete adsorption of antibodies. When antibodies were to be eluted with urea, the adsorbent was maintained in an alkaline form, and it was observed that a large amount of unbound material absorbing at 257mμ passed through the column. When this material was concentrated and tested in gel diffusion for antibody activity, strong precipitation lines developed. If, however, the adsorbent was converted to its acid form as outlined in Materials and Methods, very little absorbing material passed through and the yield of antibodies was increased by 10%. One could speculate that in the alkaline form when the conjugate is swollen, some of the antigenic

sites are blocked. Yield was calculated as the percentage of precipitable antibodies recovered from the amount applied to the column and did not take into account that amount which had not been adsorbed. Had calculations been made on the basis of the amount adsorbed, yield would have been higher and would perhaps be a more accurate index of the efficiency of the adsorbent.

A portion of antibodies not recovered could possibly be accounted for by incomplete elution from the adsorbent. In no instance has there been a report of complete recovery of antibodies, and it has been suggested that a small amount of antibodies always remains adsorbed to an immunoadsorbent. These antibodies presumably represent molecules having a high affinity for the antigen. Our findings seem to support this view. We found, in our system, that the ease with which antibodies were eluted and the extent of recovery of bound antibodies were influenced by the length of time antibodies remained complexed with antigen. Antibodies which remained complexed for more than two and one-half hours were more difficult to elute and this was more pronounced with those antibodies which were eluted at pH 2.0

and 2.5, and presumed to represent antibodies of high affinity. If an adequate flow rate was achieved, antibodies eluted after two hours came off in sharp peaks as compared to a flat, spreading pattern at three and one-half to four hours. On occasions there was a complete absence of the third and sometimes second peaks.

A similar observation was reported by Campbell, Luescher and Lerman, 1951) for cellulose immunoadsorbents. Isliker (1957) suggests that only that portion of antibodies which is combined with antigen in the last stages of adsorption and whose attachment is less firm due to steric hindrance, might be recovered. In one of our experiments in which antibodies were complexed for about six hours a much lower than usual recovery was obtained. In all previous experiments there was evidence for complete absorption of antibodies, and so we had reason to believe that the antibodies had not been eluted. It was reasoned that it might be possible to determine this if the antibodies could be removed by some other eluting agent, even if antibody activity were destroyed. Weliky et al (1964) had reported that anti-dinitrophenyl (DNP) antibodies which could not be

eluted from a DNP-cellulose adsorbent at pH 2.3, were removable with 0.1N sodium hydroxide pH 12.0. Antibody activity, however, was lost. When our adsorbent was treated with 0.1N sodium hydroxide a substantial amount of protein material as measured by the Biuret reagent, was recovered. It had no antibody activity but at least part was suspected to be antibody protein. Similar treatment of the EMA-BSA conjugate alone did not release a measurable amount of protein so that the protein in the test sample was probably not due to dissolution of the antigen. Furthermore, the appearance of the conjugate after this treatment and after subsequent treatment with acid, was similar to that observed under normal conditions of use. We concluded then, that for best results with this system the time between loading and eluting should not exceed three hours.

It is tempting to suggest that part of the loss of antibodies was due to the conditions of elution. It has been documented that most eluting agents which will dissociate antibodies with high efficiency will also destroy antibody activity. The use of acid has been criticized for this reason. We have no quantitative data for comparison, but by spectrophotometric scanning we observed that acid released

a greater amount of protein than did urea. However, frequently a significant amount of precipitate formed during dialysis when the antibodies were dissociated by acid. Under normal conditions, globulins have a tendency to aggregate, and it is possible that in a purified state when the physical environment has been greatly altered this tendency may be more pronounced.

The possibility that the precipitate seen may not have been due to denaturation but to formation of antigen-antibody complexes due to the presence of contaminating antigen cannot be dismissed. There are numerous reports of this occurrence. Although this happens more frequently in methods in which insoluble precipitates were dissociated, the authors of the EMA method expressed the view that under conditions of low antigen concentration the conjugate might not be extensively crosslinked and would have a tendency to become soluble. It was found that dialysing the antibody preparations against a buffered 0.3 to 0.4M sodium chloride solution would substantially reduce the amount of precipitate formed. This suggested that aggregation might have been the principal cause of precipitation. A high concentration of salt, however,

would also inhibit lattice formation of any soluble complexes that might have been present and add impurity to the preparation.

Yields could also be reduced if in quantitative tests precipitation of antibodies was incomplete. This could happen if there were a high concentration of non-precipitating antibodies, or if the treatment altered the combining sites so that they could no longer be precipitated. Immunoadsorbents are capable of removing non-precipitating antibodies from solution since the isolation of antibodies by this technique does not depend on precipitation. In a preparation containing precipitating and non-precipitating antibodies, the latter could combine with antigen to form soluble complexes and block the reaction of antigen with the precipitating antibodies. If this happened, one would expect that either no zone of equivalence would be reached, or the zone of equivalence would be at a high concentration of antigen. The results of tests on the supernatant fluids from quantitative precipitation gave no indication that precipitation was inhibited by non-precipitating antibodies. Moreover, Carter and Harris (1967) showed that rabbit non-precipitating

antibodies could largely be coprecipitated with antigen when mixed with precipitating antibodies.

Precipitation of a very small amount of antibodies may have been inhibited by the concentration of salt in the preparation. Heidelberger et al (1936a) showed that at high salt concentrations precipitation of rabbit antibodies was inhibited. The concentration of salt used in these experiments was lower than those which were reported to cause inhibition. When the preparation was dialysed against 0.15M sodium chloride before use, it was found that there was an increase at equivalence of about 20% over that obtained at the higher salt concentration. This represents an increase in yield of only 5%, so that it is not likely that the salt by itself could have accounted for the proportion of antibodies not recovered.

The purity of our preparation was far below that reported for this method. There are numerous reports of immunoadsorbents giving preparations of purity approaching 100%. Our figure of 50% is, therefore, well below average.

Some of the factors that might have accounted for the low yield could also have affected the purity. Non-

precipitating antibodies for example would add to the total nitrogen content but would not have been included in the yield which was based on precipitable antibodies. The same would be true of any antibodies which might have lost their ability to precipitate with antigen. Marrack and Orlans (1954) have shown that acetylated antibodies lose their ability to precipitate with their homologous antigens but were still able to bind haptens. This was confirmed by Nisonoff and Pressman (1958) who suggested that the effect was probably due to the attachment of the acetyl group to a portion of the antigen combining site. If this is so, then it is conceivable that any treatment which might cause alteration in the combining site might be expected to interfere with the precipitability of the antibody.

As suggested previously, dissociation of antigen could occur under certain conditions. Antigen could then form soluble complexes and in this way add to the total nitrogen of the preparation. Studies on the action of urea on BSA (Ferris and Katz, 1966, Katz and Dennis, 1967) revealed that treatment of BSA with 8M urea for as little as four minutes resulted in the appearance in electrophoresis of at least three fractions. Although the attachment of BSA to a matrix

might alter its susceptibility to this action of urea, the possibility of this happening has to be considered. We thought that if antigen were present in the antibody preparation we might have been able to detect it by immunoelectrophoresis. The preparation was therefore submitted to electrophoresis and developed with anti-BSA. However, we were unable to detect any antigen. This might have been due to the lack of sensitivity of the method, the concentration of anti-BSA or to the fact that the antigenic sites were destroyed or unavailable to combine with the anti-serum. Moreover, if the electrophoretic mobility of the soluble complex were similar to that of free antibody it would not be detected in electrophoresis.

Another point for consideration is the possible presence of complement. Complement is adsorbed to antigen-antibody complexes formed on immunoadsorbents, and although an ammonium sulphate precipitated globulin fraction of inactivated serum was used, at least some of the components of complement might be expected to be in this preparation. However, complement represents only about 0.78% of the total serum protein in guinea pigs and probably less in rabbits

(Kabat and Mayer, 1961). It would therefore, not be expected to contribute significantly to the total nitrogen of the preparation.

There is more concern about the amount of non-specific materials that might be present as a result of direct adsorption to the immunoadsorbent. The results of experiments designed to determine this were inconclusive and no estimation has been possible. This is unfortunate since there is no other way to determine if and how much non-specific globulin might be present. The methods of ultracentrifugation and immunoelectrophoresis do not differentiate between specific and non-specific globulins.

On the basis of precipitable nitrogen, some increase in purity was obtained over DEAE-cellulose chromatography by the use of the immunoadsorbent. The results obtained from quantitative precipitation on the DEAE-cellulose preparation indicate that about 25% of the globulins represented precipitable antibodies. This means that the immunoadsorbent gave about 25% increase in purity

Apart from high yield and purity, it is desirable that an immunoadsorbent should have a high capacity for anti-

bodies. The adsorbent, as prepared by us displayed a rather low capacity, again much lower than reported. Our preparations could bind only about 20 milligrams antibody nitrogen per gram of adsorbent as opposed to a reported 700 mg. On the basis of a publication (Levin et al 1964), two possible explanations are offered. One concerns the activity of the ethylene maleic anhydride copolymer. Levin and his co-workers found that EMA DX-840 undergoes spontaneous hydrolysis when it absorbs moisture to produce maleic acid, and that the amount of active protein (trypsin) bound was proportional to the content of anhydride in the polymer. About 90% of the hydrolysed groups could be converted back to anhydride by heating to 110°C for 24 hours in vacuo.

Since we had not previously been aware of this (Levin et al, 1964) no special precaution was taken to avoid adsorption of moisture. There is suggestive evidence that we might have been using EMA which had lost some activity. In the latter stage of experimental work we found that conjugates which had been prepared in the usual way with the same proportions of reactants failed to bind significant amount of antibodies from a preparation of globulin

which was the same as used in previous experiments. This suggested that either antigen was not bound or antigenic sites were being blocked.

The next aspect concerns the proportion of copolymer to protein. The same workers found that adsorbents of highest capacities were obtained when the ratio of protein to copolymer was 4:1 (w/w). Preparations with a low protein content had low capacities. A 1:1 ratio of protein to copolymer was used by us, and this may have been the reason for the low capacity of our immunoadsorbent.

In the reaction between protein and copolymer cross-linking of the carrier is effected by the protein molecules themselves, so that antigenic groups of the protein could possibly be blocked by unfavourable proportions of the reactants. In addition, a residue of EMA has four sites capable of entering into covalent bonding under proper conditions. If there is an excess of copolymer one might envisage free carboxyl radicals which could probably bind other molecules by electrostatic forces. Although the BSA system might not be directly comparable to the enzyme system of Levin's the principle is the same and therefore these two

factors might be well worth investigating.

The results obtained by us suggest that the immunoadsorbent used was not very efficient. However, in view of the recent information evaluations have to be made with reservations. If the observations of other workers are confirmed, and if those discussed above were the basic problems involved in our system, then it is possible that a highly efficient immunoadsorbent might yet be produced once these factors are corrected.

SUMMARY

1. Rabbit antisera to bovine serum albumin were prepared, pooled and the antibody content determined by quantitative precipitation. Nitrogen was determined by the Kjeldahl procedure.

2. A 33% ammonium sulphate fraction was obtained from the pooled antiserum and the antibody content determined. A portion of the preparation was fractionated on DEAE-cellulose and a portion was purified by immunoadsorbent.

3. The immunoadsorbent was prepared by reacting BSA with ethylene maleic anhydride copolymer at $0^{o}C$. It was washed extensively, mixed with Sephadex G 25 (coarse) and used in column form. Antibodies were eluted with phosphate buffered 8 M urea pH 7.4 or stepwise with 0.35 N glycine-HCl buffer pH 3.0, 2.5 and 2.0. The effluent was dialysed against 0.3 M sodium chloride buffered with phosphate at 7.4, concentrated by ultrafiltration and the antibody content estimated.

4. Double diffusion in agar was used to assay for antibody activity, while immunoelectrophoresis, ultracentrifugation and disc electrophoresis were used to characterize the

antibodies, and to establish their purity.

5. Based on the amount of precipitable nitrogen recovered compared to that contained in the sample applied to the immunoadsorbent, a yield of 42% was obtained. This represented 50% of the total protein recovered from the immunoadsorbent.

6. The purity of the antibody preparation obtained from the immunoadsorbent was compared with that of one obtained by a non-specific method. A preparation obtained by fractionation of an ammonium sulphate fraction on DEAE-cellulose, and containing gamma globulin only as detected by immunoelectrophoresis was found to contain 25% precipitable antibody nitrogen compared to 50% in the preparation from the immunoadsorbent.

7. Non-specific adsorption was determined by substituting normal rabbit globulin for anti-BSA. The results of the experiments were not conclusive but suggested that a negligible amount of non-specific protein was bound by the adsorbent.

8. Columns of immunoadsorbent were reused and it was established that the immunoadsorbent was stable with re-use and would give reproducible results when used for up to four times.

9. The results suggest that the efficiency of the immunoadsorbent as prepared for use in these experiments was somewhat lower than that reported. Possible explanations for the relatively low yield and purity of antibodies, as also the low capacity of the immunoadsorbent were offered and discussed.

APPENDIX

Solutions, Stains and Reagents

1. Barbital Buffer for Agar-gel Electrophoresis

(Hirschfeld, 1960)

(a) Barbital Buffer for Agar - Stock Solution

Barbitone Sodium	10.51 g
Barbitone	1.66 g
Calcium Lactate	1.54 g
Distilled water to	1000.00 ml

Adjust pH to 8.6

Before use, the buffer is diluted by mixing two parts with one part distilled water.

(b) Barbital Buffer for Electrodes

Barbitone Sodium	8.76 g
Barbitone	1.38 g
Calcium Lactate	0.38 g
Distilled water to	1000.00 ml

Adjust pH to 8.6

2. Borate-buffered Saline

Boric Acid	6.184 g
Sodium Tetraborate (Borax)	9.536 g
Sodium Chloride	4.384 g
Distilled water to	1000.00 ml

Adjust pH to 8.4

For use, 5 parts of the buffer is mixed with 95 parts saline.

3. Glycine-Hydrochloric Acid Buffer (0.35M)

Stock A:

Glycine	7.5 g
Sodium Chloride	8.5 g
Distilled water to	1000.00 ml

Stock B:

Hydrochloric Acid$_{(con.)}$	8.6 ml
Sodium Chloride	8.5 g
Distilled water to	1000.00 ml

Appropriate volumes of A and B are mixed to obtain the desired pH.

pH	A*	B*
3.0	80.8 ml	19.2 ml
2.5	66.8 "	33.2 "
2.0	52.3 "	47.7 "

*Approximation

4. Biuret Reagent

Sodium or Potassium Tartrate	9.0 g
$CuSO_4.5H_2O$	3.0 g
Potassium iodide	5.0 g
0.2 N NaOH (carbonate-free)	1000.00 ml

The tartrate is dissolved in 400 ml NaOH. Cupric sulphate is then added and dissolved, followed by potassium iodide. The solution is made up to 1000 ml with the NaOH.

Test

1.5 ml Biuret reagent is added to 1.0 ml protein sample and blanks, incubated at 37°C for 30 minutes and read at 555 mu in a Beckman spectrophotometer.

5. Phosphate Buffer for washing Immunoadsorbent

Stock A:

Potassium Dihydrogen Phosphate (KH_2PO_4)	68.05 g
Sodium Chloride	5.85 g
Distilled water to	1000.00 ml

Stock B:

Disodium Hydrogen Phosphate (Na_2HPO_4)	71.00 g
Sodium Chloride	5.85 g
Distilled water to	1000.00 ml

For use, mix 10.3 ml of A with 30 ml of B and make up to 1000 ml with 0.1 M sodium chloride solution. Adjust pH to 7.4

6. Phosphate buffered 8 M Urea

Urea	480.47 g
Phosphate buffer as prepared for use in (5) above to	1000.00 ml

Adjust pH to 7.4

7. Sodium Chloride Solution for Dialysis (0.3M)

Sodium Chloride	17.55 g
Distilled water to	1000.00 ml

Adjust pH to 7.4 with stock solution B in (5).

8. Boric Acid-Methyl Red Indicator for Nitrogen Determination - Kjeldahl procedure

Saturated Boric Acid	1000.00 ml
Saturated Solution of Methyl Red (2x crystallized)in 50% Ethanol	20.00 ml

For use, 5 ml of boric acid-methyl red solution are mixed with 5 ml glass distilled water for each sample analysed.

9. N/70 Hydrochloric Acid

Hydrochloric acid was prepared from a concentrated volumetric solution (British Drug Houses) which gives a 0.1 N solution when made up to 500 ml according to directions. The 0.1 N solution was diluted 1:7 using volumetric flasks. Glass distilled water was used.

10. Saturated Sodium Hydroxide

Saturated NaOH was prepared and filtered by gravity through Whatman filter paper. Glass distilled water was used.

11. Folin-Ciocalteu Phenol Reagent for Protein Determination

0.1 N Sodium Hydroxide	500.00 ml
Sodium Carbonate (Anhydrous)	2.00 g
$CuSO_4.5H_2O$	0.50 g
Sodium or Potassium Tartrate	1.00 g
Distilled water	100.00 ml
Folin reagent titrated and diluted to 1.0 N	0.10 ml per sample

Dissolve sodium tartrate in distilled water. Add cupric sulphate and dissolve.

Dissolve sodium carbonate in 100 ml 0.1 N NaOH.

Just before use, 1 ml of the copper solution is added to 50 ml of the carbonate-sodium hydroxide solution. For details of the test see Materials and Methods, Section C.

12. Disc Electrophoresis

(a) Tris-glycine Buffer for Reservoir

Tris*	6.0 g
Glycine	28.0 g
Distilled water to	1000.0 ml

Adjust pH to 8.3

For use dilute 1:10

(b) Stock Solutions

Solution A		Solution B	
1.0 N HCl	48.00 ml	1.0 N HCl	48.00 ml
TRIS	36.60 g	TRIS	5.98 g
TEMED**	0.23 ml	TEMED	0.46 ml
Water to	100.00 ml	Water to	100.00 ml
pH 8.9		pH 6.7	

Solution C		Solution D	
Acrylamide	28.00 g	Acrylamide	10.00 g
BIS***	0.735 g	BIS	2.50 g
Water to (Brown Bottle)	100.00 ml	Water to (Brown Bottle)	100.00 ml

Solution E		Solution F	
Riboflavin	4.00 mg	Sucrose	40.00 g
Water to (Brown Bottle)	100.00 ml	Water to	100.00 ml

(c) Working Solutions

Small Pore Solution No.1	Small Pore Solution No.2
1 part A	Ammonium persulphate 0.14 g
2 parts C	Water to 100.00 ml
1 part water	
pH 8.9	

For use mix equal parts of solutions 1 and 2.

Large Pore Solution for Wash	Large Pore Solution
1 part B	1 part B
1 part E	2 parts D
6 parts water	1 part E
	4 parts F
	pH 6.7

Fixative-Stain

Amidoblack	1.00 g
Acetic acid 7%	100.00 ml

Destaining Gel

Acrylamide	6.00 g
Riboflavin	0.50 mg
TEMED	0.05 ml
Water to	100.00 ml

Expose to fluorescent light in a 100 ml beaker at a distance of 3 inches for 1½ hour. Dilute with equal volume of water. Store in dark bottle.

Destaining and Storing Solution

Glacial acetic acid	70.00 ml
Water to	1000.00 ml

* Tris (Hydroxymethyl) Aminomethane

** N,N,N'N' - Tetramethylethylenediamine

*** N'N' - Methylenebisacrylamide

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